PART 3: GENERAL THEMES

Chapter 16: The Biogenesis of Membranes and Organelles

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Abstract

The field of organelle biogenesis has expanded so much in the last few years that it seemed impossible to include a chapter as comprehensive as that found in the last edition of this text without extending the length of the chapter inordinately. Nonetheless, the editors feel that the chapter from the last edition provides an excellent foundation for understanding more recent developments which are now covered throughout the rest of the book where they are relevant to understanding specific diseases of lysosomes, peroxisomes, mitochondria, and membrane receptors. For this reason, we include this chapter from the 7th edition.

1. The eukaryotic cell is surrounded by a plasma membrane and is characterized by the presence of a nucleus and many cytoplasmic organelles, which are functionally specialized and also delimited by membranes. The nucleus is the site of storage and initial decoding of genomic information. Completion of this decoding, however, requires the translation of mRNA in ribosomes present in the cytoplasm and the delivery of each newly synthesized polypeptide to its site of function. This chapter considers the sorting and targeting processes by which newly synthesized proteins are transferred to their sites of function, which may be in the nucleus, in the cytomatrix that occupies the space between organelles, within a membrane, in the luminal cavity of an organelle, or outside the cell. Many genetic diseases lead to defects in specific organellar functions and, in some cases, this results from faulty protein targeting.

2. The plasma membrane and several cytoplasmic organelles, including the ER, Golgi apparatus, secretory granules and vesicles, lysosomes, and endosomes, form an integrated endomembrane system that serves to transfer macromolecules and membrane components from one part of the cell to another, as well as to and from the cell exterior. Many of the proteins of these organelles are synthesized in ribosomes bound to membranes of the ER and are inserted into these membranes, or translocated into the lumen of the ER, during their synthesis. These proteins can then either remain within the ER or be transported along the endomembrane system by a process that involves their incorporation into vesicles. These vesicles are formed by budding from the membrane of a donor compartment and deliver their content by fusing with a membrane of the acceptor compartment. We discuss in detail the mechanisms that: (1) select specific mRNA for translation in ribosomes associated with the ER, (2) determine the transmembrane disposition of integral membrane proteins, (3) mediate the retention of proteins in a specific organelle, or their transport within the endomembrane system, and (4) lead to the production of vesicles in a donor membrane and determine their fusion with a specific acceptor membrane.

3. Mitochondria and peroxisomes are organelles that do not communicate with the endomembrane system through vesicular flow and acquire their protein contents by direct uptake of newly synthesized, but not yet fully folded, polypeptides from the cytoplasm. The uptake into mitochondria involves molecular chaperones, as well as receptors on the surface of the organelle, and sorting processes that address the proteins to the various submitochondrial compartments.
THE ORGANIZATION OF THE EUKARYOTIC CELL

The eukaryotic cell shows an extraordinary degree of organizational complexity. Macromolecular components that carry out different metabolic processes are segregated in distinct subcellular compartments, and these must act in concert to sustain the various cellular functions. The membranes bounding all cellular organelles not only control the passage of substances between the various compartments and the surrounding cytoplasmic matrix, but also provide a framework for the functional integration and assembly of many of the organelar macromolecules into higher-order complexes. This is also true for the plasma membrane, which surrounds the entire cell and regulates its interactions with the extracellular milieu.

The presence of a nucleus, a compartment limited by a membranous envelope, is the defining feature of the eukaryotic cell. In this compartment, the genome is stored and replicated and the process of decoding the genetic information begins. In the cytoplasm, several membrane-bound organelles form an integrated endomembrane system (sometimes referred to as the “vacuolar system,”1) which, together with the plasma membrane, is organized for the transfer of macromolecules and membrane components from one part of the cell to another, as well as to and from the cell’s exterior. This transport takes place by means of membrane vesicles, which bud from one organelle and fuse with another, although in some cases tubular connections may be established that carry material between organelles. The set of intercommunicating organelles that constitutes the endomembrane system (Fig. 16-1) includes: (1) the endoplasmic reticulum (ER), which may be regarded as an extension of the nuclear envelope and serves as a major site of protein synthesis and biosynthetic activity; (2) the Golgi apparatus, which modifies many of the proteins it receives from the ER and transfers them to other sites in the cell; (3) secretory vesicles and granules, which contain proteins that have traversed the Golgi apparatus and will be released at the cell surface; (4) endosomes, which receive materials taken in from outside the cell within plasma membrane invaginations; and (5) lysosomes, which degrade the exogenous material from endosomes as well as endogenous cellular components. Because the luminal cavities of the several membrane-bound compartments of the endomembrane system can communicate with each other and with the extracellular space via transport vesicles or tubular connections, all luminal faces of the membranes in this system can be regarded as topologically equivalent to each other (Fig. 16-2).
Subcellular compartments that constitute the cellular endomembrane system. The major organelles of the endomembrane system are: the endoplasmic reticulum (ER), with its rough (RER) and smooth (SER) components; the Golgi apparatus (GA); endosomes (endo); lysosomes (lys); secretory vesicles (sec. ves.) and granules (SG); and the plasma membrane (PM). The outer membrane of the nuclear envelope (NE) is studded with ribosomes and is continuous with...
Topologic equivalence of the membrane faces in the different compartments of the cellular endomembrane system. Intracellular traffic mediated by membrane vesicles (MV) first involves the generation of a vesicle from the membrane bounding one compartment by a budding process (also known as fission). The vesicle is then transported through the cytoplasm and its membrane fuses with the membrane of the receiving compartment. During the fusion an...

Two other membrane-bound organelles that do not directly communicate with the endomembrane system are found in animal cells: (1) mitochondria, which generate most of the ATP required to sustain cellular activity, but also play a major role in many other aspects of intermediary metabolism; and (2) peroxisomes, in which several oxidative reactions that generate hydrogen peroxide are carried out, as are...
several important steps in the degradation of long chain fatty acids and in the synthesis of plasmalogens and bile acids.

The portion of the cytoplasm that extends from the nuclear envelope to the plasma membrane and surrounds the membrane-bound organelles is known as the cytoplasmic matrix (or cytomatrix). It contains filamentous elements such as microtubules, microfilaments, and intermediate filaments, which constitute the cytoskeleton. The cytoskeleton serves to organize the cytoplasm and controls the location and movement of the different organelles, and of the cell itself. The cytomatrix also contains ribosomes that function in protein synthesis, as well as numerous soluble enzymes that carry out myriad biochemical reactions. Several ribosomes are usually engaged in the translation of a single mRNA molecule, thus forming a polyribosome or polysome. The term cytosol is sometimes applied to the soluble components of the matrix which during cell fractionation are recovered in high-speed supernatants.

**Organization of Protein and Lipid Components in Membranes**

Membranes are lipoprotein structures that consist of amphipathic lipids disposed in a bilayer arrangement and of proteins that penetrate the bilayer or are attached to its surfaces (Fig. 16-3). The most abundant lipid components of membranes are phospholipids, cholesterol, and glycolipids, all of which have their polar groups facing the aqueous environment on the membrane surfaces and their hydrophobic fatty acid chains (in phospholipids and glycolipids), or the sterol ring (in cholesterol), oriented toward the membrane interior. The hydrophobic interior of cellular membranes makes them effective barriers to the passage of highly polar or charged molecules from one compartment to another. The lipid molecules within the bilayer cannot easily flip-flop from one monolayer to the other, but can undergo extensive rotational and lateral translational movements. The resulting membrane fluidity permits the lateral displacement of proteins within the plane of the membrane, which is important in membrane function.
Relationship of integral and peripheral membrane proteins to the membrane phospholipid bilayer. Integral
membrane proteins (a) have portions of their mass embedded in the membrane that interact directly with
the hydrophobic tails of the phospholipids. Other portions of these proteins are exposed on the
cytoplasmic or luminal membrane faces. The extent of exposure on each side of the membrane may vary
substantially from one protein to another...

Proteins associated with membranes fall into two categories. Those that are embedded in the
phospholipid bilayer and, therefore, interact directly with the hydrophobic lipid phase are known as integral
membrane proteins (see Fig. 16-3). They can only be removed from the membrane by procedures that
disrupt the bilayer, such as treatment with detergents. Proteins that do not interact directly with the
membrane interior and are bound to the surface of the membrane only via interactions with other proteins
or, possibly, with the polar groups of the lipids are known as peripheral membrane proteins (see Fig.
16-3). They can be removed from membranes by treatment with mediums of high ionic strength or extreme
pH, or that contain chelating or chaotropic agents.

In general, the membrane-embedded portions of integral membrane proteins consist of peptide segments
that are rich in hydrophobic amino acids and are approximately 20 amino acids in length, just sufficient to
span the thickness of the bilayer in an α-helical configuration. In some cases (see below) proteins are
anchored in the membrane solely by a covalently bound lipid moiety. These may be the only integral
membrane proteins exposed on only one membrane surface.

Proteins that fully traverse the lipid bilayer may cross the membrane only once and therefore have only
one hydrophobic membrane-anchoring domain (Fig. 16-4). This is the case with several
well-characterized hormone receptors of the plasma membrane—such as the epidermal growth factor5, 6
(and see ref. 7) and insulin receptors8, 9 (and see ref. 10)—in which the ligand binding portion of the
molecule is exposed on the extracellular membrane surface while the signal-transducing domain is
located in the cytoplasm. Integral membrane proteins that cross the membrane only once and have
portions of their mass exposed on each surface are called bitopic proteins.11 Such proteins have one of
two possible transmembrane orientations. Type I proteins (see Fig. 16-4) have their C-terminal ends in the
cytoplasm and their N-terminal ends exposed on the extracellular surface of the plasma membrane, or on
the (topologically equivalent) luminal surface of an organelle within the endomembrane system, such as
the ER. Type II proteins (see Fig. 16-4) have the reverse disposition, traversing the membrane with an N
(cytoplasmic) to C (extracellular or luminal) orientation. As discussed in detail below, the different
transmembrane orientations of the two classes of proteins can be explained as a consequence of the
mechanism by which polypeptides are inserted into the ER membrane during their synthesis.
Transmembrane disposition of different types of integral membrane proteins. Types I and II membrane proteins cross the membrane only once. Type I proteins, such as red-cell glycophorin, the LDL and EGF receptors, the heavy chain of the class I histocompatibility antigen, and the viral envelope glycoproteins HA of influenza and G of VSV have their N-termini exposed on the extracellular (or luminal) face of the membrane and their C-termini on ...

Some transmembrane proteins, in particular ion channels, cross the phospholipid bilayer several times (type III or polytopic proteins) (see Fig. 16-4), and the N- and C-terminal ends of such proteins may be found on the same or opposite sites of the membrane. The transmembrane domains of these proteins may also be hydrophobic or may be capable of forming amphipathic helixes, whose existence within the membrane may be maintained by lateral interactions with other similar helixes within the same polypeptide or within other subunits of a multimeric protein. In the final configuration, a hydrophilic channel is formed by the polar faces of several helixes, which have their hydrophobic faces interacting with the interior of the membrane bilayer.

Many membrane proteins are glycoproteins that contain carbohydrate moieties linked to the polypeptide backbone via either N-glycosidic bonds to asparagine residues, or O-glycosidic bonds to serine or threonine residues. Carbohydrate moieties may also be linked to membrane lipids (glycolipids). In all cases, the carbohydrates of membrane components are located only on the extracellular or luminal side of the membrane (see Fig. 16-4). Since the enzymatic system responsible for the formation of N-glycosidic bonds is present only in the ER, only proteins that reside in this organelle or pass through it during their biosynthesis can bear asparagine-linked oligosaccharide chains.
An Overview of Organelle Biogenesis

Because of the organizational complexity of eukaryotic cells, the implementation of their genetic programs requires not only the transcription of sets of specific genes and the translation of the resulting messenger RNAs, but also the operation of mechanisms that ensure that the encoded polypeptides are transferred from their sites of synthesis to their sites of function, which may be in the cytomatrix, in a membrane, within a space enclosed by an organellar membrane, or outside the cell.

Aside from a very small number of polypeptides that are synthesized on special ribosomes found within mitochondria, the bulk of protein synthesis in mammalian cells takes place in the cytoplasmic matrix—either on ribosomes that appear to be free in the matrix but could be associated with cytoskeletal elements or on ribosomes that during their synthetic activity are attached to the membranes of the ER. The part of the ER to which these ribosomes are attached is called the rough ER (RER), on account of the appearance of its cytoplasmic surface in electron micrographs. Portions of the ER devoid of attached ribosomes constitute the smooth ER (SER).

As previously noted, the universal structural feature of all cellular membranes is the presence of a phospholipid bilayer with a hydrophobic interior that constitutes a barrier to the passage of polar molecules. In particular, proteins—which normally fold with their charged and polar residues exposed on their surfaces—cannot freely traverse a phospholipid bilayer. Therefore, special mechanisms have evolved that facilitate the incorporation of polypeptides into specific membranes, and when necessary, assist them in their passage across the hydrophobic barrier. In many cases one or more molecular chaperones associate with the polypeptide to be incorporated into or to be transported across the membrane and serve to maintain it in a conformation that is compatible with these processes.

Proteins destined for the nucleus, mitochondria, or peroxisomes are synthesized in ribosomes that are free in the cytoplasmic matrix, and are directly targeted to their respective organelles (Fig. 16-5). Specific receptors for the newly synthesized organellar proteins are present in the surface of mitochondria and probably also in the surface of peroxisomes. Those receptors must recognize structural features of each polypeptide and participate in a process that leads either to its insertion into the membrane or its translocation across it. The latter process may require the expenditure of energy and entail conformational changes or extensive structural modifications of the polypeptide. Proteins destined to the interior of the nucleus must pass through the nuclear pores of the nuclear envelope.
Different fates of polypeptides synthesized on free polysomes. On release from the ribosome, these polypeptides may remain in the cytomatrix (as soluble proteins or as components of the cytoskeleton) or may be posttranslationally incorporated into different organelles.

Some proteins of the ER and of the plasma membrane are also synthesized on free polysomes and become embedded in the membrane only after their synthesis is completed and they are discharged into the cytoplasm (see Fig. 16-5). A similar mechanism could, in principle, also lead to the insertion of proteins in the cytoplasmic surfaces of other organelles.

In contrast to the direct targeting of nuclear, mitochondrial, and peroxisomal proteins to their sites of function, proteins destined for secretion or for incorporation into lysosomes, as well as most proteins of the plasma membrane and the Golgi apparatus, are initially incorporated into the ER, and reach their sites of function by transfer through the cellular endomembrane system. Such proteins, like most proteins of the ER itself, are translocated across or inserted into the ER membrane cotranslationally, that is, during the course of their synthesis in ribosomes bound to the RER membrane (Fig. 16-6). Although these proteins may later undergo extensive posttranslational modifications, it is during or immediately after their synthesis in bound polysomes that they are either transferred to the lumen of the endomembrane system or incorporated into the membrane with a characteristic disposition with respect to the phospholipid bilayer.
Different fates of polypeptides synthesized in ribosomes bound to the ER membrane. Proteins synthesized in membrane-bound ribosomes are inserted into the ER membranes or translocated into the ER lumen by a process that begins during their synthesis. After their synthesis is completed, these proteins either remain in the ER or are transferred to the membranes or luminal cavities of other organelles within the endomembrane system.

After discharge into the ER lumen or incorporation into the ER membrane, proteins synthesized in membrane-bound ribosomes are subjected to sorting processes, just beginning to be understood, which ensure that certain polypeptides are retained in the ER while others are transferred to the Golgi apparatus and either remain there or, on exit from this organelle, are transported to lysosomes, secretory vesicles or granules, or the plasma membrane. As already mentioned, transport within the endomembrane system and to and from the plasma membrane is effected by membrane vesicles that bud from one organelle, traverse a portion of the cytoplasm, and fuse with another. Throughout this movement, luminal proteins remain segregated within the successive organelar cavities, and membrane proteins retain the characteristic transmembrane disposition that they acquired in the ER.

In the past few years much has been learned about the molecular machinery and intermolecular interactions involved in the formation, targeting, and fusion of the vesicles that mediate interorganellar transport. This has resulted from both the development of in vitro systems that reproduce these phenomena under controlled conditions and from the isolation of yeast mutants defective at specific stages of vesicle formation or consumption. The latter approach has led in many cases to the determination of the biochemical nature and role of the products of the defective genes.

**Yeast as a Model Organism to Study Intracellular Protein Trafficking**

Although the emphasis in this chapter is on membrane and organelle biogenesis in the mammalian cell, it will frequently be necessary to discuss information on these processes obtained from studies with the yeast *Saccharomyces cerevisiae*. This organism serves as a useful and widely employed model to study fundamental functions of the eukaryotic cell since with yeast one can combine the powerful approach of molecular genetics with physiological and biochemical analyses. Using a variety of elegant but relatively simple selection procedures, a large number of conditionally lethal (temperature-sensitive) yeast mutants have been identified that, at the nonpermissive temperature, fail to grow because they are defective in various steps along the pathway of protein secretion or in the transport of newly synthesized proteins to a
The availability of such mutants has permitted cloning of the respective genes by complementation in which mutant cells are transfected with a wild-type yeast genomic library and transformants containing a plasmid with the gene of interest are selected by virtue of their growth at the restrictive temperature. In addition, the isolation of extragenic suppressor mutations has led to the identification of other genes whose products, usually when overexpressed or mutated, can compensate for the defect caused by the original mutation. The products of suppressor genes function in the same pathway and probably interact with the product of the original mutant gene. These and other genetic approaches have facilitated the elucidation of the role of many gene products within the protein targeting and transport machinery of the cell. Moreover, analysis of the sequence of a cloned yeast gene has sometimes revealed the existence of a mammalian homologue and, in some instances, it has even been possible to correct the yeast defect by expressing in the yeast cell a gene encoding the mammalian protein (e.g., ref. 19). Conversely, in some cases, the yeast product has been shown to substitute for the mammalian one in an in vitro transport reaction.20 Yeast homologues of mammalian proteins involved in transport have also been identified, or cloned, on the basis of their expected sequence homology to known mammalian proteins (e.g., the clathrin heavy and light chains, and the ADP ribosylation factor, Arf1p; see ref. 18). Finally, proof that a specific yeast protein whose gene has been cloned is essential can be ascertained from the viability of cells in which the gene has been “knocked out” (disrupted) by homologous recombination procedures, which are easy to carry out in this organism.

Studies on a large set of mutants defective in secretion, designated sec mutants,

*Uppercase letters (e.g., SEC4) indicate the wild-type gene and lower-case letters (e.g., sec4) the mutant gene. The protein product of the normal gene has only the first letter capitalized and is followed by a lower-case p (e.g., Sec4p).

have illuminated aspects of essentially all steps of transport along the endomembrane system. Sec mutants were first recognized because they accumulated precursor forms of exported proteins. It is now clear that some of the corresponding gene products are required for protein insertion into the ER (e.g., Sec61p, Sec62p, Sec63p, Sec65p), while others participate in transport from the ER to the Golgi apparatus (e.g., Sec12p, Sec13p, Sec17p, Sec18p, Sec20p, Sec21p, and Ypt1p), through the Golgi cisternae (e.g., Sec7p, Sec14p, Arf1p, Arf2p), or from the Golgi to the cell surface (e.g., Sec2p, Sec4p, Sec15p). A large number of mutants (vps) defective in transport to the vacuole, the yeast equivalent of the mammalian lysosome, are also available, as are mutants in genes (ERD1 and ERD2) required for retention in the ER of proteins that normally reside in this organelle. Other classes of mutants defective in the importation of proteins into the nucleus, mitochondria, or peroxisomes have also been isolated.

**GTP-Binding Proteins Control Many Steps along the Secretory Pathway**

A very important class of yeast genes (e.g., YPT1 and SEC4) involved in protein traffic encode proteins that bind and hydrolyze GTP (guanine nucleotide binding proteins). Studies of the corresponding mutants have contributed greatly to focus attention on the role of GTP-binding proteins as “molecular switches” that control the directionality of a wide variety of individual steps in intracellular protein transport.

GTP-binding proteins constitute a superfamily that include the heterotrimeric (Goβγ) G proteins that transduce extracellular hormonal and sensory signals into intracellular changes; the protein synthesis elongation factor EF-Tu, that delivers aminoacyl-tRNA to the ribosome;28 the tubulin subunits of
microtubules; a large number of low-molecular-weight (20- to 25-kDa) proteins related to the product of the ras oncogene, many of which are now known to be involved in protein transport; and subunits of the signal recognition particle (SRP) and its ER membrane receptor (SR) (see below) that participate in the targeting of nascent polypeptide chains containing insertion signal sequences to the protein translocation apparatus in the ER membrane.

The essential feature of GTP-binding proteins, that allows them to serve as molecular switches, is that they have slow or latent GTPase activity and, therefore, can exist in two different conformational states, depending on whether they contain bound GTP or GDP, with the GTP-bound form being referred to as the “active” one. In general, the conformational state of a GTP-binding protein determines its capacity to associate with a downstream effector. In the case of the heterotrimeric G proteins (Gαβγ) that are coupled to plasma membrane receptors with seven transmembrane domains, such as rhodopsin and the β-adrenergic receptor, activation of the receptor catalyzes the exchange of GDP by GTP in the Gα subunit, which in this “activated state” dissociates from the β and γ subunits, and exerts its effect on an effector, such as a channel (e.g., the muscarinic receptor-activated potassium channel) or an enzyme (e.g., phosphodiesterase or adenylcyclase). There are two main types of G proteins that associate with different receptors, those that contain stimulatory α subunits (Gsα) and activate the effector and those that contain inhibitory α subunits (Giα) and exert the opposite effect. In both cases, the Gα subunit remains active until the switch is turned off by the spontaneous hydrolysis of its bound GTP. Although the best-understood role of heterotrimeric G proteins is in signal transduction at the plasma membrane, evidence has indicated that proteins of this class, not yet fully characterized, are also associated with intracellular membranes and function in regulating various steps of intracellular vesicular transport (see refs. and our discussion below).

The ras-related low-molecular-weight GTP-binding proteins involved in intracellular protein transport, and probably the GTP-binding proteins in the SRP and its cognate receptor (SR) that function in the targeting of newly synthesized polypeptides to the ER (see below), do not appear to control an enzymatic reaction but rather to serve as switches that confer unidirectionality to a sequence of molecular associations. The paradigm for this mode of action is the elongation factor EF-Tu, which in its GTP-bound or “active” form binds aminoacyl-tRNA in the cytosol, but releases it when GTP hydrolysis takes place (Fig. 16-7). In this case, the molecular switch associated with GTP hydrolysis controls the unidirectional transport of the aminoacyl-tRNA from the site of its charging with the amino acid (the cytosol) to its site of utilization (the ribosome). The cyclic function of EF-Tu requires that the released factor be recharged with GTP by another cytosolic elongation factor, EF-Ts, which functions as a guanine nucleotide exchange protein. The EF-Tu GTPase paradigm has served to inspire hypotheses for the role of small GTP-binding proteins in the vectorial insertion of proteins into the ER, and in the vesicular transport of proteins from one intracellular compartment to another, in which many ras-related proteins of the rab family have been found to play key regulatory roles.
Polypeptide chain elongation factor Tu (EF-Tu) functions as a molecular switch in the delivery of aminoacyl-tRNA to the ribosome. Elongation factor Tu can exist in two different conformations depending on whether it contains bound GTP or GDP. In its active GTP-containing form, Tu binds aminoacyl-tRNA in the cytosol, forming a ternary complex (Tu.GTP.aatRNA). This in turn binds to the A site in a ribosome that is engaged in the translation of...

The low-molecular-weight (20- to 29-kDa) GTP-binding proteins of the \textit{ras} superfamily are divided into several families (\textit{ras}, \textit{rap}, \textit{rab}, \textit{rho}, \textit{rac}, \textit{ran}, and \textit{arf}), based on sequence similarities. Many proteins of the \textit{rab} family (for \textit{rat} \textit{brain}, from which the original DNAs were cloned), of which at least 25 members have now been identified, have been localized to several intracellular organelles in a variety of cell types and have been shown to be involved in different steps of transport at the endomembrane system (see refs. \textit{26}, \textit{26a}, and \textit{27} and our discussion below). In some of these processes \textit{arf} proteins (for \textit{ADP ribosylation factor}, the name given to the original member of this family, identified as a cofactor in the cholera toxin-induced ADP ribosylation of Gs\textsubscript{x} proteins) have also been implicated.

GTP-binding proteins of the \textit{rab} and \textit{ras} families share a common domain structure, which is reflected in their function. In addition to the cysteine-containing C-terminal region that is required for their prenylation and membrane binding (see below), they contain three highly conserved segments that participate in GTP binding and a segment (residues 32 to 40 in the \textit{rab} proteins) known as the \textit{effector domain} (because it corresponds to the effector domain of \textit{ras}, that is, the region on the surface of \textit{ras} that interacts with the protein, called \textit{raf}, that regulates its GTP hydrolysis). Studies with \textit{ras} have shown that its effector domain...
undergoes a conformational change during the GTP cycle and, therefore, is part of the switch mechanism essential for the function of the protein. Finally, a hypervariable region near the C-terminus of the rab proteins appears to determine their specific distribution in different organelles (see refs. 27 and 37). Many different amino acid substitutions in the GTP binding or effector domains of the ras protein have been shown to lead to its activation, independently of nucleotide binding,38 and the same effects are expected to result from similar mutations in other ras-related proteins.

With the exception of the ran family members, which are nuclear proteins, the different ras-related proteins are characterized by specific C- or N-terminal posttranslational modifications that are essential for their function. Thus, proteins in the ras and rho families are characterized by the C-terminal CAAX sequence motif (where A is an aliphatic residue and X any other amino acid) and ras proteins (in which X is M or S) are modified by the addition of a C-15 isoprenoid moiety, farnesyl, to the cysteine residue. The modified proteins then undergo proteolytic removal of the AAX sequence, followed by carboxymethylation of the new C-terminal cysteine residue (see ref. 39). Some of these proteins also undergo palmitoylation (which is a reversible modification) at a neighboring cysteine residue and together these modifications participate in anchoring the proteins to a membrane. Proteins in the rab family contain dicysteine motifs near their C-termini (CC, CXC, CCX, CCXX, CCXXX), which receive the C-20 isoprenoid moiety, geranylgeranyl.40–42 These proteins do not undergo proteolysis, but those with the CXC sequence are carboxymethylated.43

In contrast to those in the ras and rab families, the arf proteins are modified only at their N-termini by removal of the initiator methionine and addition of a myristoyl group (a tetradecanoic acyl group) to a glycine residue that immediately follows it.44 The myristoyl moiety serves as a membrane anchor, but only if the arf protein carries bound GTP. An arf protein, therefore, cycles on and off a membrane (e.g., a Golgi membrane) during its GTP cycle.45

The capacity of ras-related GTP-binding proteins to switch between their off (GDP-bound) and on (GTP-bound) states is controlled by a set of regulatory proteins that interact with them (Fig. 16-8). These include (see ref. 25) GTPase-activating proteins (GAP) that, when the protein binds to its effector (which may be the GAP itself), increase the GTPase activity up to 100 times (e.g., ref. 46); guanine nucleotide-exchange proteins (GEF), also known as GDP/GTP dissociation stimulators (GDS), that accelerate the release of GDP, which in the GTP-rich cellular environment promotes the binding of GTP;47–49 and GDP dissociation inhibitors (GDI) that prevent the release of GDP and, at least in some cases, after GTP hydrolysis, remove the cognate GTP binding protein from the membrane, or prevent its membrane association.50, 51 GDI proteins may, therefore, maintain in the cytosol a pool of inactive GTP-binding proteins, ready to be activated by GDS proteins that may themselves be membrane-associated. In a plausible scheme (Fig. 16-9), activation of a GTP-binding protein by a specific guanine nucleotide exchange factor (GEF or GDS) located in a donor membrane would trigger the association of the GTP binding with the membrane through its interaction with other membrane proteins, which would promote the formation of a carrier vesicle. On vesicle docking on the correct acceptor membrane GTP hydrolysis would occur, releasing into the cytosol the GTP-binding protein in its GDP state. Docking of the vesicle would be followed by activation of the components of the molecular machinery that leads to membrane fusion.
Factors that regulate the activity of GTP-binding proteins of the ras superfamily. In the active GTP-containing state (represented by a triangle) the protein binds to the effector that mediates its physiological action. Binding to the effector markedly increases the latent GTPase activity of the GTP-binding protein which, on hydrolysis of GTP, switches its conformation to the GDP-containing inactive state (represented by an oval). In this ca...
A scheme for the role of a low-molecular-weight GTP-binding protein such as a rab protein, in directing the vectorial delivery of a membrane vesicle. In this scheme it is assumed that, in its active form, a rab protein promotes vesicle formation in the donor membrane (by a mechanism not depicted) and confers to a membrane protein that becomes incorporated into a forming vesicle the conformation necessary to ensure docking of the vesicle on t...
The involvement of a GTP-binding protein in an intracellular transport process is most easily assessed through the effect on that process of the nonhydrolyzable GTP analogue, GTP-\(\gamma\)-S, which, when bound to a GTP-binding protein, maintains it in the “active” conformation. If a step in the transport process requires this conformation, addition of the analogue should stimulate it. If, on the other hand, the step requires hydrolysis of the bound GTP, it should be inhibited by GTP-\(\gamma\)-S. Of course, hydrolysis of GTP is the step required for the GTP-binding protein to complete a cycle of function and to allow for its reutilization, as well as for the reutilization of the other components to which the protein binds specifically when in the activated state. Therefore, if a transport process—observed in a cell-free system or in permeabilized cells—is regulated by a GTP-binding protein and the result of many cycles of function is being observed, addition of GTP-\(\gamma\)-S should lead to inhibition of transport. Similarly, a GTP-binding protein carrying a mutation that maintains it in the active conformation, after binding to its effector, would not be able to function cyclically. Such a protein would block effector sites and inhibit subsequent cycles in the process, exerting a dominant negative effect.

AlF\(_3\)-5 is also a useful reagent that can be used to assess specifically the participation of a heterotrimeric G protein in a cellular process.\(^{52}\) This complex ion can mimic the \(\gamma\) phosphate of GTP and directly activate the G\(\alpha\) subunit of a G protein that contains bound GDP, but is not able to activate the low-molecular-weight GTP-binding proteins. The specific role of a G protein may also be revealed by the effects of mastoparan, a cationic amphiphilic peptide from wasp venom that mimics the polypeptide segment of plasma membrane receptors that interacts with G proteins. This peptide preferentially binds to the C-terminal region of G\(\alpha\) subunits, uncouples them from their receptor, and triggers the replacement of GDP by GTP, thus leading to activation of the inhibitory G protein.

Two toxins capable of catalyzing the ADP ribosylation of G\(\alpha\) subunits, the pertussis toxin and cholera toxin, have also been very useful in revealing the involvement of a heterotrimeric G protein in a cellular function. The pertussis toxin modifies (by ADP ribosylation) the C-terminal ends of some inhibitory G\(\alpha\) subunits. This uncouples them from the receptors and, by preventing their activation through the GDP-GTP exchange, inhibits their function. The modification induced by pertussis toxin also prevents mastoparan from exerting its effect.\(^{53, 54}\) In contrast, cholera toxin ADP ribosylates the stimulatory G\(\alpha\) subunits and leads to their constitutive activation.\(^{55}\)

**PROTEIN SYNTHESIS IN THE ROUGH ENDOPLASMIC RETICULUM**\(^{11–13, 30, 31, 56–59}\)

The ER is a complex system of intercommunicating membrane-bound flattened sacs (cisternae) and tubules that is present in all eukaryotic cells and in many cases permeates large regions of the cytoplasm. Membranes of the rough portions of the ER contain receptors for ribosomes and for nascent polypeptides, as well as proteins that are involved in the cotranslational incorporation of these polypeptides into the organelle, or in their processing during or soon after their synthesis. In addition, both RER and SER membranes contain enzymatic systems that carry out functions essential to all cells, such as steps in the synthesis of triglycerides, phospholipids, and cholesterol, as well as systems that carry out specialized metabolic or biosynthetic functions and, therefore, are present only in specific cell types.

The RER is most prominent in cells engaged in protein secretion, such as pancreatic acinar and anterior pituitary cells, which synthesize digestive enzymes and polypeptide hormones, respectively; plasma cells, which produce immunoglobulins; and hepatocytes, which manufacture a wide variety of serum proteins. Because the RER plays a major role in the synthesis and assembly of membrane proteins, this organelle is prominent in cells, such as neurons, which maintain greatly expanded plasma membranes.
The degree of development of the SER in different cell types usually reflects the participation of ER membrane enzymes in specialized activities of the cell. Thus, the SER is very well developed in cells of steroid-secreting tissues, where it contains enzymes that catalyze several of the hydroxylation reactions that modify the steroid nucleus. It is also highly developed in skeletal muscle cells, in which it is known as the “sarcoplasmic reticulum,” an organelle equipped to sequester calcium ions into its lumen and to release them when the cells are stimulated to contract. In fact, the ER appears to play a major role in the control of cytoplasmic Ca\(^{2+}\) levels in almost all cell types.

Although the membranes of the rough and smooth portions of the ER are continuous, they usually adopt different morphologic configurations within the same cell that must reflect differences in their protein and/or lipid composition. The rough cisternae are frequently arranged in stacks of interconnected flattened disks, whereas the smooth portions usually form an extensive system of thin convoluted tubules. EM of grazing sections of rough cisternae reveal that the ribosomes attached to the membranes form rosettes, hairpins, or spiral patterns, which correspond to membrane-bound polysomes. Individual ribosomes within bound polysomes contact the membrane via their large subunits, which are known to contain the nascent polypeptide chains.

Much information on the biochemical composition and function of the ER has come from the analysis of rough and smooth microsomes, subcellular fractions derived from rough and smooth portions of the ER, respectively. Extensive fragmentation of the ER takes place during the tissue homogenization that must be carried out before cell fractionation. The broken ER membranes, however, rapidly reseal to form microsomal vesicles that still contain a large part of the luminal content of the intact organelle. The rough microsomes retain the ribosomes bound to their membranes and can be separated from the smooth microsomes on the basis of their greater density.

The structural and compositional differences between rough and smooth ER membranes have been best studied in liver cells, where both portions of the organelle are well developed and can be isolated as rough and smooth microsomes, respectively, with high yields and relative purity. Many of the most abundant ER membrane proteins are present in both rough and smooth membranes, but rough microsomes contain several specific membrane polypeptides that are likely to be involved in functions associated with the synthesis and processing of proteins made in bound ribosomes, or with maintaining the characteristic structure of the rough cisternae.

Although cellular phospholipids are synthesized in the ER, the phospholipid composition of the ER membranes is not a simple reflection of their biosynthetic capacity. They are rich in phosphatidycholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI), but contain very small amounts of sphingomyelin (SM) and cholesterol, which are abundant in the plasma membrane. The fatty acids of ER phospholipids are usually highly unsaturated, which accounts for the high fluidity of the ER membranes (see ref. 4).

**Cotranslational Insertion of Polypeptides into ER Membranes: Role of Insertion Signals in Determining the Association with the ER Membranes of Polysomes Synthesizing Specific Proteins**

Ribosomes that are part of polysomes found free in the cytoplasmic matrix are structurally and functionally identical to those within polysomes bound to ER membranes. Indeed, within the cell, after completion of each polypeptide chain, both polysomal populations may exchange ribosomal subunits. The attachment to the ER membrane of the ribosomes that synthesize secretory, lysosomal, or certain classes of membrane proteins is determined by information contained within the nascent polypeptide chains. Extensive studies with a wide variety of secretory proteins have demonstrated that nascent secretory
polypeptides almost invariably contain N-terminal peptide segments that are not present in the mature proteins. These segments serve to determine the attachment of the ribosome bearing the nascent chain to the ER membrane.\textsuperscript{70–74} They consist of 15 to 30 amino acid residues and characteristically include a central hydrophobic core of at least eight amino acids (see refs. \textsuperscript{75 to 78}). Similar N-terminal peptides are found in nascent lysosomal proteins and in many membrane proteins. These peptide segments are known as \textit{transient insertion signals} or \textit{signal sequences} or \textit{presequences}. They serve to trigger the association of the ribosome with the membrane and initiate the complete or partial translocation of the nascent polypeptide through it, but are removed by proteolytic cleavage before synthesis of the polypeptide is completed. The translocation of proteins across the ER membranes initiated by signal sequences (Fig. 16-10) is frequently referred to as the \textit{vectorial discharge of nascent polypeptides}.\textsuperscript{79}

Vectorial discharge of a secretory polypeptide across the ER membrane mediated by a cleavable N-terminal insertion signal. The cotranslational passage of a polypeptide, such as a secretory or lysosomal protein, into the ER lumen is represented in a simplified form that does not include any molecular components of the translocation machinery. The translocation is initiated by an N-terminal signal that is cleaved during the course of polypepti...

\textbf{Process of Assembly of a Membrane-Bound Polysome (Fig. 16-11)}

As is the case with the assembly of a free polysome, the assembly of a membrane-bound polysome begins in the cytoplasm with the binding of a small ribosomal subunit to the 5′ end of the mRNA. After the large ribosomal subunit joins the small subunit at the initiation codon of the mRNA, synthesis of the polypeptide begins. It is only after elongation is in course and the polypeptide is long enough for the signal segment to emerge from the large ribosomal subunit, which normally encloses a 40-amino-acid segment of the nascent chain,\textsuperscript{80, 81} that the mechanism that leads to translocation begins to operate. This mechanism, illustrated schematically in Fig. 16-11, includes fail-safe features that ensure not only that the nascent polypeptide is inserted into the ER during its synthesis but also that, if insertion cannot take place, synthesis of the polypeptide is halted soon after the signal segment emerges from the ribosome.
Process of assembly of a membrane-bound polysome and the mechanism for the cotranslational translocation of a nascent polypeptide. An ordered series of molecular recognition events leads to the insertion of a nascent chain in the ER membrane. This involves an initial interaction of SRP with the ribosome (1) and with the emerging signal sequence (2 and 3), followed by binding of SRP to its receptor (4), which in turn leads to release of the S...

The process of targeting the ribosome to the membrane begins with the recognition of the emerging signal by a soluble macromolecular complex, the SRP, which consists of six distinct polypeptides and a small RNA molecule (7SL RNA) of approximately 300 nucleotides in length. The SRP interacts not only with the signal but also with the ribosome in such a way as to lead to a temporary block in polypeptide chain elongation. This block is relieved only in a subsequent step, when the SRP binds to its cognate receptor (SR), also known as the docking protein, which is an integral membrane protein exposed on the cytoplasmic surface of the ER. The pause in translocation caused by SRP ensures that continued growth in the cytoplasm and subsequent folding of the polypeptide, which could prevent insertion in the membrane, do not take place.

RER membranes also contain sites with high affinity for ribosomes, which may be regarded as ribosome receptors. Following docking of the SRP on the membrane, a firm attachment of the ribosome to its receptor takes place, which allows for the coupling of the processes of translation and membrane insertion. The exact sequence of events that occurs next is not known, but it is clear that binding of the SRP to its receptor displaces it from the signal and from the ribosome. The signal sequence must then
enter the membrane, where it is thought to interact with protein components of a translocation apparatus.\textsuperscript{96–100}

Since neither the SRP nor its receptor appear to remain associated with the membrane-bound ribosome at the site of translocation (the number of SRP receptors in the ER membrane is much smaller than the number of active bound ribosomes), the essential role of the SRP/SRP receptor system appears to be simply to target the ribosome and its incipient chain to the ER, without participating in the translocation process itself.

Although it is not yet fully understood how the polypeptide actually traverses the ER membrane, it is clear that an interaction of the nascent chain with membrane proteins is necessary for translocation to occur. Considerable evidence supports a model in which passage of the nascent polypeptide across the hydrophobic barrier within the membrane occurs through the aqueous environment of a proteinaceous channel.\textsuperscript{73, 79, 101–104} The role of the insertion signal would be to open the channel or to trigger its assembly from dispersed membrane protein subunits. The open or assembled state of the channel could be stabilized by the interaction of its components with the large ribosomal subunit, but the channel would be closed or disassembled when the ribosome is released following polypeptide chain termination, or a halt in translocation caused by a stop transfer signal (see below). In alternative models,\textsuperscript{105, 106} now not in favor (see below), following targeting to the membrane, the insertion signal and the nascent chain would interact directly with the membrane bilayer, and no specific membrane proteins would be required to mediate translocation itself.

It is clear that, in addition to proteins that may participate directly in the translocation process, several membrane enzymes are also located near the site of translocation and are able to interact with the growing chain to modify it as it emerges on the luminal side of the ER membrane. Thus, a signal peptidase cleaves off the signal, a protein oligosaccharyl transferase links preformed mannose-rich oligosaccharide chains to selected asparagine residues within the nascent polypeptide, and a protein disulfide isomerase (PDI) enzyme catalyzes the formation of intramolecular disulfide bonds. These modifications, however, are not required for translocation to occur.

**Experimental Analysis of Translocation\textsuperscript{107}**

The process of cotranslational insertion of nascent polypeptides into ER membranes has been best studied utilizing cell-free systems in which messenger RNAs obtained from natural sources, or produced by in vitro transcription of cloned cDNA templates, are translated in the presence of rough microsomes. The most commonly used translation systems are derived from rabbit reticulocytes or wheat germ, and the most frequently used rough microsomal membranes are obtained from dog pancreas.\textsuperscript{108}

When messenger RNA encoding secretory proteins are translated in the absence of membranes, primary translation products are obtained, which contain the signal sequences and are devoid of any modifications of their primary structure (Fig. 16-12Aa). Such artificial products of in vitro translation, which are generally not produced in vivo, are called presecretory proteins or preproteins. These are not true precursors of the secretory proteins, since, in vivo, their signals are actually removed before synthesis of the polypeptide is completed. When rough microsomal membranes are present during the in vitro translation, a large fraction of the product synthesized is translocated into the lumen of the microsomes and undergoes signal removal. If the translocated protein does not contain sites for N-linked glycosylation, its electrophoretic mobility is greater than that of the primary translation product (the presecretory protein) by the 2 to 3 kDa that corresponds to the cleaved signal (compare Fig. 16-12Aa and Ab). The sequestration of the processed polypeptides in the lumen of the microsomes is demonstrated by the fact that they are
protected from proteolysis when proteases are added to the reaction mixture after translation is completed (compare Fig. 16-12Ab and Ac). On the other hand, the presecretory proteins (pre-GH in Fig. 16-12Ab), which remain outside the microsomes, are completely digested (Fig. 16-12Ac). Destruction of the membranes by detergent solubilization, of course, leads to digestion of the translocated products by the exogenous proteases.

Experimental analysis of the translocation of in vitro synthesized polypeptides: demonstration of signal cleavage, sequestration in the microsomal lumen, and glycosylation. Messenger RNAs encoding pregrowth hormone (A) or the hemagglutinin (HA) of influenza virus (B) were translated in vitro in the absence (a) or presence (b and c) of dog pancreas microsomal membranes, using a cell-free system for protein synthesis derived from wheat germ. I...

When the messenger RNA utilized for in vitro translation experiments encodes a protein with sites for N glycosylation, translocation of the nascent chain is accompanied by both signal cleavage and addition of N-linked oligosaccharides (see below). In this case, the apparent size of the translocated product, when assessed by gel electrophoresis, may be higher than that of the primary translation product since the contribution of the added oligosaccharide chains may more than compensate for the size reduction resulting from signal cleavage (compare Fig. 16-12Ba and Ab). On treatment with proteases, only the glycosylated polypeptide remains undigested. The absence of the signal sequence in the translocated glycoproteins becomes apparent when, after dissolution of the microsomal membrane, they are treated with a glycosidase (endoglycosidase H) that removes the oligosaccharide chains (see below).

In vitro translocation experiments of the type just described, with mRNA that encode lysosomal enzymes (most of which are glycoproteins) or type I transmembrane proteins, have demonstrated that these polypeptides also contain transient N-terminal signals that mediate their cotranslational insertion into the ER.
Characterization of Insertion Signals\textsuperscript{75–78, 109–111}

Insertion signals are necessary to initiate the translocation of nascent polypeptides across the ER membrane. Secretory polypeptides from which the signal is deleted by modification of the corresponding cloned gene can no longer be translocated across ER membranes, in vivo or in vitro. Moreover, in some cases, attachment of a cleavable insertion signal to the N-terminus of a cytosolic protein has been shown to be sufficient to confer on it the capacity to be translocated. Although interaction of the signal with the membrane is necessary to initiate translocation, it is clear that covalent attachment of the signal to the rest of the polypeptide need not be maintained throughout translocation, since signal cleavage generally occurs much before elongation of the nascent chain is completed.\textsuperscript{73} However, it is possible that the signal could, even after it is severed from the body of the nascent chain, be necessary for translocation to continue. If this were the case, degradation of the cleaved signal segments by the yet to be discovered signal peptidase would occur only after translocation is completed.

Comparison of the amino acid sequences of different insertion signals shows that there is considerable variation in their primary structure. This suggests that general properties of the signals, including conformational features, rather than specific sequence information, are recognized by the various components of the translocation machinery (such as the SRP and the signal peptidase) that interact with the signal. Indeed many random sequences of the human genome that encode peptide segments of relatively high but varying degrees of hydrophobicity were shown to be capable of serving a signal function when linked to the yeast secretory protein invertase, from which the N-terminal signal was removed.\textsuperscript{112}

Insertion signals are usually 15 to 30 amino acids in length. Preprotein sequences are conventionally numbered so that the first residue after the cleavage site of the signal is designated +1 and the last residue of the signal is −1. Three segments can be recognized in all cleavable signals\textsuperscript{76} (Fig. 16-13): (1) a hydrophobic core (the h-region) 8 to 16 residues in length, which generally ends at residue −6, (2) a hydrophilic N-terminal segment that precedes the core (the n-region) and usually contains, in addition to the positively charged N-terminus, one basic amino acid, (3) an approximately five-residue-long C-terminal segment (the c-region), which defines the cleavage site and usually begins with a helix-breaking glycine or proline residue. In some signals, such as the one in rat growth hormone, the N-terminal segment bears no net charge due to the presence of a negatively charged amino acid residue.
Sequences of transient insertion signals of secretory proteins. The amino acid sequences of the transient insertion signals of numerous proteins are known, and general consensus features for the signals have become apparent. N-terminal segments of the sequences of three presecretory proteins are shown, and the point of cleavage by the signal peptidase is indicated in each case by a downward arrow. The amino acid residue within the signal adj...

The charges in the n-region of the signal are likely to play a role in initiating the association of the signal with the membrane that triggers the insertion of the nascent polypeptide. Deletion of the n-region from preproparathyroid hormone does not prevent the elongation arrest caused by SRP or its relief by the SRP receptor, but translocation of the nascent polypeptide is impaired. These observations are in accord with the notion that, as the signal begins to enter the membrane itself, the charges in the n-region associate directly with the polar head groups of the phospholipids. If this association is maintained as translocation proceeds, the nascent chain would adopt a loop disposition in the membrane (Fig. 16-14), with its N-terminus on the cytoplasmic face, the hydrophobic core of the signal within the membrane, and the cleavage site on the luminal surface. The formation of this loop would be facilitated by the helix-breaking nature of the residues immediately following the hydrophobic core.
Loop model for the disposition during translocation of the cleavable signal of a polypeptide synthesized in a membrane-bound ribosome. In this model, the extreme N-terminus of the signal remains on the cytoplasmic face of the membrane and the nascent chain has a looped disposition during the initial stages of translocation. In most secretory, lysosomal, and type I transmembrane proteins, the signal is N-terminal and is cleaved during translo...

Direct evidence for the loop model has been obtained from an analysis of the behavior of a genetically engineered protein expressed in transfected cells whose N-terminal cleavable insertion signal was extended by the addition of upstream sequences and whose cleavage site was abolished by mutation. Translocation of this protein proceeded normally but, since the signal was not removed, it served as a membrane anchor for the final product, which was a transmembrane protein with the N-terminal extension preceding the signal exposed on the cytoplasmic surface of the ER. The cytoplasmic exposure of the N-terminal extension implies that throughout the course of translocation the signal was maintained in the loop configuration.

From the analysis of numerous insertion signal sequences, certain rules have emerged that allow the prediction of the site of cleavage of the signal within the sequence of a preprotein with a fair degree of certainty. The $-1, -3$ rule states that the $-1$ position is almost always occupied by small neutral amino acids (such as alanine, glycine, or serine) and that the residue at $-3$ must not be aromatic (Phe, His, Tyr, Trp), charged (Asp, Glu, Lys, Arg), or large and polar (Asn, Gln). It is also apparent that residues following the cleavage site may contribute to its recognition by the signal peptidase. Thus, some point mutations, produced by genetic engineering techniques, that affect residues following the cleavage site have been shown to prevent cleavage. A role of the sequence following the cleavage site in determining signal cleavage may account for the fact that some secretory proteins, such as parathyroid hormone and albumin, contain a second transient N-terminal peptide segment, the propiece, that is removed from the proprotein during or after passage through the Golgi apparatus. In these instances, the N-terminal sequence of the mature protein, which may be important for the function of the protein, might not have permitted cleavage of the signal had it been immediately adjacent to the $-1$ residue. One function of the propiece could, therefore, be to satisfy the requirements for the creation of a signal peptidase cleavage site in the nascent preprotein.
The conformation that the signal segment attains within the interior of the membrane has not been established. Within a hydrophobic environment, an $\alpha$-helical conformation would be favored for the core region. However, the core is usually shorter than the approximately 20 amino acid residues that would be required for an $\alpha$ helix to completely span the membrane thickness of 2.5 to 3 nm, whereas in a fully extended configuration a peptide segment of only eight residues could span the membrane. Therefore, it has been suggested\footnote{110} that the hydrophobic core of the signal may exist within the membrane partially as an $\alpha$ helix and partially as a fully extended structure. The important role played by the hydrophobicity of the central core of the signal in translocation is apparent from the deleterious effects of mutations in bacterial secretory proteins replacing some of the hydrophobic residues by charged ones, or introducing partial deletions covering core residues.\footnote{119,120}

Even though insertion signals are usually removed by cleavage from nascent secretory and lysosomal polypeptides and from many nascent membrane proteins (see below), signal cleavage is not required for translocation. Indeed, one secretory protein, ovalbumin,\footnote{121,122} and several viral envelope glycoproteins\footnote{123–125} are known to contain signals near their N-termini that mediate translocation, but are not cleaved and are themselves transferred with adjacent portions of the polypeptide into the ER lumen.

**Signal Recognition Particle**\footnote{30, 31, 58, 59}

The SRP plays a central role in selecting ribosomes for binding to the ER and in delivering the nascent chains to a receptor within the membrane. The distribution of SRP within the cell reflects its cyclic participation in these processes. SRP may be found free in the cytoplasm, weakly bound to inactive ribosomes or attached to its receptor in the ER membranes\footnote{126} (see Fig. 16-11). The affinity of SRP for ribosomes, however, increases at least 6000 times when the ribosome contains a nascent chain with an exposed signal sequence, to which the SRP also binds.\footnote{84}

The most commonly used source of SRP for in vitro studies of its role in translocation is dog pancreas microsomes, from which SRP can be released by treatment with media with high salt concentrations. Indeed, microsomes treated with high levels of salt (KRM) are inactive in translocation unless supplemented with SRP.\footnote{82} The pause in translation caused by SRP is best observed when SRP is added to a wheat-germ translation system, which lacks endogenous SRP. In the absence of added microsomes, SRP leads to an effective block in the elongation of nascent proteins that contain a signal peptide, such as preprolactin, but the synthesis of cytosolic proteins, such as globin, proceeds unaffected.\footnote{86} In several cases, it has been shown that in the presence of SRP, a ribosome-associated arrested fragment of the preprotein of approximately 80 amino acids accumulates in the translation system. The SRP-mediated arrest of polypeptide chain elongation is relieved by the addition of microsomes to the system, which leads to signal cleavage and translocation,\footnote{85,86} or even by the addition of purified SRP receptor.\footnote{88}

The SRP obtained from dog pancreas microsomes by washing with a high-salt medium is a particle with a sedimentation efficient of 11S that contains, in addition to the 7SL RNA molecule, six polypeptide chains of molecular weights 9, 14, 19, 54, 68, and 72 kDa.\footnote{83,127} The 7SL RNA is an abundant and metabolically stable molecule that contains at its 5’ and 3’ ends sequences of the Alu family (one of the most highly repeated families of sequences in the genome) and in its middle region a core segment of 150 nucleotides, termed the S sequence, that is much less frequently repeated.\footnote{128} Both protein and RNA components of SRP have been shown to be required for its function. SRP has been disassembled into its RNA and protein components by the removal of Mg$^{2+}$ ions, which normally stabilize its structure, and it has been possible to reassemble a functional particle from the dissociated components.\footnote{129} This has allowed studies on the role of the individual proteins on the different aspects of SRP function.\footnote{130,131}
It is noteworthy that 7SL RNA from such evolutionarily distant species as *Drosophila melanogaster* and *Xenopus laevis* can replace the canine RNA in the reassembled particles. Reconstitution experiments have shown that the two smallest polypeptides of SRP are required for translational arrest but are not necessary for translocation, which, of course, demonstrates that the arrest in translation is not essential for translocation to occur. In fact, SRP may cause only a slowdown in the translation of certain mRNAs for which arrested peptides have not been detected in the wheat-germ system.

Treatment of SRP with nucleases generates two subparticles that may correspond to domains exerting the functions of SRP. One particle contains the two smallest polypeptides bound to the two ends of the 7SL RNA, and the other the four remaining ones bound to the central region of the RNA.

SRP can be purified by hydrophobic chromatography, which suggests that it interacts with the hydrophobic core of signal sequences. Indeed, replacement of leucine residues in a presecretory protein with β-hydroxyleucine, a polar analogue, abolishes the high-affinity binding of SRP for the ribosome, and hence the translational arrest and subsequent translocation. Moreover, it has been demonstrated that in a ribosome carrying SRP, the nascent chain is in close proximity to the 54-kDa polypeptide of SRP, since the two can be crosslinked through a photoactivatable group incorporated into the nascent chain. In this case, the elongation arrest was maintained after crosslinking and was relieved on binding to the SRP receptor, but translocation could not occur.

Much progress has been made in identifying within the SRP structural domains that carry out its three distinct sequential functions: signal sequence recognition, elongation arrest, and delivery of the nascent chain to the translocation machinery within the ER membrane. The two smaller SRP polypeptides (9 kDa and 14 kDa) form a heterodimer and are required for the elongation arrest to occur, whereas the two largest ones (68 kDa and 72 kDa), which also form a heterodimer, are required for the binding of SRP to its membrane receptor (SR). The 54-kDa polypeptide SRP54 is a GTP-binding protein that contains a putative GTPase segment. Its primary function is in the recognition of the insertion signal in the nascent polypeptide. The C-terminal portion of SRP54 constitutes a methionine-rich “M domain,” which by itself shows affinity for the signal sequence, although in the native protein this appears to be regulated by the N-terminal “G” domain.

It is noteworthy that genes encoding homologues of the SRP54 and of the α subunit of the SRP receptor have been identified in yeast and that, although their deletion leads to poor growth and to the accumulation of precursors of some secretory and membrane proteins in the cytosol, the cells, nevertheless, remain viable. This suggests that another mechanism, independent of SRP, can function in this unicellular eukaryote and effect the translocation of most essential proteins incorporated into the ER. Indeed, the posttranslational translocation of the α-mating factor precursor had previously been observed in yeast, both in vivo and in vitro. Evidence has also been presented that “molecular chaperones,” encoded by the yeast Ssa1 and Ssa2 genes, facilitate the SRP-independent uptake of the α-factor precursor into the ER. Molecular chaperones are members of a family of proteins that mediate the proper folding of other polypeptides and sometimes their assembly into oligomeric complexes. The chaperones involved in posttranslational translocation in the ER belong to the heat-shock hsp70 family of proteins and utilize ATP to confer on the polypeptide a conformation compatible with its transport across the membrane. These chaperones have also been shown (see below) to facilitate the uptake of polypeptides into mitochondria.
It should be noted that a yeast protein in the lumen of the ER (Kar2p) that can be crosslinked to the nascent chain\textsuperscript{142} is also necessary for translocation.\textsuperscript{143} Kar2p is the yeast homologue of the mammalian protein Bip (Grp 78), which is a heat-shock protein of the hsp70 family that serves as a molecular chaperone in the lumen of the ER and was first identified as an \textit{immunoglobulin heavy chain binding protein} that functions in the assembly of immunoglobulin molecules.\textsuperscript{144, 145}

**Signal Recognition Particle Receptor or Docking Protein\textsuperscript{30, 31, 59}**

The SR is a heterodimeric (SR\textsubscript{α}, 72 kDa; SR\textsubscript{β}, 30 kDa) protein complex exposed on the cytoplasmic surface of the ER membrane, where it receives the SRP bound to a ribosome containing an exposed signal sequence (see Fig. 16-11). This binding displaces the SRP from the ribosome and from the signal and allows the signal to insert into the membrane.\textsuperscript{95} The SR is present in the ER in low amounts (0.1 percent of the total membrane protein) and functions catalytically, remaining associated with SRP for only the very brief period required to displace it from the ribosome and to establish the ribosome-membrane junction. These reactions can occur at 0°C in the absence of any polypeptide chain elongation.\textsuperscript{95}

The SR has been purified from solubilized rough microsomal membranes by affinity chromatography to immobilized SRP, using the relief of the translation arrest of preprolactin as an assay to detect functional receptor.\textsuperscript{87, 88} The SR obtained in this manner consists of two subunits, a 69-kDa glycoprotein α subunit and a 30-kDa β subunit.\textsuperscript{146} Treatment of rough microsomes with the protease elastase renders the membrane inactive in translocation and leads to the release of a 52-kDa fragment of the α subunit, which can be added back to the proteolyzed membranes at low ionic strength to restore translocation competence.\textsuperscript{90, 91}

The complete primary structures of the SRP receptor subunits have been derived from the nucleotide sequence of cDNA clones.\textsuperscript{31, 147} Comparison of the sequence of the α subunit with the N-terminal sequence of the 52-kDa fragment released by proteolysis shows that the protein is anchored to the membrane via a 155-amino-acid N-terminal segment that contains two hydrophobic domains. The portion of the molecule exposed on the cytoplasmic surface contains three extremely hydrophilic regions rich in charged amino acids, with a predominance of basic residues that may interact directly with the 7SL RNA component of the SRP. This portion of the molecule also contains several additional hydrophobic segments that do not interact permanently with the membrane and are probably buried within the protein.

Both the α and β subunits of the SR are GTP-binding proteins and, surprisingly, SRα and the 54-kDa subunit of SRP are sufficiently related in sequence to constitute a new subfamily of GTPases. The exact roles of these three GTPases (SRP54, SRα, and SRβ) have not yet become clear, but most likely, a series of GTP-dependent changes in the conformational states of these proteins controls the sequential macromolecular interactions that confer directionality to the polypeptide targeting and insertion processes. Using a nonhydrolyzable GTP analogue (GppNHp) it has been established\textsuperscript{148} that binding of GTP, but not its hydrolysis, is required for the displacement of the signal sequence from the SRP and for the insertion of the nascent chain into the membrane that takes place after docking on the SR. On the other hand, GTP hydrolysis is required for the dissociation of SRP from the SR,\textsuperscript{149} which allows both components to function cyclically. Although it is not known how many and which bound GTP molecules are hydrolyzed in each round of targeting and nascent chain insertion, it has been shown that a mutation in the GTP-binding consensus sequence of the SRα subunit reduces the efficiency of the GTP-dependent insertion of the nascent chain into the membrane and prevents the formation of the stable SRP-SR complex that occurs in the presence of the GppNHp.\textsuperscript{150}
Several plausible models can be proposed for the concerted action of the SRP and SR GTP-binding proteins in the targeting and membrane insertion processes. One of these assumes (Fig. 16-15) that binding of ribosome-associated SRP to an emerging signal sequence leads to exchange of GDP for GTP in SRP54 and that in this “active conformation” SRP binds more tightly to the ribosome in a manner that arrests translation. In its active conformation GTP-containing SRP would also have a higher affinity for the SR, to which it binds through its 68- and 72-kDa subunits. Unoccupied SR has at least its α subunit (SRα) in the GDP-bound state and the docking of SRP promotes the exchange of GTP for GDP in this subunit which, in some way, leads to release of the signal sequence from SRP54 and detachment of SRP from the ribosome. These events would not be followed immediately by hydrolysis of the GTP bound to SRP54, or else SRP would rebind to the signal at this point. Hydrolysis of the GTP in the SRα subunit would follow, leading to the dissociation of the SRP-SR complex and release of SRP into the cytosol, where, after hydrolysis of its GTP, it would undergo another cycle of function. It has also been proposed that the GTPase activity of the SRβ subunit, and the accompanying conformational changes, regulate the association of the SR with other components of the translocation machinery in the membrane, to which the nascent chain is delivered after its release from SRP.

Hypothetical model for the role of GTP hydrolysis cycles within the GTP-binding subunits of SRP and the SRP receptor (SR) in the delivery of a ribosome bearing a nascent polypeptide with a signal sequence to the translocation apparatus in the ER membrane. This tentative scheme assumes that SRP with its 54-kDa subunit (SRP54) containing bound GDP binds to the ribosome, albeit weakly (1), and that binding
of the signal sequence that emerges fr...

**Interaction of the Signal Sequence and the Nascent Polypeptide with Membrane Protein Components**

After the displacement from the SRP, induced by the SRP receptor, the insertion signal and the nascent chain enter the ER membrane where they interact with protein components (see Fig. 16-11) of a putative translocation machinery, for which the term *translocon apparatus* has been proposed. An association of the nascent chain with membrane proteins was first demonstrated by the observation that partially translocated, incomplete, nascent polypeptides could be removed from the microsomal membrane by treatment with agents, such as urea, that perturb protein-protein interactions but do not remove integral membrane proteins from membranes. Attempts to identify components of the translocation apparatus in the ER membrane have employed crosslinking agents to link a radioactive nascent chain to dog pancreas microsome membrane proteins that are in its close proximity when it traverses the membrane. A 35- to 39-kDa glycoprotein, first thought to be a signal sequence receptor and termed SSRα, was identified in this manner. However, it was later shown that this protein, also termed mp39, can be crosslinked to various other portions of the translocating polypeptide and is therefore unlikely to serve only as a signal sequence receptor. Moreover, although SSRα/mp39 clearly resides in the neighborhood of the site of passage of the nascent chain throughout the course of elongation, this protein does not appear to be necessary for translocation, since translocation-competent microsomes can be reconstituted with detergent extracts from which it was removed. Other microsomal polypeptides have also been crosslinked to nascent chains, including a 34-kDa (imp34) nonglycosylated protein and a 39-kDa multispansing membrane glycoprotein, termed translocating chain-associating membrane glycoprotein (TRAM), that appears to be required for the translocation activity of reconstituted vesicles. TRAM could only be crosslinked to short nascent polypeptide chains, which indicates that it is near the nascent chain only at the beginning of its insertion and that cleavage of the signal sequence may displace it from the passageway in the membrane.

Several translocation-deficient yeast mutants have been identified in genes that encode transmembrane glycoproteins (Sec61p, Sec62p, Sec63p) that are part of a multisubunit complex of the type expected to function in translocation. Of these, Sec61p and Sec62p could be crosslinked to nascent chains, the latter only when the nascent chains are short. The mammalian homologue of Sec61p (40 kDa) has been purified and its sequence, derived from the cloning of its cDNA, reveals that it is likely to have 10 transmembrane domains that contain a number of hydrophobic and charged amino acid residues. Sec61p is the major membrane component that can be crosslinked to long nascent chains in both mammalian and yeast cells. This protein is also homologous to the SecYp product of *Escherichia coli* that, together with SecE, are the only two integral membrane proteins required to effect translocation in a system of reconstituted liposomes. Moreover, Sec61p becomes tightly associated with ribosomes during the course of translocation. Taken together, these findings raise the strong possibility that Sec61p represents a major constituent of the channel through which the nascent chain traverses the membrane.

Further characterization of Sec61p, Sec62p, and Sec63p and other recently identified SEC gene products (Sec70p, Sec71p, Sec72p) whose mutations affect translocation and/or membrane protein integration, and of their mammalian homologues, may soon yield a more complete picture of the molecular assembly that constitutes the translocation apparatus in the ER membrane.
The Signal Peptidase

The signal peptidase activity of microsomes can be demonstrated in detergent solubilized preparations,\textsuperscript{157} using as substrates certain completed preproteins synthesized in vitro, such as preprolactin and pregrowth hormone. Most preproteins, however, cannot be processed posttranslationally by microsomal extracts, presumably because they are folded in such a way as to sequester the signal cleavage site. This sequestration of the signal may occur before synthesis of the preprotein is completed, and the incapacity of the masked signals to interact with SRP when this is added late in translation would account for the fact that, beyond a certain length, nascent polypeptides are no longer “translocation-competent.”\textsuperscript{158}

The solubilized signal peptidase is active only in the presence of phospholipids,\textsuperscript{159} and its activity can be inhibited by agents such as chymostatin that inhibit zinc metallopeptidases.\textsuperscript{160} Because the peptidase activity cannot be demonstrated without detergent solubilization, and it is not destroyed by proteolysis of intact microsomes,\textsuperscript{157, 161} it can be concluded that the active site of the signal peptidase is located on the luminal side of the ER membrane (see Fig. 16-11). This location is consistent with the observation that signal cleavage does not take place before the polypeptide attains a minimal length of 70 to 90 residues, which are required to bring the cleavage site to the luminal face of the ER.

A protein complex with signal peptidase activity has been purified from solubilized dog pancreas microsomes.\textsuperscript{162} It contains five polypeptide chains of apparent molecular weights ranging from 12 to 25 kDa, one of which is glycosylated. It is likely that only one of these polypeptides carries out the signal cleavage and that the remaining ones participate in other aspects of the translocation process, such as the degradation of the cleaved signal peptide (signal peptide peptidase) or cotranslational modifications of the nascent chain. One or more of the polypeptides in the complex may be part of the channel in the membrane through which translocation is likely to take place. It is noteworthy that neither the SSRα (mp39) nor proteins that have been implicated in ribosome binding (see below) are part of the signal peptidase complex. Two of the protein subunits of the mammalian signal peptidase display substantial sequence homology to the yeast sec11 protein,\textsuperscript{163} which is also part of a complex with signal peptidase activity\textsuperscript{164} and when mutated leads to defective signal cleavage in vivo.\textsuperscript{165}

Ribosome Binding Sites on the ER Membrane

After displacement of the SRP by its receptor, binding of the ribosome to the ER membrane takes place (see Fig. 16-11). During the subsequent translocation, the ribosome remains associated with the ER membrane via two types of bonds—one directly linking the large ribosomal subunit and a receptor in the membrane and an indirect link that is provided by the nascent polypeptide chain.\textsuperscript{166} The latter is broken on termination of polypeptide growth, or when a prematurely terminated polypeptide is released from the ribosome as a result of the incorporation at the C-terminus of the nascent chain of the chain-terminating peptidyl-tRNA analogue puromycin. Ribosomal subunits can then be detached from the membrane by exposure of the microsomes to mediums of high ionic strength, which disrupt electrostatic interactions between the large ribosomal subunit and its receptor. Ribosomes not containing nascent chains rebind in vitro and at low ionic strengths to rough microsomal membranes stripped of ribosomes but not to other cellular membranes, including those of smooth microsomes.\textsuperscript{94, 167, 168} The number of ribosome binding sites detected by this method is equivalent to the number of ribosomes originally present in the rough microsomes.\textsuperscript{169}

The ribosome receptors present in the rough ER contain proteinaceous components, since ribosome binding is abolished by mild proteolysis or heat treatment of the membranes.\textsuperscript{94} The specific polypeptides involved in ribosome binding, however, have not been definitively identified. Two transmembrane
glycoproteins, ribophorins I and II, are present only in rough microsomes, are found in amounts stoichiometrically related to the number of ribosomes, and appear to be associated with the binding sites. These proteins, and only a few other membrane polypeptides, are recovered with the ribosomes when they are sedimented after certain nonionic detergents are used to solubilize the membranes. The proteins recovered in the membrane residue appear to form a two-dimensional network bearing ribosomes. On this basis, it has been proposed that ribophorins also play a structural role in the rough ER, providing a scaffolding within the ER membrane that restricts the ribosome binding sites and their associated translocation apparatus to the rough domains and confers on the rough cisternae their characteristic morphology. The cDNAs for both ribophorins have been cloned. The primary structure of the polypeptides indicates that both proteins are type I monotopic proteins that cross the membrane only once, and have C-terminal segments of 150 and 70 amino acids, respectively, exposed on the cytoplasmic face of the membrane. It now seems clear that proteins other than the ribophorins must contribute to the ribosome binding sites in the ER membrane, since mild proteolysis that does not appear to cleave the cytoplasmically exposed portions of the ribophorins abolishes the ribosome binding capacity of the membrane. Moreover, it has been reported that membrane vesicles that are capable of efficient ribosome binding can be reconstituted from purified lipids and a microsomal protein fraction from which all glycoproteins (including ribophorins) were removed by lectin chromatography. However, a function of the ribophorins associated with translocation has been suggested by the finding that a protein complex consisting of both ribophorins and a 48-kDa polypeptide isolated from microsomal membranes manifests oligosaccharyl transferase activity, that is, it catalyzes the transfer of a high-mannose oligosaccharide from a dolichol pyrophosphate donor to asparagine residues in the peptide sequence Asn-X-Ser/Thr. This process (see below) normally occurs as the nascent glycoprotein chain emerges on the luminal side of the ER membrane.

Two other candidate proteins for ribosome receptors, a 34-kDa and a 180-kDa protein, have also been identified, but definitive evidence for their ribosome binding roles is yet to come.

**Protein-Conducting Channel in the ER Membrane**

It was originally proposed that a transient or permanent aqueous channel through the ER membrane, just under the ribosome, provides a passageway for the nascent polypeptide into the ER lumen. It seems likely that at least some of the ER membrane proteins that have been crosslinked to the nascent polypeptide chain are part of such a channel. Electrophysiological studies of the properties of microsomal membranes fused to planar lipid bilayers have, in fact, demonstrated the existence of large ion-conducting channels in the ER membrane, which appear to be occupied, and therefore occluded, by the nascent polypeptide chain. The conductance of the membrane increased dramatically on addition of puromycin, a drug that releases the nascent chain. The channel also appeared to be stabilized by the bound ribosomes. The conductance was markedly reduced when the ribosomes were detached from the membrane after nascent chain release. Similar electrophysiological studies with *E. coli* plasma membranes—through which translocation in vivo can occur cotranslationally, as well as posttranslationally (i.e., without ribosome binding)—have demonstrated the existence of channels with a conductance comparable to that of those in the mammalian ER in these membranes. In this case, opening (or assembly) of the channels could be triggered by addition of a synthetic signal peptide that appears to function as a physiological ligand for channel opening. These exciting findings provide hope that the full set of molecular components that constitute the prokaryotic and eukaryotic protein conducting channels will be identified within the next few years. The molecular interactions that control channel function may begin to be understood soon thereafter.
Biosynthesis of Membrane Proteins: Role of Insertion and Halt Transfer Signals in Determining Their Transmembrane Disposition

All membrane glycoproteins and transmembrane proteins that are not contained within mitochondria or peroxisomes are synthesized in ribosomes bound to the RER and are cotranslationally inserted into the ER membrane.

Type I membrane proteins (see Fig. 16-4), which cross the membrane only once and contain their N-termini exposed on the extracellular or luminal surface of the membrane, such as the low-density-lipoprotein (LDL) and EGF receptors, the heavy chain of the histocompatibility antigen, and the much-studied hemagglutinin and G envelope glycoproteins of the influenza and vesicular stomatitis viruses, respectively, are generally synthesized with transient N-terminal insertion signals that are totally equivalent to those in secretory proteins (Fig. 16-16a, b, and c). These signals, via their interaction with SRP, initiate passage of the polypeptides across the membrane and their cleavage results in generation of new N-termini exposed in the lumen of the ER. In contrast to secretory proteins (Fig. 16-16d), translocation of a type I membrane polypeptide is interrupted by a highly hydrophobic segment, the halt- or stop-transfer signal. This sequence constitutes the sole transmembrane segment or membrane anchoring domain in the mature protein (Fig. 16-16e). The relative lengths of the luminal and cytoplasmic domains of type I polypeptides are determined by the position of the halt-transfer signal within the polypeptide. In rare instances, such as in the envelope glycoproteins of Sindbis and SFV virus, type I proteins contain uncleaved N-terminal insertion signals which, after initiating translocation, are themselves translocated and form part of the luminal domain of the mature protein.
The transmembrane disposition of a type I membrane polypeptide is established by the sequential action of a cleavable N-terminal insertion signal and an interior halt-transfer signal. The early stages of insertion of a type I membrane protein into the ER membrane (a, b, and c) are identical to those of a secretory protein. After signal cleavage takes place, translocation continues until a halt-transfer signal in the interior of the polypepti...

Segments that serve as halt-transfer signals consist of approximately 20 hydrophobic or uncharged amino acids, usually followed by several basic residues (Fig. 16-17). The halt-transfer signal must allow for the reutilization of the components of the translocation apparatus in other rounds of translocation. Therefore, if cotranslational translocation occurs through a protein channel, the halt-transfer signal must open this channel laterally or disassemble it to allow for the hydrophobic segment in the signal to become directly associated with the membrane phospholipids and serve as a membrane anchor. In many instances, the halt-transfer segment is located so close to the C-terminus that it must enter the membrane only after synthesis of the polypeptide has been completed and the chain released from the ribosome. In other cases, however, the halt-transfer signal is distant from the C-terminus, and elongation must continue in the cytoplasm after translocation has been halted. It is possible that in these instances the ribosome is
dislodged from the membrane by the growing polypeptide chain.

Segments that contain the halt-transfer signal-membrane anchoring domains of type I membrane proteins. The regions of three type I membrane proteins that contain the hydrophobic segments that traverse the phospholipid bilayer in the membrane are presented. The exact borders of the transmembrane domains of these polypeptides have not been determined. Hydrophobic residues are marked with a circle, basic ones with a rectangle, and acidic residu...

The fact that the presence of the halt-transfer segment is the only feature of type I membrane proteins that distinguishes them from secretory proteins is strikingly demonstrated during B lymphocyte maturation, when the cells shift from the production of a membrane-bound form of IgM to a secretory form with the same antigen specificity.181, 182 This shift simply involves a modification in the processing of the RNA-transcript (primary transcript) from which the µ heavy chain messenger RNA is generated.183 In pre-B cells, in which the immunoglobulin heavy chain (µm) is membrane-bound, the extreme 3' portion of the coding region of the mRNA encodes the transmembrane and cytoplasmic domains of the polypeptide. At later stages, a different messenger RNA is generated from the same primary transcript. This mRNA encodes a polypeptide (µs) that does not contain the membrane-anchoring or cytoplasmic domains of µm but contains instead a short segment that includes the cysteine residue that, within the pentameric secreted IgM, forms a disulfide bond with the immunoglobulin J chain.
The exclusive role of the hydrophobic halt-transfer signal in maintaining the association of type I proteins with the membrane has been demonstrated experimentally by using genetic engineering techniques to convert membrane proteins into secretory proteins by deleting the region encoding the halt-transfer signal from the mRNA. Moreover, in reciprocal experiments, secretory proteins have been converted into type I membrane proteins simply by introducing in them a halt-transfer signal (e.g., ref. 186). Other genetic engineering experiments have shown that 12 to 16 consecutive hydrophobic residues suffice to maintain the anchoring of a type I protein to the membrane and that the charges that follow the hydrophobic segment are not essential for this purpose.

The mechanism just described cannot, of course, account for the transmembrane disposition of type II membrane proteins, such as the asialoglycoprotein and transferrin receptors, the sucrase-isomaltase of the intestinal brush border, and the neuraminidase of influenza virus. These proteins have their C-termini exposed on the outer surface of the membrane and the N-termini on the cytoplasmic side (see Fig. 16-4). The biogenetic origin of the transmembrane disposition of these proteins can be understood, however, in the context of the loop model for the configuration of the signal and the nascent chain during translocation, as depicted in Fig. 16-18. In all these cases, the cotranslational insertion is initiated by a signal within the polypeptide that serves to mediate the translocation of its downstream portions but does not undergo cleavage and remains membrane-associated as the anchoring domain of the mature protein. A signal with these properties is frequently termed a signal/anchor sequence (S/A) type II. The N-terminal portion of the polypeptide that precedes the signal, therefore, remains exposed on the cytoplasmic surface of the membrane. It should be clear that the process of insertion of a type II protein would be identical in all respects to that of a secretory protein, except that the signal does not undergo cleavage and is sufficiently hydrophobic to remain membrane-anchored after polypeptide chain termination. When the interior insertion signal-membrane anchoring domain of a type II protein is located at some distance from the N-terminal end, it is, of course, necessary that folding of the N-terminal portion does not mask the signal and prevent its interaction with SRP.

The transmembrane disposition of a type II membrane protein results from the action of a permanent insertion signal that remains as a membrane anchor in the mature protein. The permanent insertion signal
that initiates translocation of type II membrane proteins may be near the N-terminus or, as shown in the figure, in the interior of the polypeptide. Insertion begins only after the signal emerges from the ribosome and, therefore, the portion...

Membrane-anchoring domains of type I and type II proteins differ, therefore, not only in their N-to-C orientation within the membrane, but also in that those in type I proteins enter the membrane as part of a translocating polypeptide and serve to arrest translocation, whereas those in type II proteins initiate the membrane insertion process and promote the translocation of following portions of the nascent polypeptide chain.

It should be apparent that a series of alternating insertion and halt-transfer signals within a single polypeptide chain, functioning in succession, could explain the transmembrane disposition of many type III (polytopic) proteins (Fig. 16-19). In these cases, the location of the N-terminus would be determined by whether the first signal is N-terminal and transient (Fig. 16-19A) or internal and permanent (Fig. 16-19B), and the location of the C-terminus by whether the last signal is an insertion (Fig. 16-19A) or a halt-transfer signal (Fig. 16-19B). It must be emphasized, however, that the two types of transmembrane domains could be, and frequently are, very similar in their primary structure, which is what determines the stability of their interaction with the phospholipid bilayer. Whether or not a sufficiently hydrophobic segment in a polypeptide functions as an insertion or as a halt-transfer signal could therefore depend on its relative position with respect to other signals within the primary translation product, since this determines how the segment is presented to the membrane. If preceded by an insertion signal, cleaved or uncleaved, the hydrophobic segment would enter the membrane directly as it exits the ribosome and, therefore, would halt transfer of a translocating polypeptide. If, on the other hand, the segment is preceded by a halt-transfer signal, it would emerge from the ribosome into the cytoplasm and behave as an insertion signal, promoting the translocation of downstream sequences. The insertion of this type of insertion signal, which emerges from a ribosome already targeted to the membrane, can, however, take place without the requirement for SRP, which highlights that the primary function of SRP is in the initial targeting of a ribosome bearing an appropriate nascent chain to the membrane. It should be noted, however, that the hydrophobicity of a typical cleavable insertion signal, such as that in the hemagglutinin of influenza, may not be sufficient to halt transfer when the signal is paced in the interior of the translocating polypeptide.
The transmembrane disposition of proteins that traverse the membrane several times can result from the sequential action of a series of alternating insertion and halt-transfer signals. It is presumed that interior insertion signals can reinitiate translocation after this has been interrupted by the action of a preceding halt-transfer signal. Consequently, a series of insertion and halt-transfer signals leads to multiple crossings of the membrane...

**Combined Insertion Halt-Transfer Signals in Membrane Proteins**

Several membrane proteins have been characterized (such as rhodopsin,\(^{202}\) the evolutionarily related \(\beta_2\)-adrenergic receptor,\(^{203, 204}\) and the M2 protein encoded in the influenza virus genome\(^{205, 206}\)) that have glycosylated N-terminal segments located on the luminal side of the membrane and yet do not undergo signal removal during their membrane insertion. In the case of rhodopsin, which is a polytopic protein, it has been shown that the first transmembrane segment, which begins 35 residues from the N-terminus, has the capacity to initiate the insertion of the nascent polypeptide into the membrane.\(^{207, 208}\) Although this segment can, therefore, be regarded as an insertion signal, it is clear from the disposition of the polypeptide with respect to the membrane that it must also become a membrane anchor and act to halt translocation of following polypeptide sequences while, paradoxically, promoting the translocation of the preceding portion into the ER lumen, which in fact becomes glycosylated. The first transmembrane domain of rhodopsin is, therefore, a combined insertion-halt-transfer signal whose final orientation in the membrane is that characteristic of the regular halt-transfer signals found in type I monotypic proteins—that is, with the N-terminus in the luminal surface of the membrane. Signals of this type are frequently referred to as signal/anchor sequences, type I (S/A type I).
If, as it may be reasonable to assume, the signal in rhodopsin enters the membrane in a loop configuration, which would place the N-terminal portion of the protein in the cytoplasm, then the signal must later reorient to effect the transfer of the preceding peptide segment across the membrane, where its glycosylation occurs. This would lead to the dissipation of the loop and, hence, halt the translocation of amino acids following the signal, which consequently remain on the cytoplasmic side of the membrane (Fig. 16-20A). Alternatively, the S/A type I signal may never form a loop within the membrane but on insertion may orient itself in a way that leads to translocation of preceding sequences (Fig. 16-20B).
Fig. 16-20:

A

B

C
How a combined insertion-halt transfer signal (signal/anchor I) may lead to a type I transmembrane disposition. A. The signal is assumed to enter the membrane in the “normal” loop configuration, but because of the absence of positive charges in the N-flanking segment and their presence in the C-flanking segment, it reorients within the membrane to translocate preceding sequences. The dissipation of the loop halts translo...

A comparison of the sequences of proteins containing S/A types I and II signals\textsuperscript{208} and extensive mutagenesis studies indicate that an important determinant of the behavior of a signal anchor in determining the type II or I disposition is the presence of positive charges in the N-flanking segment of the signal. A prevalence of positive charges tends to retain the N-terminus on the cytoplasmic side of the membrane, and therefore confers to the protein the type II disposition (Fig. 16-20C). On the other hand, a prevalence of positive charges on the C-flanking segment of the signal tends to retain that portion of the polypeptide on the cytoplasmic side of the membrane, leading to the type I disposition. Of course, in the case of rhodopsin the other membrane crossings would require the action of a succession of following insertion and halt-transfer signals.

A very-well-characterized S/A type I is that present in the M2 protein encoded by influenza virus, which crosses the membrane only once with a type I transmembrane disposition. This protein lacks a cleavable signal but has a 19-amino-acid hydrophobic sequence preceded by a 24-amino-acid N-terminal ectodomain.\textsuperscript{205, 206} Replacement of the N-terminal portion of a type II integral membrane protein, comprising its type II signal/anchor, with the N-terminal portion of the M2 protein, comprising its hydrophobic S/A type I and preceding segment, conferred to the chimeric protein the type I disposition.\textsuperscript{209}

Signals with properties similar to those in the M2 protein and in the first transmembrane segment of rhodopsin are also present in several proteins of the ER membrane, such as cytochrome P450\textsuperscript{210, 211} and its NADPH-dependent reductase,\textsuperscript{212} which are synthesized in membrane-bound ribosomes and have uncleaved hydrophobic N-terminal segments, but remain almost completely exposed on the cytoplasmic surface of the ER. In the case of cytochrome P450, the insertion-halt-transfer function (S/A type I) of a segment in the N-terminal portion of this protein has been directly demonstrated by genetic engineering experiments. When linked to the N-terminus of a secretory protein, such as growth hormone, this segment confers on the latter the same disposition with respect to the membrane as that of P450 itself.\textsuperscript{210, 211, 213, 214}

**Cotranslational Modifications of Polypeptides Synthesized in the ER\textsuperscript{215–219}**

Glycoproteins are characterized by the presence of oligosaccharide chains that are linked either to nitrogen atoms of asparagine residues or to oxygen atoms of serines and threonines. In collagen, hydroxylsine residues may also bear O-linked sugars. The attachment of N-linked oligosaccharide chains to proteins takes place during the course of polypeptide chain elongation, while the nascent chains are still traversing the ER membrane (see Fig. 16-11). In this reaction, a preformed oligosaccharide (Glc3Man9GlcNAc2) (Fig. 16-21) is transferred by an oligosaccharyltransferase from a membrane-bound dolicholpyrophosphate lipid carrier to asparagine residues in the polypeptide backbone. The active site of this enzyme is located on the luminal face of the ER membrane and recognizes asparagine residues within the triplet sequence Asn-X-Ser/Thr, where X can be any amino acid, except proline or aspartic acid.\textsuperscript{220, 221} As previously mentioned, oligosaccharyltransferase activity has been found to be associated with a protein complex that contains the two ribophorins and a 48-kDa polypeptide.\textsuperscript{176} It was noted that the transmembrane segment of ribophorin I contains a sequence that resembles a proposed recognition sequence for the dolichol moiety of the oligosaccharide donor.
structure of the lipid-linked oligosaccharide precursor transferred en bloc to nascent glycoproteins. The lipid carrier dolichol pyrophosphate consists of 22 5-carbon isoprene units that are embedded in the ER membrane. After the sugars are added to the lipid carrier, by a series of reactions that are mentioned in the text, the oligosaccharide is transferred en bloc to the nascent polypeptide chain. In this process, the N-acetylglucosamine (...)

The process by which the oligosaccharide is assembled on the dolichol pyrophosphate carrier involves enzymatic reactions that take place on both sides of the membrane. The first seven sugar residues (two GlcNAc and five Man) are transferred directly in a stepwise fashion from the nucleotide sugars UDP-GlcNAc and GDPMan to the membrane-bound dolichol phosphate, which has its acceptor site
exposed on the cytoplasmic face of the membrane. It is believed that the resulting intermediate (Man$_5$GlcNAc$_2$-PPDol) is then reoriented so that its acceptor site is exposed on the luminal side of the membrane where it receives the remaining four mannose and three glucose residues from dolichol phosphate sugars that are formed on the cytoplasmic side but are capable of flipping across the membrane. The synthesis of the oligosaccharide chain on the lipid carrier can be inhibited by the drug tunicamycin, which thereby completely prevents the addition of oligosaccharides to asparagine residues.

Only about 30 percent of all the potential asparagine acceptor sites in a polypeptide actually carry oligosaccharide chains. This may reflect the fact that glycosylation can take place only within a narrow window of time during polypeptide chain elongation, after the acceptor asparagine reaches the luminal side of the membrane but before folding of the polypeptide may mask it. In addition, certain asparagine-containing triplet sequences are better acceptors than others, depending on the X residue and the sequences flanking the triplet. Thus, the extent of glycosylation that can be achieved in this narrow window may vary considerably for different potential acceptor sequences.

Soon after its transfer to the polypeptide, and even before the polypeptide is completed, the oligosaccharide chain begins to undergo a trimming process in which the three glucose residues and one or two mannoses are removed (Fig. 16-22). These reactions are catalyzed by $\alpha$1-2 and $\alpha$1-3 glucosidases (glucosidases I and II) and by an $\alpha$1-2 mannosidase, respectively, that are located in the ER membrane and are likely to be closely associated with the translocation apparatus. Oligosaccharides in glycoproteins that reside permanently in the ER, such as the ribophorins or HMG-CoA reductase, or traverse it slowly on their way to the Golgi apparatus, may lose two additional mannose residues in the ER. Oligosaccharides in proteins that are transferred to the Golgi apparatus usually undergo further trimming of mannose residues and the addition of terminal sugars in that organelle.
Cotranslational transfer of an N-linked oligosaccharide to a nascent polypeptide and initial trimming in the ER. The oligosaccharyltransferase recognizes the triplet ASN-X-SER in the nascent chain and transfers the high-mannose oligosaccharide from the lipid donor to the asparagine residue. While the polypeptide chain is still growing, α-glucosidases and an α-mannosidase catalyze the sequential removal of the three glucose and ...

As a consequence of this biosynthetic pathway, which allows for different degrees of processing of individual oligosaccharides by the ER and Golgi enzymatic enzymes, N-linked oligosaccharide chains in the mature glycoproteins fall into three groups (Fig. 16-23). These all share a common pentasaccharide core structure,$^{217}$ Man-3(Man-6)Manβ1-4GlcNAcβ1-4GlcNAc-Asn, which reflects their derivation from the same high-mannose precursor. One group, the high-mannose oligosaccharides, consists of oligosaccharides that generally retain two to six mannose residues linked to the pentasaccharide core. Oligosaccharides in glycoproteins that are permanent residents of the ER belong to this group. A second group consists of complex oligosaccharides, in which the core is generally followed by two to four branches that most frequently consist of sialyllactosamine (SA2-3 or 6Galβ1-4GlcNAc) sequences linked to the two outer mannoses of the core. In addition, in complex oligosaccharides, a “bisecting” GlcNAc residue may be bound to the first mannose in the core and a fucose may be linked to the innermost GlcNAc. The outer branches in complex oligosaccharides can vary significantly in length, and in many cases contain fucose residues and polysialic acid chains. A third group of oligosaccharides consists of hybrid structures in which one branch retains some of the outer mannose residues characteristic of the high-mannose chains and other branches resemble those in complex oligosaccharides. In hybrid oligosaccharides, the bisecting GlcNAc may also be attached to the first mannose.
Structures of representative high-mannose, complex, and hybrid oligosaccharides that are N-linked to glycoproteins: a common pentasaccharide core is present in all types of chains. The core pentasaccharide structure that is enclosed in the rectangles is present in all N-linked oligosaccharide chains.

High-mannose oligosaccharides in glycoproteins may have from five to nine mannose units and result from the removal of the glucose residues, as...

High-mannose, but not complex or hybrid, oligosaccharide chains can be removed from glycoproteins by treatment with the enzyme endoglycosidase H,\(^{226, 227}\) which cleaves the core between the two GlcNAc residues. Another endoglycosidase, endo D,\(^{228, 229}\) hydrolyzes the same bond only when the Man residue in the core that is linked by an \(\alpha 1–3\) bond to the first mannose is unsubstituted in the 2 position, that is, when the chain has been processed to the Man\(_5\)GlcNAc\(_2\) species. However, all N-linked oligosaccharides can be removed by treatment with endoglycosidase F.\(^{230}\) An increase in the electrophoretic mobility of a protein caused by treatment with any one of these enzymes demonstrates the presence of oligosaccharide chains. The acquisition of endo D sensitivity can be used as a criterion to establish the transfer of a newly synthesized glycoprotein from the ER to the cis Golgi, where the \(\alpha 1–2\) manniosidase I that reduces the number of mannoses to 5 is located. The acquisition of endo H resistance indicates that further progress of the protein through the Golgi apparatus has taken place to sites where the terminal sugars characteristic of complex oligosaccharides are added.

**Retention of Resident Proteins in the Endoplasmic Reticulum**\(^{231, 232}\)

Whereas most proteins synthesized in membrane-bound ribosomes are transferred to the Golgi apparatus for incorporation into this organelle or for distribution to other sites of the cell, others remain as permanent residents of the ER, where they carry out their functions either as membrane or luminal proteins. The notion was originally suggested,\(^{12}\) because of its parsimony, that retention of these proteins in the ER is an active process that results from the presence of specific signals (retention signals) within the polypeptides that lead them to interact with other components of the ER and prevent them from flowing.
into the vesicles that carry proteins to the Golgi apparatus. It has long been known (reviewed in ref. 233) that in rodent liver the enzyme β-glucuronidase has a dual localization—being present in lysosomes as well as a peripheral membrane protein in the lumen of the ER—and that a microsomal glycoprotein, egasyn, is responsible for retaining β-glucuronidase in the ER, since the latter is not detected in microsomes from egasyn-deficient mutant animals.234–236 Moreover, in vivo dissociation of the egasyn-β-glucuronidase complex, induced by organophosphorous compounds, leads to massive and rapid secretion of β-glucuronidase into the plasma.235 It can, therefore, be concluded that the microsomal β-glucuronidase has a retention signal that mediates its interaction with egasyn. Of course, this raises the question of how egasyn itself is retained in the ER.

The fact that specific information within a protein is not required for it to be exported from the ER and, therefore, proteins that lack retention signals exit from the ER by default is strikingly demonstrated by the observation that β-lactamase, a protein of bacterial origin that could not possibly contain information for passage out of the ER, is secreted when it is synthesized in amphibian oocytes that are microinjected with the corresponding mRNA.237

In recent years much evidence has accumulated demonstrating that many luminal proteins of the ER, such as protein disulfide isomerase, microsomal esterases, reticulin and two glucose-regulated proteins (Grp74 and Grp 78 or Bip), and some membrane proteins, are actively concentrated in this organelle by a mechanism that involves a retrieving receptor that, in one or more post-ER compartments, recognizes a signal in the escaped ER proteins and returns them to their site of primary residence. This retention signal consists of a C-terminal tetrapeptide sequence (Lys-Asp-Glu-Leu; KDEL; or a closely related one) that is both necessary and sufficient to cause retention of the protein in the ER, and for which a recycling receptor has been identified.238–240 For several luminal proteins it has been shown that deletion of the tetrapeptide signal leads to their secretion,241, 242 whereas the addition of the signal to the C-terminus of a secretory protein (lysozyme) leads to its retention in the ER.242

The first evidence that proteins containing a KDEL sequence continuously escape from the ER to a salvage compartment, which may be an intermediate compartment between the ER and the Golgi apparatus, or the cis region of the Golgi apparatus itself (see below), but are returned to the ER, was obtained from the behavior of a chimeric protein consisting of a lysosomal enzyme, cathepsin D, to which a KDEL signal was added.243 Immunofluorescence studies showed that this protein accumulated in the ER but, nevertheless, it was modified by the enzyme N-acetylglucosamine-1-phosphotransferase, which participates in the synthesis of the lysosomal targeting marker, mannose 6-phosphate, and is believed to be located in the cis Golgi (see below). Further evidence that resident ER luminal proteins escape from the ER and are retrieved was obtained in yeast. A chimeric protein containing an HDEL C-terminal tetrapeptide (which for certain strains of yeast is functionally equivalent to the mammalian KDEL) also underwent a Golgi-specific glycosylation reaction. However, it did so only if the cells did not carry mutations in the secretory machinery (a sec mutation) that made them defective in transport of proteins out of the ER under the experimental conditions used.244 An elegant selection procedure was devised to identify mutants deficient in the ER retention mechanism.238 This allowed the cloning of the yeast HDEL receptor gene (ERD2).238 This in turn led to the subsequent cloning of the cDNA for its human (KDEL binding) counterpart (hERD2).245 Both the yeast and the mammalian receptors were found to reside primarily in the Golgi apparatus normally,238–245 as would be expected if the receptors have a “retrieving” rather than a direct “retaining” function. Even more convincing evidence for the recycling function of the receptor came from the demonstration that, in mammalian cells, overexpression of proteins containing the KDEL signal leads to redistribution of the receptor molecules to the ER.240 This redistribution did not occur when the receptor protein was modified to reduce its affinity for the signal. Interestingly, a C-terminal, luminally exposed, HDEL signal that mediates retention in the ER has also been identified in a
yeast transmembrane ER protein, the product of the SEC20 gene. This type II membrane protein, itself, is thought to function in vesicular transport from the ER to the Golgi apparatus and it is, therefore, possible that its receptor-mediated shuttling between these compartments is essential for its function.

The yeast ERD2 gene that encodes the HDEL receptor is essential for growth. Its role in sustaining retrograde transport is apparently essential for maintaining a balance in the bidirectional traffic of membrane constituents between the ER and the Golgi apparatus. Genes that suppress the ERD2 deletion (SED genes) when overexpressed have been identified. One of these (SED5) encodes a 39-kDa protein that is required for ER-to-Golgi transport. This protein has a C-terminal membrane anchor that is largely exposed on the cytoplasmic side of the membrane and has a region of similarity with syntaxin, a presynaptic membrane protein that appears to play a role in synaptic vesicle docking at the plasma membrane (see below). The capacity of overexpressed SED5 to suppress the deleterious effect of the ERD2 mutation is likely to be due to an inhibitory effect that it has on anterograde ER-to-Golgi protein flow for a yet unknown reason.

Clearly, the major mechanistic questions about the function of the retrieving KDEL receptor concern what determines that the proteins to be retrieved bind to it in the salvage compartment but are released in the ER. How does the movement of the receptor from one compartment to another take place, and how is the movement regulated? These are related questions, since the binding of the ligand to the receptor in the salvage compartment must somehow trigger its transport back to the ER. It would seem likely that the different environmental conditions in the lumen of the ER and post-ER compartments, such as decreasing Ca\(^{2+}\) concentration and pH, play important roles in regulating the association of the receptor and its ligands (Fig. 16-24). In fact, recent in vitro binding studies have demonstrated that optimal binding of the receptor to its ligands occurs at the acid pH (pH 5 to 6) that prevails in the Golgi apparatus, whereas very little binding occurs at pH 7, thought to be characteristic of the ER. It is interesting to note that in some cell types, such as hepatocytes and pancreatic exocrine cells in which the Golgi environment is neutral, a variety of KDEL-containing proteins escape from the ER.
A receptor retrieves escaped ER proteins that contain the KDEL signal from a salvage compartment or from Golgi cisternae. Transmembrane KDEL receptor molecules are normally found in the salvage compartment or Golgi cisternae, where they avidly bind to any escaped luminal ER proteins (right side) that bear the C-terminal KDEL tetrapeptide. The complexes are incorporated into vesicles that bring them back to the ER (left side), where the condi...

The retention of most membrane proteins in the ER appears to depend on other types of signals and retention mechanisms. A short cytoplasmic sequence at the C-terminus of the adenovirus membrane glycoprotein E19 is required for its retention in the ER. At early times during viral infection, the E19 glycoprotein helps the infected cells to escape from immune surveillance by cytotoxic T lymphocytes. It does so by interacting in the ER with newly synthesized class I major histocompatibility antigen molecules and preventing their transport out of the ER. Similar cytoplasmic C-terminal retention signals have been demonstrated to function in several other ER membrane proteins, including different forms of UDP-glucuronyltransferase, 3-hydroxy-3-methylglutaryl CoA reductase, and a 53-kDa membrane protein of the sarcoplasmic reticulum. In all of these cases a double-lysine motif, such as XKKXX, KXXX, or XXXKX, is present at the extreme C-terminus, and addition of one of these sequences to another protein was sufficient to cause its retention in the ER. Moreover, replacement of the lysine at position −3,
as well as deletion or addition of even a single amino acid to the C-terminus of the ER protein, abolished
the function of the retention signal.\textsuperscript{253,254} A study of the subcellular distribution and the posttranslational
modifications undergone by chimeric proteins containing the dilysine motif expressed in transfected cells
indicated that a retrieving mechanism also contributes to the ER localization of these proteins.\textsuperscript{255}

Other ER transmembrane proteins do not contain “double lysine” motifs, and their retention in the
organelle appears to be determined by other features of the polypeptides. For example, the C-terminal
segments of the cytoplasmic domains of ribophorins I and II and of a 22- to 23-kDa subunit of the signal
peptidase do not cause retention of other reporter proteins to which they have been transferred.\textsuperscript{253} One
plausible mechanism\textsuperscript{256} for the retention of these and other components of the translocation apparatus in
the ER is their association with other proteins. Their incorporation into the extensive proteinaceous
network which exists within the ER membrane and is associated with the ribosome binding sites may
prevent entrance of the proteins into the carrier vesicles that emerge from the ER. The formation of
multiprotein complexes may also account for the retention in the ER of proteins that have large portions
exposed on the cytoplasmic side of the ER membrane, such as cytochrome \( P_{450} \) and its reductase, the
major components of the microsomal monooxygenase system. Indeed, the formation of multiprotein
complexes may be a general mechanism for the retention of proteins within organelles.

**Protein Folding, Oligomerization, and Quality Control in the ER\textsuperscript{145, 257–260a}**

In addition to undergoing covalent modifications, polypeptides synthesized in the ER must fold and, in
many cases, associate with other subunits to form a mature protein. For both luminal and membrane
proteins, these processes must take place in the ER, where conditions are very different from the strongly
reducing environment and low calcium concentration of the cytosol. Completion of the folding and
assembly processes is necessary for the resulting protein to be transported out of the ER. In this way, the
ER exercises quality control on its own products. The fate of the misfolded or unassembled polypeptide
subunits is usually degradation within the ER itself,\textsuperscript{259, 261, 262} although in some instances partially
assembled polypeptide complexes may leave the ER to be degraded within lysosomes.

The fact that proteins must have a “normal conformation” to be transported out of the ER is most strikingly
demonstrated by the behavior of certain multimeric proteins, when their assembly in the ER is perturbed
(e.g., ref. \textsuperscript{263}). Thus, immunoglobulin heavy chains are not transported out of the ER unless the light
chains are also present.\textsuperscript{264} Similarly, in some cases, the heavy chain of the class I major
histocompatibility antigen, which is a type I transmembrane protein, does not exit from the ER unless it
becomes associated with \( \beta_2 \)-microglobulin.\textsuperscript{265, 266} Moreover, the formation of a transport-competent MHC
class I molecule that on the surface of the cell presents antigenic peptides to cytotoxic T lymphocytes is
dependent on its acquisition of the antigenic peptide in the ER. The peptides are generated by proteolysis
in the cytosol and are transported into the lumen of the ER by specific transporters (the products of the
TAP1 and TAP2 genes) in the ER membrane (see ref. \textsuperscript{267}). The influenza HA and the VSV G viral
envelope glycoproteins must also form oligomers (trimers) in the ER to be transported to the Golgi
apparatus.\textsuperscript{268–270} Numerous examples also exist of proteins that are normally secreted or transferred to
the plasma membrane, but remain in the ER when altered by genetic engineering techniques. The fact
that a “normal” conformation that makes the protein “soluble and transportable” is required for exit from
the ER would explain the accumulation in this organelle, and the failure to be secreted, of a mutant form of
the human serum protein \( \alpha_1 \)-antitrypsin,\textsuperscript{271, 272} as well as of nonsecreting variants of immunoglobulin
chains in certain myelomas.\textsuperscript{273} Similarly, many human LDL receptor mutations leading to familial
hypercholesterolemia are characterized by the failure of the receptor to be transported out of the
ER,\textsuperscript{274, 275}
The retention in the ER of defective luminal or membrane proteins may not be simply due to their insolubility within the organelle but, at least in some cases, may also be due to their specific recognition by Bip (or Grp78), a resident protein of the ER that belongs to the class of molecular chaperones. In B lymphocytes, this 78-kDa protein normally binds to the free heavy chains, or to incompletely assembled immunoglobulin molecules, preventing their premature exit from the ER and, therefore, their secretion from the cell. In the lymphoproliferative heavy chain disease, in which heavy chains are secreted in the absence of light chains, a deletion mutation in the heavy chain constant region gene that eliminates the CH4 domain of the protein reduces its affinity for Bip. Although Bip was first recognized in pre-B-cell lines that do not yet synthesize light chains, its presence has now been demonstrated in many other cell types in which it was found to form complexes with abnormal or incompletely assembled secretory and membrane proteins. Bip is a member of the heat-shock (or stress) family of proteins (hsp), and it accumulates at high levels in the lumen of the ER of cultured fibroblasts when these are subjected to the stress of glucose starvation, or are treated with tunicamycin to prevent N-glycosylation. It was originally proposed that by binding to and blocking the transport to the cell surface of abnormally folded polypeptides, Bip may play a role in protecting the organism from the adverse effects that could result from the recognition by the immune system of denatured “self” polypeptides as foreign antigens. On the other hand, as the concept of “molecular chaperone” has evolved, the prevalent view has emerged that, in many instances, the normal function of Bip—rather than to simply prevent exit from the ER—is to stabilize a newly synthesized polypeptide in a form that allows it to achieve its final structure more efficiently, which may require additional folding steps or assembly with other subunits. This is in accord with the fact that the CH1 domain of the immunoglobulin heavy chain, to which Bip binds, is the same portion of the polypeptide that interfaces with the light chain.

Another ER protein that appears to function as a molecular chaperone is an 88-kDa transmembrane protein, calnexin (p88), that associates with the MHC class I heavy chain in the ER and may remain associated with it during the formation of the complex with β2-microglobulin and the antigenic peptide. Binding of the antigenic peptide to MHC molecules may lead to release of the chaperone and export of the complex to the Golgi apparatus. The same protein also associates with incompletely assembled T-cell antigen receptors and membrane-bound immunoglobulins.

Protein Degradation in the ER

As part of its quality-control function, the ER is capable of carrying out the degradation of many improperly folded or incompletely assembled polypeptides that are retained within it. In addition, the capacity of the ER for protein degradation allows it to play a role in the regulation of some metabolic pathways that utilize ER enzymes, as exemplified by the cholesterol-controlled degradation of HMG-CoA reductase, an enzyme whose activity is rate limiting for sterol biosynthesis.

Known substrates for ER degradation include unassembled subunits of the T cell, acetylcholine and asialoglycoprotein receptors, as well as mutant forms of the LDL receptor and of α1-antitrypsin, the cystic fibrosis transmembrane regulator, and β-hexosaminidase. The degradation of these polypeptides begins soon after their synthesis is completed and proceeds with a half-life of 1 h or less.

The ER degradation system is highly discriminatory with respect to substrate selection. This is best illustrated by studies of the fate of unassembled or partially assembled subunits of the T-cell receptor (TCR). This receptor is composed of at least eight (α, β, γ, δ, ε2, ζ2) transmembrane polypeptides, which normally form a complex in the ER that is rapidly transported through the Golgi apparatus to the cell surface. In a T-cell hybridoma in which the TCR ζ chains were made in much lower amounts than the other subunits, incomplete multimeric complexes lacking ζ chains were formed and were transported to
the Golgi apparatus, but were then diverted to lysosomes for degradation. On the other hand, in a hybridoma in which the \( \delta \) chains were not synthesized, \( \alpha \) chains and \( \alpha-\beta \) disulfide-linked heterodimers were rapidly degraded within the ER itself. Similarly, when individual subunits of the TCR were expressed in transfected fibroblasts, the \( \alpha \), \( \beta \), and \( \delta \) chains underwent rapid degradation in the ER, although under the same conditions the \( \zeta \) and \( \epsilon \) subunits were stable. Strikingly, the murine \( \gamma \) chain, when expressed by itself, was rapidly degraded, but it was markedly stabilized after it formed a complex with the \( \epsilon \) chain.

Studies done primarily with the \( \alpha \) chain have attempted to identify the structural features of the isolated TCR subunits that determine their pre-Golgi degradation. The observation that a truncated \( \alpha \) chain that consisted only of its luminal domain was retained in the ER but was much more stable than the intact polypeptides, suggested that the transmembrane domain of this polypeptide (this is unusual in that it contains two positively charged amino acids) is responsible for the rapid rate of degradation of the \( \alpha \) chain. This hypothesis was substantiated by the finding that transfer of a nine-amino-acid segment of the \( \alpha \)-chain transmembrane domain, containing the charged residues, into the transmembrane domain of the Tac antigen (the IL-2 receptor) was sufficient to cause retention and rapid degradation of the chimeric polypeptide containing the TCR \( \alpha \)-chain transmembrane domain. These experiments demonstrate that the positively charged residues in the transmembrane domain constitute a critical determinant for the retention and degradation of the \( \alpha \) chain in the ER. The availability of this determinant to the degradative system appears to be controlled by the state of assembly of the polypeptide. Thus, association of the \( \alpha \) chain with the \( \delta \) chain, which contains two negatively charged residues in its transmembrane domain, leads to masking of the determinant and to formation of a stable dimeric complex. It thus appears that the charged residues have the mutually exclusive roles of targeting the polypeptide for ER degradation or participating in subunit assembly, so that quality control on the assembly process can be exerted by a mechanism that senses the exposure of the charged residues within the ER membrane.

Little is known about the nature of the proteases that carry out the degradation of abnormal or unassembled polypeptides in the ER, or even if the same proteases are involved in the normal turnover of ER resident proteins. The degradative enzymes, however, must be able to function at the high calcium concentration characteristic of the lumen of the ER. Indeed, exposure of cells to calcium ionophores in the presence of EGTA, which leads to the depletion of intracellular \( \text{Ca}^{2+} \), inhibited the ER degradation. This observation, together with the finding that calcium depletion accelerated the already rapid degradation of the TCR \( \delta \) and \( \beta \) chains, indicates that changes in \( \text{Ca}^{2+} \) concentration alter the susceptibility of the substrates to the proteases. Most likely, ER degradation involves a constellation of enzymes, since the degradation of different substrates has been found to be blocked by protease inhibitors of different specificities.

**TRANSFER OF PROTEINS FROM THE ER TO THE GOLGI APPARATUS**

ER-to-Golgi transport was first demonstrated in pancreatic acinar cells, where the vast majority of the newly synthesized proteins are digestive enzymes to be stored in secretory granules (see ref. 56). Autoradiographic analysis of pulse-labeled slices of pancreatic tissue and cell fractionation studies established that the newly synthesized proteins exit from the ER at specialized ER cisternae, known as transitional elements or transitional cisternae, that are located close to the receiving (cis) face of the Golgi apparatus (see Fig. 16-1). Transitional cisternae are partly “rough” and partly “smooth.” Nonclathrin-coated coated vesicles and/or tubular elements appear to emerge from their smooth portions. Early cytochemical and immunocytochemical studies demonstrated the presence of specific secretory proteins in the transitional elements and in the vesicles located near the cis side of the Golgi apparatus. It has therefore been assumed that the tubulovesicular elements seen near the
transitional cisternae serve as carriers that transport newly synthesized proteins to the Golgi apparatus. It is now clear, however, that the ER-Golgi interface is a complex region and at least some of the elements that it contains may also be functioning in a retrograde transport that retrieves to the ER constituents that either escaped the organelle or are to be reutilized in new rounds of anterograde transport.

ER-to-Golgi transport is an energy-requiring process and, therefore, can be halted by anoxia or by drugs, such as azide or antimycin, that inhibit respiration, or by inhibitors of oxidative phosphorylation, such as dinitrophenol, oligomycin, or carbonylcyanide m-chlorophenylhydrazone (CCCP). When the energy supply is exhausted, the proteins accumulate in the transitional elements or in vesicles between them and the cis face of the Golgi apparatus.

The kinetics of transport of specific proteins from the ER to the Golgi apparatus have been followed in tissues and in cultured cells by analyzing the acquisition of endo H resistance in polypeptides purified by immunoprecipitation with specific antibodies. Studies with both secretory and integral membrane proteins destined for the cell surface have shown that different proteins are transported from the ER to the cell surface at different rates, with half times for transport varying from 10 min to 1½ h. The rate limiting step appears to be passage from the ER to the Golgi apparatus, with transport from this organelle to the cell surface occurring at the same fast rate for all proteins in a given cell. Using cells infected with a temperature-sensitive vesicular stomatitis virus, the transport of the G glycoprotein through the Golgi apparatus has been visualized directly by immunoelectron microscopy. At the nonpermissive temperature, the envelope glycoprotein accumulates in the ER, but within 5 to 10 min after the infected cells are shifted to the permissive temperature, it appears in the Golgi cisternae concomitantly with its acquisition of endo H resistance.

Two possible interpretations can be given to the different rates observed for the transport of proteins out of the ER. One is that, in a manner analogous to receptor-mediated endocytosis at the plasma membrane (see below), rapid transport of the proteins requires their interaction with a receptor or carrier in the ER that is incorporated into transport vesicles and that those proteins with highest affinity for the receptor are transported most rapidly. This implies that the transported proteins contain a sorting signal for interaction with the receptor and, hence, that proteins that lack such signals would move at a uniform slow rate, unless they contain a retention signal that completely excludes them from the transport vesicles. In this view, proteins that are transported by default (i.e., lack transport or retention signals) would be free to diffuse in the fluid phase that exists within the luminal cavities of the transporting vesicles and of all the organelles within the endomembrane system and to finally reach the cell surface. A similar default pathway to the plasma membrane could be followed by membrane proteins lacking signals that would be capable of moving by diffusion within the phospholipid bilayers of all the membranes and vesicular carriers that compose the endomembrane system.

In another interpretation, a rapid bulk flow, of soluble as well as membrane components, would have its source in the ER and would take place constantly toward the surface of the cell. Proteins destined to become permanent residents of the various organelles of the endomembrane system, including the ER itself and the Golgi cisternae, would contain retention signals for local assembly that are recognized by other components of those organelles, with which the newly arrived proteins form oligomeric complexes. In this view, the rate of transport of a protein out of the ER would be determined by the time required for its proper folding, oligomerization and maturation in that organelle. Once secretory, lysosomal, or plasma membrane proteins reach the trans-Golgi region, a process that sorts them from each other would take place.
The concept of a rapid bulk flow that begins in the ER is based on studies of the kinetics of uptake and reexport by mammalian cells of simple synthetic peptides that contain an acceptor site for N-glycosylation. After entering the cell, these peptides cross the ER membrane, become glycosylated, and reappear in the medium within 10 min. It was thought that after their glycosylation the peptides could no longer cross the membranes of the endomembrane system and, therefore, could exit from the cells only by traversing the entire secretory pathway. Hence, the rapid export of the peptides was taken as reflecting the rapid rate of bulk flow. The validity of this conclusion has been brought into question by experiments showing that the glycosylated peptides do not all exit with the same kinetics from cultured mammalian cells, and that they do not exit at all from Xenopus oocytes, which are capable of secreting almost any secretory protein. Moreover, studies with yeast indicate that the ER contains an ATP-dependent pump capable of extruding glycosylated peptides directly into the cytosol, from which the peptides could conceivably be secreted from the cells by an equivalent pump present in the plasma membrane.

Insights into ER-to-Golgi Transport Obtained from Studies with Yeast

The study of ER-to-Golgi transport in yeast has been greatly advanced by a combination of biochemical and genetic approaches and has greatly illuminated the equivalent process in mammalian cells. Temperature-sensitive mutations in more than 20 different yeast genes defined by complementation analysis have been obtained that disrupt this transport step at the nonpermissive temperature. In most cases, the corresponding wild-type gene could be isolated by complementation and, frequently, extragenic suppressor genes that compensate for the specific mutation when expressed at high levels were identified using libraries constructed in high copy plasmid vectors. At the nonpermissive temperature, mutations in genes involved in ER-to-Golgi transport lead to the expansion of the ER and the accumulation of core-glycosylated forms of secretory protein (e.g., pro-α-factor and invertase) or vacuolar proteins (e.g., carboxypeptidase Y) in this organelle. Many sec mutants of this type could be categorized in two classes: those that are defective in vesicle formation (class I; e.g., sec12, sec13, sec16, sec23) and those in which vesicles (=50 nm in diameter) are formed but accumulate within the cell at the nonpermissive temperature (class II; e.g., sec17, sec18, sec22). Apparently, class II mutants have a defect in a subsequent step required for targeting or fusion of the vesicle to the acceptor organelle, the Golgi apparatus.

The assignment of the various mutant strains to one class or the other was facilitated by an epistasis test, in which vesicle accumulation was assessed in haploid yeast strains in which two of the mutations were present. This test showed that class I mutations are epistatic with respect to class II, that is, no vesicles accumulated when a mutation in a class I gene was present together with a mutation in a class II gene, and, therefore, that the formation of the vesicles that accumulate at the nonpermissive temperature in the class II mutants depends on the function of class I genes. In addition, the phenomenon of synthetic lethality allowed the recognition of genes whose products function in the same step, and may even interact with each other. In such cases, the cells containing both mutations may fail to grow or may secrete at a lower temperature than cells containing either single mutation. In this test, the combination of sec17 and sec18, as well as five of the six possible pairwise combinations between sec12, sec13, sec16, and sec23, exhibited synthetic lethality.

The specific functions of many of the products of the genes involved in vesicle formation and fusion are currently being gleaned from: (1) an analysis of the cloned sequences, (2) the behavior of cell extracts derived from mutants, or depleted of specific gene products, in in vitro assays for overall ER-to-Golgi transport or for the partial steps of vesicle formation or vesicle delivery, (3) the effects in these assays of adding either specific antibodies to purified proteins, or the products themselves of the normal or mutant SEC genes, which can be produced in large amounts by recombinant DNA procedures, and (4) the
identification and characterization of the gene products of suppressor genes.

The availability of experimental systems that utilize disrupted or perforated yeast or mammalian cells, or cell fractions derived from them, to reconstitute interorganellar transport steps in vitro has permitted the study of the requirements for cytosolic components, as well as of the effects of various impermeant inhibitors, nucleotides, antibodies, peptides, and proteins on transport. The main systems used to investigate ER-to-Golgi transport in yeast employ disrupted spheroplasts or microsomal fractions that contain both donor ER and Golgi acceptor membranes. Transport is measured from the extent to which a secretary protein, pro-α-factor, undergoes Golgi-specific outer-chain modifications of its oligosaccharides. S-labeled prepro-α-factor is first synthesized in vitro and introduced into the yeast microsomes, or into the ER of permeabilized cells, in a posttranslational translocation reaction (that occurs in yeast and not in mammalian cells). Transport leads to removal of the signal sequence and core glycosylation at three sites within the polypeptide. Vesicular transport from the ER to the Golgi apparatus takes place only when the system is incubated at physiological temperatures in the presence of cytosol and a continuous supply of ATP. With these in vitro systems it has been possible to separate transport into the successive stages of vesicle formation, targeting or docking, and fusion. Indeed, these systems have provided the only direct evidence available for the role of vesicles as mediators of protein transport between organelles.

Much has been learned of the functions of proteins required for vesicle formation in the ER and the intermolecular interactions that take place during this event. Some class I gene products (sec12p and sec23p) appear to regulate the cyclic function of a low-molecular-weight ras-related GTP-binding protein, Sar1p, during the process of vesicle formation. Gene cloning and the use of specific antibodies permitted the demonstration that Sec12p is a type II integral membrane protein of the ER whose large N-terminal cytoplasmic domain interacts with Sar1p and recruits it from the cytosol. Sar1p was first identified as a suppressor gene product that, when present at high levels, compensates for a mutation in Sec12p. Genetic studies showed that it too was necessary for ER-to-Golgi transport. The suppressor effect of Sar1p was also observed in vitro when the protein was added in excess to a transport system containing ER membranes defective in Sec12p. It now appears that the exchange of GDP for GTP that leads to the activation of Sar1p is catalyzed by Sec12p. This explains why overexpression of Sar1p, with a concomitant increase in the amount of its active GTP-containing form, can compensate for a partial deficiency in Sec12p. The GTPase activity of Sar1p is not required for its function in vesicle production, but is required to complete a round of transport, since when the protein is precharged with the nonhydrolyzable analogue GTP-γ-S instead of GTP, it cannot support transport in a Sar1p-depleted extract. The completion of the cycle of GTP utilization by Sar1p seems to involve another class I Sec gene product, Sec23p, that, acting as a GAP protein, stimulates the GTPase activity of Sar1p and leads to its release from the vesicle, which now can enter into the targeting phase of vesicular transport in which Ypt1, another low-molecular-weight GTP binding protein, plays a critical role (see below). Sec23p is an 85-kDa cytosolic protein that forms a complex with a 105-kDa protein, identified by commounprecipitation and subsequently cloned, now designated Sec24p, that is also required for vesicle budding from the ER. Antibodies to the yeast Sec23p localize the homologous mammalian protein in exocrine and endocrine pancreatic cells to the cytoplasmic region between the transitional cisternae and the Golgi apparatus, where at least some of the protein appears to be bound to the membranous tubulovesicular elements that characterize that region. The restricted localization of the mammalian homologue of Sec23p led to the suggestion that it is associated with an organized structure within the transitional zone of the cell that serves to facilitate vesicular flow from the ER toward the Golgi and permits the efficient reutilization of the protein in many rounds of vesicular transport.
Another class I gene product, Sec13p, is also a cytosolic protein (33 kDa) that is peripherally associated with the ER membranes, and is required for vesicle formation and release in vitro. Strikingly, Sec13p is normally a component of a very large (700-kDa) cytosolic macromolecular complex, which includes other proteins. When its gene is overexpressed, however, the monomeric form of Sec13p that accumulates is also active in a Sec13p-dependent vesicle formation assay. The sequence of Sec13p consists of a series of repeating polypeptide segments that show homology to a segment that is also repeated in other proteins involved in a variety of cellular processes, including signal transduction (e.g., the β subunit of the heterotrimeric G protein transducin) and cell cycle progression. Since Sec13p shows synthetic lethal interactions with Sec12 and Sec23, whose products function in the GTP cycle of Sar1p, it is possible that Sec13p also participates in that cycle, either as an upstream regulator at the guanine nucleotide exchange event, as one might expect for a G protein, or as a downstream effector of Sar1p that promotes the budding process itself. If the latter were the case, Sec13p could be a component of a coat required for vesicle budding, to promote the assembly of such a coat, to induce the deformation of the donor membrane, or to participate in the membrane fission event that completes the generation of a free vesicle.

Several proteins involved in vesicle docking at the Golgi apparatus have also been studied extensively. One of these is Ypt1p, a member of the rab family of GTP-binding proteins that shows extensive homology to Sec4p, a protein required for vesicle delivery from the Golgi apparatus to the plasma membrane. Ypt1p and Sec4p were the first low-molecular-weight GTP-binding proteins shown to have essential functions in protein traffic. From their discovery stems most of our recent knowledge of the role of rab family members in vesicular transport. Under restrictive conditions, temperature-sensitive mutants in Ypt1p manifest a defect in ER-to-Golgi transport, both in vivo and in vitro, and vesicles accumulate in the intact cells. In addition, antibodies to Ypt1p block transport in vitro, but do not inhibit vesicle formation. A mammalian homologue of Ypt1p, rab1a, has been identified that can functionally replace Ypt1p in a yeast temperature-sensitive strain, and it was shown to be required for ER-to-Golgi transport in a mammalian cell-free system.

It is currently thought that Ypt1p action leads to a loose docking of vesicles on the acceptor membranes, and it is known that other Sec gene products, such as Sec18p and Sec17p, as well as Ca\(^{2+}\), act subsequently to trigger the fusion step that completes the delivery of the protein to the Golgi apparatus. Ca\(^{2+}\) is required for ER-to-Golgi transport in both mammalian cells and yeast, and it is known to act at a late step in this process. That Ypt1p participates in the transport reaction, becoming a vesicle component at a step prior to that requiring Ca\(^{2+}\), is demonstrated by the fact that when the in vitro yeast transport reaction is carried out in the presence of EGTA, completion of transport will occur on the readdition of Ca\(^{2+}\), even in the presence of anti-Ypt1p antibodies that would have blocked transport if added initially.

Sec18p is the yeast equivalent of NSF20—an N-ethylmaleimide (NEM) sensitive factor, originally purified from mammalian tissues, that was first shown to be necessary for intra-Golgi transport and later found to play a widespread role in membrane fusion events in mammalian cells, including ER-to-Golgi transport and endocytic vesicle-endosome fusion. In fact, yeast Sec18p could replace NSF in a mammalian transport assay. Studies with the mammalian system have shown that NSF binds to membranes and that this binding is mediated by a soluble NSF attachment protein (SNAP), of which three different gene products, (α, β, and γ SNAP), related in sequence, have been identified. The Sec17 yeast gene product is functionally equivalent to the mammalian αSNAP.

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From a variety of studies carried out in vitro, primarily with mammalian systems, it appears that NSF, which is a tetramer of 76-kDa subunits, serves as a bridge between SNAPs that specifically bind to receptors in the donor and target membranes. Although it has been proposed that NSF plays a direct role in membrane fusion in mammalian intra-Golgi transport, evidence has been obtained that in yeast ER-to-Golgi transport NSF acts after Ypt1p, but before the Ca\(^{2+}\)-requiring step that leads to membrane fusion. Thus, when transport is carried out at the permissive temperature, with a lysate from a Sec18p temperature-sensitive mutant, in a Ca\(^{2+}\)-depleted medium to arrest transport at the Ca\(^{2+}\)-sensitive step, fusion will proceed normally when Ca\(^{2+}\) is restored, even if the temperature is raised to inactivate Sec18p.

The actual vesicles that mediate ER-to-Golgi transport have not yet been purified and characterized biochemically. It is not known what type of coat, if any, they acquire during their formation. However, it has been shown that the Sec21 gene product, which is required for ER-to-Golgi transport, is the yeast homologue of \(\gamma\)COP, a subunit of the coat of the non-clathrin-coated vesicles that mediate intra-Golgi transport in mammalian cells (see below). Moreover, as is the case with its mammalian counterpart, Sec21p is a subunit of a very large (700- to 800-kDa) complex that in polypeptide composition closely resembles that of the mammalian coatomer (see below). In fact, it has been shown that another polypeptide within the complex containing Sec21p is the yeast equivalent of \(\beta\)COP, the best-characterized subunit of the mammalian coatomer.

Like other members of the ras superfamily of GTP-binding proteins, Ypt1p undergoes prenylation in a cysteine residue at its C-terminus, at which a geranylgeranyl group is added, and it may also undergo palmitoylation at another neighboring cysteine. Prenylation allows Ypt1p to become membrane-anchored and is necessary for its activity in transport. Accordingly, a temperature-sensitive mutation (bet2) that inactivates the geranylgeranyl transferase that effects these modifications impairs attachment of Ypt1 to membranes and blocks the delivery of ER-derived transport vesicles to the Golgi apparatus.

Because in yeast many mannose residues are added to glycoproteins on their arrival to the Golgi apparatus, it was possible to use a \(^3\)H-mannose suicide technique to isolate conditional lethal mutants that are blocked early in transport (defining the genes BET1 and BET2) and, therefore, survive incubation at the nonpermissive temperature in medium containing highly radioactive mannose. Other genes that act early in the secretory pathway have been identified as suppressors of the loss of YPT1 (SLY1, SLY2, SLY12, and SLY41) function, or suppressors of the bet1 mutation (BOS1, for bet one suppressor; 27 kDa). The products of all these genes are involved in ER-to-Golgi transport and, in fact, SLY12 is identical to BET1, and SLY2 to the previously identified SEC22 gene. Bos1p and Bet1p are class II genes that are required for vesicle consumption and not for vesicle formation. The products of these genes (Bos1p, 27 kDa; SLY12p/Bet1p, 142 amino acids, and SLY2p/Sec22p, 214 amino acids) lack insertion signal sequences and are associated with the ER membrane through their hydrophobic C-terminal regions. Therefore, they have most of their masses exposed on the cytoplasmic membrane surface. This disposition, together with the fact that Sly2p/Sec22p and SLY12p/Bet1p show sequence similarity to the mammalian synaptic vesicle membrane protein synaptobrevin (or VAMP)—which is currently thought to play a role as a targeting molecule in the docking of the synaptic vesicle at the presynaptic membrane during neurotransmitter discharge (see refs. and strongly suggests that the latter two proteins play a role in vesicle docking during ER-to-Golgi transport. It is noteworthy that Sly2p/Sec22p and Ypt1p, like Bos1p, are components of the transport vesicles that accumulate in an in vitro assay in the absence of acceptor Golgi membranes.
Given the role of Ypt1p in targeting, how could it be explained that overexpression of SLY2/SEC22, SLY12/BET1, and SLY41, or a point mutation in the SLY1 gene (Sly1-20 allele) can overcome a complete absence of Ypt1p? It has been suggested that in the case of Sly1-20 this occurs because normally, an activated Ypt1p functions to induce a conformational change in Sly1p, which is mimicked by the mutation. As for Sly2p/Sec22p and Sly12p/Bet1p, it is possible that these proteins are normally present in very limiting amounts on the transport vesicles, which, therefore, rely for their efficient docking on the action of Ypt1p. However, when these synaptobrevin-like putative targeting proteins are superabundant, the guiding action of Ypt1p may become dispensable for cell viability.

**Molecular Interactions that Underlie ER-to-Golgi Transport in Mammalian Cells**

Several systems have been developed that utilize disrupted or detergent-permeabilized cultured cells to reproduce ER-to-Golgi and intra-Golgi transport in vitro under conditions that allow the penetration into the cells of normally impermeant reagents. In general, the cells are infected with a temperature-sensitive variant (tsO45) of the vesicular stomatitis virus (VSV) that produces a defective viral envelope glycoprotein (G). At the nonpermissive temperature (37°C), the mutant protein accumulates in the ER, but it rapidly exits toward the Golgi apparatus when the cells are transferred to an environment of 30°C. In these assays, the G protein is metabolically labeled with $[^{35}\text{S}]$methionine prior to permeabilization of the cells, and its passage to the Golgi apparatus is monitored from the extent to which its $N$-linked oligosaccharides are modified by Golgi enzymes. Such modifications can be detected by virtue of the changes in the electrophoretic mobility of the protein caused by digestion with endoglycosidases (endo D, endo H, endo F).

Utilizing semi-intact CHO cells, it was shown that ER-to-cis-Golgi transport is a temperature-dependent process that requires cytosolic proteins (including NSF, the NEM-sensitive factor that represents the yeast Sec18 homologue), $\text{Ca}^{2+}$ ions, and an energy supply, as well as the participation of GTP-binding proteins ($\text{rab}$, $\text{arf}$, and heterotrimeric G proteins), and the hydrolysis of GTP (e.g., refs. 341, 365, and 366). GTP-$\gamma$-S blocks ER-to-Golgi transport when added early during the course of the reaction, which indicates that a critical GTP-binding protein must be charged with GTP during an early phase, possibly during vesicle formation.

Three $\text{rab}$ proteins—$\text{rab}1\text{a}$, $\text{rab}1\text{b}$, and $\text{rab}2$—are localized in the ER and Golgi compartments and participate in ER-to-Golgi transport. $\text{rab}1\text{a}$ and $\text{rab}1\text{b}$ are closely related to each other (92 percent sequence identity) and to the yeast Ypt1p (almost 70 percent identity), which $\text{rab}1\text{a}$ can functionally replace. The addition of specific antibodies that recognize $\text{rab}1\text{a}$ and $\text{rab}1\text{b}$ has been shown to block in vitro transport, but only if the antibodies are added very soon after transport is allowed to begin. Immunofluorescence studies have shown that antibodies to $\text{rab}1\text{b}$ block vesicle formation in the ER.

Although these findings suggest that the $\text{rab}$ proteins complete their function early in transport (as expected if they participate only in vesicle formation), it is possible that they are also active in a later step that can no longer be inhibited by antibodies once these proteins are assembled onto a vesicle. In fact, independent evidence indicates that the $\text{rab}$1 proteins interact with their effectors (which leads to GTP hydrolysis) very late, much later than the time at which addition of GTP-$\gamma$-S to the system no longer has an effect on transport or the time at which anti-rab antibodies no longer are effective (see below).

As may be expected from the role of $\text{Ca}^{2+}$ in many membrane fusion events, this ion is also required to complete ER-to-Golgi transport. Thus, chelation of $\text{Ca}^{2+}$ with EGTA at any time during the course of the incubation almost instantly and reversibly blocks transport to the Golgi apparatus. The rapid cessation of transport on depletion of $\text{Ca}^{2+}$ indicates that $\text{Ca}^{2+}$ is required at a very late step in the chain of events that leads to transport. This is also apparent from the finding that after prolonged incubation in EGTA,
restoration of Ca\(^{2+}\) leads to the rapid resumption and completion of transport, with an initial rate considerably faster than that normally achieved. Moreover, after Ca\(^{2+}\) is restored, transport can no longer be inhibited by addition of GTP-\(\gamma\)-S or anti-rab antibodies,\(^{367}\) indicating that GTP and the rab proteins have already been incorporated into the transporting machinery. On the other hand, synthetic peptides corresponding to the highly conserved effector domain of the rab subfamily of proteins completely block transport, even when added at the time of Ca\(^{2+}\) restoration, presumably because they compete with the rab proteins for interaction with their as yet unidentified effectors.\(^{367}\) Thus, this interaction, which may occur on the acceptor membranes, may be one of the last steps in transport.

The cyclic role of rab1a, rab1b, and rab2 in ER-to-Golgi transport is demonstrated by the findings that certain mutations in these proteins designed to maintain them permanently in the active configuration (by amino acid substitutions in the effector or the GTP-binding domains) block ER-to-Golgi transport. This was shown both in cells that express those proteins after transfection with a viral vector,\(^{341}\) and in an in vitro system to which the purified proteins produced in bacteria are added.\(^{364}\) These results would be expected if the rab proteins unable to hydrolyze GTP remain bound to their effectors, or to other molecules, and prevent their reutilization in new rounds of transport.

In addition to rab proteins, GTP-binding proteins of the arf and heterotrimeric G families also participate in ER-to-Golgi transport as in other interorganelar transport steps in mammalian cells. The arf proteins are members of the ras superfamily that become membrane-associated through an N-terminal myristate moiety. The yeast protein Arf1p appears to play a role in ER-to-Golgi transport, since ARF shows synthetic lethality with other yeast genes involved in that process, such as YPT1, SEC21, and BET2.\(^{368}\) The human ARF1 and ARF4 gene products (hARFp) have been shown to be capable of replacing Arf1p in mutant yeast cells.\(^{45}\) Moreover, synthetic peptides corresponding to the N-terminal region of the human ARF irreversibly inhibited ER-to-Golgi transport in semi-intact cells, apparently by preventing vesicle formation in the ER.\(^{366, 369}\) This is not surprising since studies with in vitro systems that reproduce intra-Golgi transport indicate that an ARF protein is a component of the coatamer-containing coat of the vesicles that mediate that process, and it plays a critical role in triggering the assembly of this coat (see ref. \(^{348}\)). The ARF peptides, however, were also able to inhibit transport when added at a very late stage.\(^{369}\) This suggests that ARF proteins also participate in late events, such as vesicle docking and fusion.

That heterotrimeric G proteins, like those that function in signal transduction at the plasma membrane, have a role in ER-to-Golgi transport first became apparent from studies that demonstrated strong inhibitory effects of AlF\(_3\).\(^{342}\) More recently, export of a viral glycoprotein from the ER, monitored in permeabilized cells using immunofluorescence, was found to be also blocked by the addition of purified \(\beta\gamma\) subunits of the G protein, which should bind to free G\(\alpha\) subunits and prevent them from exerting their effects. Mastoparan, the cationic peptide that mimics the G protein-binding region of plasma membrane receptors and preferentially activates G\(\alpha\) subunits, also blocked transport.\(^{366}\) The similar inhibitory effects of the \(\beta\gamma\) subunits and mastoparan on transport, despite their opposite actions on G\(\alpha\) subunits, may indicate that heterotrimeric G proteins containing both stimulatory (G\(\alpha\)) and inhibitory (G\(\beta\gamma\), or G\(\alpha\)) subunits function in this system and have opposite regulatory effects, as has been reported for the formation of constitutive secretory vesicles in the trans-Golgi network (see below and ref. \(^{370}\)).

An additional mechanism that may control vesicular transport between subcellular organelles involves the phosphorylation of specific components by cytosolic protein kinases. Phosphorylation seems to have opposite effects in ER-to-Golgi transport and in transport from the medial Golgi to the trans-Golgi network (TGN). Inhibitors of protein phosphatases, such as okadaic acid and microcystin-LR, blocked ER-to-Golgi transport, both in vivo and in vitro, and this effect was eliminated by treatment with protein kinase
antagonists such as staurosporine and H-8, reagents that, by themselves, do not affect transport.\textsuperscript{371} Therefore, the presence of phosphate groups in yet unidentified proteins that participate in ER-to-Golgi transport inhibits this process. It is noteworthy that during mitosis, vesicular transport is inhibited concomitantly with the activation of MPF (mitosis-promoting factor), which is a protein kinase. On the other hand, protein phosphorylation is essential for transport between the medial Golgi and the TGN, since the same protein kinase inhibitors mentioned above completely arrest this process.\textsuperscript{364} One can expect that phosphorylation-dephosphorylation events will be found to play a critical role in many vesicular transport processes.

**Intermediate Compartment at the ER–Golgi Interface**\textsuperscript{303, 372–374}

The area of cytoplasm between the ER transitional elements and the first (\textit{cis}) cisternae of the Golgi apparatus is rich in tubulovesicular membranous structures. EM images frequently suggest that vesicles or tubules in this region of the cell either bud from or fuse with the adjacent ER or Golgi membranes. The precise interconnections or relationships between these tubulovesicular elements, which are collectively known as the \textit{ER–Golgi intermediate compartment} (ERGIC), and the ER and Golgi have not yet been elucidated. It is known, however, that when cells are incubated at \textit{15°C} this compartment greatly expands and that, under these conditions, newly synthesized proteins that are normally transported to the Golgi apparatus, such as the Semliki Forest (SFV) and vesicular stomatitis (VSV) viral envelope glycoproteins, accumulate in it.\textsuperscript{375, 376} This indicates that the low temperature causes a specific block in transport in this region of the endomembrane system.

Elements of the ER–Golgi intermediate compartment have been regarded as constituting a specialized organelle, since they appear to contain specific resident proteins, most notably a 53-kDa nonglycosylated transmembrane protein (\textit{p53}) which, in some cell types, is also found in the first (\textit{cis}) Golgi cisternae.\textsuperscript{377} As expected from a bona fide component of the putative organelle, after incubation of VSV-infected cells at \textit{15°C}, p53 colocalizes with the arrested VSV G glycoprotein in the expanded intermediate compartment.\textsuperscript{376} A 58-kDa glycoprotein (lacking terminal sugars normally added in the \textit{trans}-Golgi and TGN) found in \textit{cis}-Golgi cisternae is also present in the intermediate-compartment elements,\textsuperscript{378} as are \textit{rab2} and \textit{rab1p}, the two small GTP-binding proteins that play an essential role in ER-to-Golgi vesicular transport.\textsuperscript{37, 378, 379}

It has been possible to isolate from Vero (African green monkey kidney) cells a subcellular fraction that, judging from its content of \textit{p53}, is highly enriched in elements of the intermediate compartment and is depleted in rough ER and \textit{cis}-Golgi markers.\textsuperscript{380} Therefore, much may soon be learned of the biochemical properties of the intermediate compartment. At this moment, however, it is not yet clear if it represents a true, separate, compartment (or organelle) that receives vesicles from ER transitional elements and produces other vesicles that are targeted to the \textit{cis} face of the Golgi apparatus, or if it consists only of expansions of one of the adjacent organelles. One opinion expressed is that it is connected to the ER transitional elements and is therefore part of what would be the equivalent of a \textit{transitional element network} (TEN).\textsuperscript{372} Another opinion is that it is continuous with the first \textit{cis}-Golgi cisterna, forming part of a \textit{cis}-Golgi network, or CGN.\textsuperscript{232, 373, 381} Furthermore, the possibility has been suggested that the intermediate compartment is simply a pleomorphic transport intermediate consisting mainly of tubulovesicular membranous elements that are formed by the aggregation and fusion of vesicles that emerge from the transitional elements of the ER or the Golgi apparatus. Such tubulovesicular elements would then coalesce to form the first \textit{cis}-Golgi cisternae.\textsuperscript{303, 376, 379} In this view (Fig. 16-25), only one round of vesicle fission (from the ER) is required for transport to the \textit{cis}-Golgi, but many fusion events would be required to form the \textit{cis}-Golgi network. It has been proposed that because the CGN would be the first way station for traffic that emerges from the ER, it is also likely to be a site of sorting, where elements that are to be returned to the ER are segregated from those that must move along the secretory
pathway. For this reason the intermediate compartment has also been termed the *exosome*. This name is intended to reflect a mode of biogenesis and function for the intermediate compartment that would be similar to that of the peripheral endosomal compartment that, as will be discussed later, is viewed by some as resulting from the coalescence and fusion of vesicles formed at the plasma membrane (see below).

![Diagram](image)

**Fig. 16-25:** Structure of the ER-Golgi intermediate compartment. Proteins exported from the ER pass through an intermediate compartment that consists of tubulovesicular elements found between the ER transitional elements and the first (cis) cisternae of Golgi stacks. In cells incubated at 15°C, the proteins accumulate in this compartment, which also greatly expands at this temperature. The term cis-Golgi network (CGN) has also been applied to the ...

Whether the intermediate compartment represents a permanent structure through which proteins pass or consists mainly of transient carriers, its morphologic and biochemical identities seem well established. Functionally, its elements may represent at least one of the *salvage* compartments from which KDEL-containing proteins are recycled back to the ER. The intermediate compartment may also be the site to which certain abnormal proteins, whose degradation begins in the ER, are transported for completion of their degradation at a faster rate. The relationship of the intermediate compartment to a subcompartment of the ER, the *calciosome*, where a Ca$^{2+}$ pump and Ca$^{2+}$ binding proteins are located and where Ca$^{2+}$ ions are stored at high concentrations, has not yet been examined.

It seems that ER transitional cisternae and the intermediate compartment share at least some biochemical properties since the mouse hepatitis coronavirus A59 assembles and specifically buds only at these sites, where, apparently, N-acetylgalactosamine residues also become O-linked to serines and threonines in the viral glycoprotein. It is possible that protein palmitoylation (see below), which in certain transmembrane proteins modifies cysteine residues located in their cytoplasmic domains, is also a function of the intermediate compartment.
Employing the 15°C block and a semi-intact cell system, it has been possible to characterize in VSV-infected cells the requirements for the viral envelope G glycoprotein to enter into and exit from the intermediate compartment. It should be noted that when VSV-infected cells in which pulse-labeled temperature-sensitive G glycoprotein molecules have accumulated in the ER are perforated and incubated for protein transport in vitro, the molecules that emerge from the ER reach the cis region of the Golgi apparatus only after a lapse of approximately 10 to 20 min. In contrast, such lag is eliminated if the cells, before perforation, are incubated for 90 min at 15°C. This would be expected if, at the low temperature, vesicles carrying the protein had progressed to the intermediate compartment, even though they may not have undergone the fusion event required to deliver their cargo. In fact, completion of transport after the 15°C block still requires an energy supply, Ca\(^{2+}\), and GTP hydrolysis and is blocked by antibodies to NSF, the factor that is thought to mediate the fusion.

**THE GOLGI APPARATUS** 18, 297, 348, 373, 386a–389

The Golgi apparatus is a complex organelle that receives both luminal and membrane proteins that are exported from the ER and pass through the intermediate compartment. It effects a wide variety of posttranslational modifications on many of these proteins, including the processing of N-linked oligosaccharide chains to complex forms, the O-glycosylation of hydroxy amino acid residues, the phosphorylation of mannoses in enzymes destined to lysosomes, the fatty acylation of cysteines, the sulfation of oligosaccharide chains and tyrosine residues in proteins, and the proteolytic processing of many precursor polypeptides. It is also the site of synthesis of glycosaminoglycans (GAG) and sphingolipids. Whereas some of the proteins that reach the Golgi apparatus from the ER remain as permanent residents of its cisternae, others traverse the organelle and are either transported to the cell surface or are segregated within distal elements of the endomembrane system, such as secretory granules or lysosomes.

In secretory cells, and perhaps in all cell types, some membrane proteins that reach the cell surface by exocytosis are retrieved by endocytosis and returned to the Golgi apparatus for reutilization in the packaging of new secretory products. The multiple destinations of proteins that emerge from the Golgi apparatus, as well as its participation in the recycling of plasma membrane proteins, make this organelle the cell’s center for the distribution and sorting of proteins addressed to various subcellular locations.

**Structure and Organization of the Golgi Apparatus** 386a, 389, 396–401

Characteristically, the Golgi apparatus consists of stacks of three to eight slightly curved, membranous cisternae, or saccules, that are platelike near their centers and dilated toward their rims. Several of these stacks may exist within a single cell, and they may be interconnected. A Golgi stack shows a polarized organization with one side, the cis face (generally the convex one), oriented toward the ER and the opposite, trans face, oriented toward secretory granules or the centrioles. The cis-most cisternae is usually fenestrated and connected to the network of tubular and tubulovesicular elements that constitutes the intermediate compartment that transfers materials from the ER to the Golgi apparatus. Together, the intermediate compartment and the cis cisterna can be viewed as forming a CGN.

Coated vesicles that are thought to transport proteins from one Golgi cisterna to the next, in a cis-to-trans direction, are also found near the periphery of the Golgi stacks and may be seen fusing with or budding from the cisternal rims. Vesicles of this type have been generated in vitro, and their coat and the process of its assembly have been extensively characterized (see ref. 348 and discussion below). In certain types of secretory cells, proteins to be packaged in secretory granules first accumulate in the dilated rims of the two or three trans-most Golgi cisternae. In other secretory cells, such as those in pancreatic acini, the
concentration of secretory products takes place within separate dilated sacs, known as condensing vacuoles, that are adjacent to the trans face of the Golgi and appear to receive material from it by vesicular transport.\(^{304, 305}\)

In its trans-most region, the Golgi apparatus extends into a network of tubulovesicular structures that have somewhat thicker membranes and were originally known as GERL,\(^{397, 398}\) but are now generally referred to as the TGN\(^{388}\) trans-Golgi reticulum\(^{404}\) or transtubular network\(^{401}\). It is in this region of the trans-Golgi that many of proteins retrieved from the plasma membrane reach the organelle. Several different types of vesicles are seen near the TGN\(^{388, 405}\) and appear to originate from it. Among these are clathrin-coated vesicles that contain either lysosomal enzymes complexed to the mannose 6-phosphate receptor (see below) or, possibly, secretory proteins destined to be stored in secretory granules. Other vesicles appear to lack a coat (but probably had one when they were formed) and are likely to ferry a constitutive flow of secretory and membrane proteins to the cell surface. In polarized epithelial cells, membrane proteins destined to the two different plasma membrane domains (apical and basolateral) are packed into different vesicles at the TGN.\(^{406-408}\) The TGN has thus emerged as the major site of sorting for proteins that traverse the endomembrane system.

The polarized organization of the Golgi apparatus is also apparent morphologically from a progressive increase in the thickness of its cisternal membranes, from the cis to the trans side, seen in EM, and from the intense staining of the cis cisternae when cells are incubated for long times with OsO\(_4\).\(^{409}\) Cytochemically, the trans-most cisternae are characterized by a thiamine pyrophosphatase activity\(^{403, 410}\) that is not present in the TGN elements. However, TGN elements do show acid phosphatase activity,\(^{388}\) presumably reflecting the presence of this hydrolase en route to lysosomes.

The cis-, medial, and trans-Golgi cisternae represent a series of subcompartments enriched in specific enzymatic activities that sequentially carry out postranslational modifications on newly synthesized proteins that traverse the organelle unidirectionally. A combination of cytochemical, immunoelectron microscopic, and cell fractionation studies has defined a general pattern of organization within the Golgi apparatus (Fig. 16-26) of the enzymes involved in the processing by N-linked oligosaccharide chains (see refs. \(^{411}\) to \(^{415}\)). The cis cisternae contain most of the N-acetylgalacosaminylphosphotransferase and the N-acetylgalactosamine-1-phospho-diester-\(\alpha\)-N-acetylgalacosaminidase that add the phosphate marker to the mannose residues of newly synthesized lysosomal hydrolases (see below). An \(\alpha\)-mannosidase I, that reduces to five the number of mannose residues in oligosaccharides partially trimmed in the ER, is also found in the cis and, possibly, the medial cisternae. As previously mentioned, the presence in a protein of N-linked oligosaccharides trimmed to five mannose residues can be detected by their sensitivity to the action of endo D, and this can be used to demonstrate the transport of a glycoprotein to the Golgi apparatus. Medial cisternae contain a number of enzymes, including the N-acetylgalacosaminyltransferase I that adds the first outer GlcNAC residue in the formation of complex oligosaccharides, the \(\alpha\)-mannosidase II that is responsible for the removal of the next two mannose residues and hence confers endo H resistance to N-linked oligosaccharides, the transferase II that adds a second outer GlcNAC, and the fucosyltransferase that modifies the innermost GlcNAC. The glycosyltransferases that add galactose and sialic acid residues to the regrowing oligosaccharides have been localized mostly to the trans-most cisternae and the TGN, respectively.
Chapter 16: The Biogenesis of Membranes and Organelles

Fig. 16-26:

\[ \text{from ER} \]

\[
\begin{align*}
\text{cis golgi} & \quad \text{phosphodiester-acetyl-glucosaminidase} \quad \text{phosphotransferase} \quad \alpha - \text{mannosidase I} \\
\text{medial golgi} & \quad \text{Glc NAc transferase I} \quad \text{Glc NAc transferase II} + \text{fucosyl transferase} \\
\text{trans golgi} & \quad \alpha - \text{mannosidase II} \\
\end{align*}
\]

\[ \text{sialyl transferase} \]

- N-acetylglucosamine
- mannose
- fucose
- galactose
- sialic acid
Sequential modifications of oligosaccharide chains as glycoproteins move through the Golgi apparatus. A glycoprotein containing a high-mannose oligosaccharide is transferred from the ER to a cis-Golgi cisterna. If the protein is a lysosomal hydrolase, it acquires the mannose 6-phosphate marker by the sequential action of N-acetylglucosaminylphosphotransferase (phosphotransferase) and N-acetylglucosamine-1-phosphodiester α-N-acetylgluc... 

The general pattern of distribution just described does not imply that Golgi enzymes that carry out successive reactions are totally segregated from each other. Some overlapping in the distribution of enzymatic activities was revealed by the simultaneous immunolocalization of GlcNAc-transferase I and galactosyltransferase. This showed that, although the bulk of the former enzyme is present in the medial Golgi, substantial amounts are also found in trans cisternae. On the other hand, galactosyltransferase is present not only in the trans cisternae, but in the TGN as well. Thus, different cisternae may contain characteristic mixtures of enzymes that may even vary with the cell type, and the ordered modification of oligosaccharide chains in glycoproteins may be as much a consequence of the restricted specificity of the various glycosidases and transferases as of their physical segregation in separate compartments. Some authors have advanced the extreme view that the Golgi apparatus is composed of only three compartments—CGN, Golgi stacks, and the TGN. The first and last of these compartments would primarily play a sorting role, while oligosaccharide processing would occur in the Golgi stacks, where the glycosyltransferases and glycosidases would be intermixed.

In addition to the different sets of processing enzymes that they encounter as they move across the Golgi apparatus, newly synthesized proteins confront environments of decreasing pH in their passage from the cis cisternae to the TGN. The presence of a proton pump has been demonstrated in isolated Golgi fractions. Studies using a probe detectable by immunoelectron microscopy, whose accumulation within membrane-bound compartments is an inverse function of the pH, have revealed that the trans cisternae and the TGN are substantially more acidic than the cis Golgi cisternae. Moreover, secretory vesicles in the trans-Golgi region were found to have a low pH, comparable to that of the TGN from which they are derived.

The drug monensin, which is an ionophore that exchanges K+ ions for protons and thus dissipates pH gradients across membranes, inhibits the secretion of many proteins and the passage of viral envelope glycoproteins through the Golgi apparatus. This drug leads to a remarkable swelling of Golgi cisternae, and the movement of the viral glycoproteins through the organelle is halted within the medial region. It should be noted that treatment of cells with lysosomotropic drugs (i.e., weak bases such as primaquine, chloroquine, and NH₄Cl), which traverse membranes in their uncharged forms but in their protonated forms accumulate within acidic compartments (raising the intravesicular pH), also affects the secretion of many proteins, albeit to different extents for different proteins within a given cell. These drugs appear to act at a late Golgi or post-Golgi stage. In fact, primaquine completely blocks the secretion of albumin from hepatoma cells, leading to its accumulation within vesicles in the trans side of the Golgi. It has a lesser effect on the secretion of other proteins, such as transferrin. The acid pH of the Golgi apparatus may actually be required for some proteins to achieve a conformation that allows them to exit from the organelle. Conversely, the aberrant conformation of some abnormal proteins generated using recombinant DNA techniques may be manifested at the acidic pH of Golgi elements, which may account for their accumulation in this organelle.
It has been suggested that a pH gradient may play a role in determining the unidirectional transport of proteins through the Golgi apparatus. It is known that the low pH of endosomes (see below) mediates the dissociation of some ligands from their receptors, which allows for the return of interiorized receptors to the plasma membrane. One could imagine that vesicles that effect the successive transfer of proteins from cisternae to cisternae contain pH-sensitive receptors for these proteins that release their ligands at different pHs, and that this is necessary for the vesicles to return to the cisternae of origin. This idea, however, is not in accord with the notion that transport across the Golgi apparatus is a non-receptor-mediated bulk flow process.

The organization of the Golgi apparatus is dependent on the integrity of the microtubular system: drugs that disassemble microtubules lead to fragmentation of the organelle into dispersed smaller cisternal stacks that remain functional and can reassemble on removal of the microtubule depolymerizing agent. This type of fragmentation and preassembly occurs naturally as cells pass through mitosis.

The remarkable effects of the drug brefeldin A (BFA) on the endomembrane system have provided insights into the dynamic relationships that exist between the Golgi apparatus on one side and the ER or the endosomal compartment on the other. This fungal metabolite (a macrocyclic lactone synthesized from palmitate), resembling an acyl group, is a powerful inhibitor of ER-to-Golgi transport and of anterograde transport through the Golgi stacks (see refs. 432 and 433). Treatment of cells with BFA leads to the disassembly of the Golgi complex and to the microtubule-dependent retrograde movement of Golgi enzymes into the ER. The TGN, however, undergoes fusion with endosomes, and its components do not undergo retrograde transport to the ER. In BFA-treated cells, therefore, resident ER proteins mix with enzymes of the Golgi stacks and may acquire modifications that are characteristic of proteins that normally reach the Golgi apparatus (e.g., refs. 262, 438, and 439).

The retrograde movement of Golgi components to the ER induced by BFA appears to take place via narrow membrane tubules (90 nm in diameter) that do not have an apparent cytoplasmic coat and during the first 10 min of incubation with the drug emerge from swollen Golgi cisternae and extend along microtubules. Although the mechanism for the resorption of Golgi components into the ER is not fully understood, it is likely that this occurs because the drug selectively inhibits the forward transport of proteins from the ER to the Golgi and through this organelle, without blocking the retrograde transport that, presumably, normally retrieves components of the transport machinery that function cyclically. The finding that tubular membrane extensions are prominent soon after BFA is added to cells suggests that such structures may be the normal vehicles for retrograde flow within the Golgi apparatus and from this organelle to the ER. The molecular basis for the inhibitory effects of the drug on anterograde transport through the Golgi is described below.

### Signals for the Localization of Resident Proteins in the Golgi Apparatus

The critical role that the Golgi apparatus plays in the posttranslational modification and sorting of a wide variety of itinerant as well as resident proteins is dependent on the segregation of the necessary enzymes, such as glycosyltransferases, glycosidases, proteases, and sugar nucleotide transporters, in specific regions of the organelle.

The behavior of chimeric constructs containing portions of Golgi resident proteins linked to segments of reporter proteins that normally completely transverse that organelle has begun to provide information on the location and nature of the signals that determine the localization of the resident proteins, as well as some insight into the mechanisms by which these signals act. Several studies indicate that the
transmembrane domains of proteins of the CGN and Golgi stacks are responsible for their localization. This first became apparent\(^{442-444}\) from the analysis of the M envelope protein of the avian coronavirus infectious bronchitis virus (IBV). This membrane protein accumulates in the CGN/cis region of the organelle, leading to budding of the virus into the lumen of the cisternae, from which the viral particles are transported to the plasma membrane within vesicles, in the same manner as a secretory protein.

Necessary and sufficient information for targeting of the M protein to the CGN was found to be contained within the first (N-terminal) of its three transmembrane domains (TMD), which consists of an amphipathic \(\alpha\) helix with uncharged polar residues (Asn, Thr, Gln) on one of its faces. When incorporated in a chimeric protein, this \(\alpha\) helix was able to confer Golgi localization to proteins normally destined to the plasma membrane, and mutations in its polar residues led to transport of the chimeras to the cell surface. It is noteworthy that the M protein of a murine coronavirus that is targeted to the TGN, rather than the CGN, contains a similar amphipathic helix, but this did not localize a reporter protein to the Golgi.\(^{441}\) In this case, a cytoplasmic segment of 18 amino acids preceding the TMD may be necessary for Golgi localization, since its deletion caused diversion of the protein to lysosomes.\(^{445}\) Thus, the different sites of budding within the Golgi of the two coronaviruses seem to be determined by two different types of sorting signals in their M proteins.

Cloning of the cDNA for several endogenous proteins of the Golgi stacks has allowed similar studies on the identification of their localization signals. It is quite striking that all the Golgi stack enzymes whose cDNAs have been cloned—\(\beta\)-1,4-galactosyltransferase (GT); \(N\)-acetylglucosaminyltransferase (transferase I); \(\alpha\)-2,6-sialyltransferase (ST); and \(\alpha\)-mannosidase II—have a type II transmembrane disposition in which a short N-terminal cytoplasmic segment is followed by a signal/anchor sequence and a large C-terminal catalytic domain located in the cisternal lumen. For the three transferases, localization signals sufficient for Golgi retention have been found in their transmembrane domains, although flanking sequences were found to enhance the efficiency of retention.\(^{446-453}\) It has been proposed\(^{440, 447}\) that the function of these signals is to mediate the formation within Golgi membranes of hetero-oligomeric complexes that would include different protein molecules that are to be retained in the same compartment and that such complexes might be stabilized by interactions with other proteins in the luminal and/or cytoplasmic sides of the membranes. As was proposed for the retention of certain proteins in the ER,\(^{256}\) the inclusion of resident membrane proteins in two-dimensional lattices formed by oligomerization or aggregation would be an effective means of preventing their entrance into the bulk flow carrier vesicles that effect constitutive transport through the organelle. That such a retention mechanism operates in the Golgi is supported by the finding that overexpression of several of the glycosyltransferases leads to their “backup” accumulation in the ER, rather than to their escape to the cell surface.\(^{447, 449, 451, 452}\) This would be expected if the signals act by mediating oligomerization, which, in principle, could occur in the ER at high enough protein concentrations. On the other hand, if the signals mediate Golgi localization by interacting with retrieving or retaining receptors, saturation of such receptors in overexpressing cells should allow the excess proteins to escape to the cell surface.

In contrast to enzymes of the Golgi stacks, all proteins of the TGN that have been studied (one mammalian and several yeast proteins) have a type I transmembrane disposition and contain localization signals in their cytoplasmic tails. In TGN38, a mammalian protein whose function is not yet known, the signal consists of a short tyrosine-containing segment (YQRL) closely resembling the endocytic signals that mediate the internalization of plasma receptors or lysosomal membrane proteins at the plasma membrane (see below). Indeed, in a chimera, the sorting signal within TGN38 was able to function as an internalization signal in the fraction of molecules that were present in the plasma membrane. Clearly, however, the function of the signal in TGN38 is not limited to triggering internalization since a mutation that changed it to YQDL (the internalization signal in the asialoglycoprotein receptor) did not diminish its
endocytic ability, but markedly reduced its capacity to cause TGN localization. The YQRL signal appears to be recognized by a saturable receptor responsible for the TGN localization of the protein, since the overexpression of chimeric proteins containing the signal led to their accumulation in the plasma membrane and caused the disappearance of the endogenous protein from the TGN. This would be expected if the chimera and the endogenous protein competed for interaction with a saturable receptor.

Critical sorting information was also found to be contained within the cytoplasmic tails of several yeast proteins of the trans-Golgi: Kex1p, Kex2p, and dipeptidylaminopeptidase A (DPAP A). These enzymes effect the proteolytic processing of precursor proteins, and comparable enzymes [the furin/PACE (paired basic amino acid residue cleaving enzyme)] are present in mammalian cells, probably in the TGN. Deletion of the cytoplasmic tails of the yeast proteins leads to their default transport to the vacuole. In the case of Kex2p, a specific tyrosine residue in the cytoplasmic tail was shown to be essential for Golgi localization. It appears that clathrin, the major structural protein of the coat of vesicles that mediate endocytosis at the plasma membrane and ferry lysosomal enzymes to the incipient lysosome, is involved in retaining Kex2p and DPAP A in the yeast trans-Golgi, since a temperature-sensitive mutation in the clathrin heavy chain causes missorting of the proteins to the cell surface at the nonpermissive temperature. This suggests that retention of the proteases in the TGN is mediated by an interaction of their cytoplasmic tails with the adaptor polypeptides that assemble clathrin coats (see below).

**Intercisternal Traffic within the Golgi Apparatus**

The sequential passage of a protein across a stack of Golgi cisternae was first strikingly demonstrated in immunoelectron microscopic studies with cells infected with a temperature-sensitive mutant of VSV, in which it is possible to synchronize the transport of the envelope glycoprotein G out of the ER. It was clearly shown that the G glycoprotein enters the Golgi apparatus at the cis cisterna, traverses the organelle vectorially in approximately 10 min, and exits at the opposite face. When VSV-infected cells are incubated at low temperatures (20°C), large amounts of G glycoprotein accumulate in the TGN, which becomes greatly expanded at this temperature. When the temperature is raised to 32°C, G glycoprotein molecules exit from the TGN toward the plasma membrane within vesicles that are not coated by clathrin.

The demonstration that transfer of newly synthesized proteins through the Golgi stack is a vectorial process involving vesicular carriers that bud from a donor cisterna and fuse with an acceptor one, rather than the flow of proteins along permanent physical connections between cisternae, relied both on cell fusion experiments and on in vitro systems in which vectorial transfer between cisternae of different stacks was shown. These experiments were possible because of the availability of mutant cell lines defective in some of the oligosaccharide processing enzymes. In these cells, the processing of the oligosaccharide chains is arrested at specific points, but the glycoproteins continue to be transferred to the cell surface. Transfer from the cis to medial cisternae was demonstrated when cells deficient in the medial Golgi enzyme GlcNAc transferase I were infected with VSV and fused with wild-type cells. The final mature G glycoprotein produced was shown to carry the normal terminal sugars and therefore had been transferred from the Golgi apparatus of the infected mutant cell to that of the uninfected wild type, where the normal transferase was found. In a similar experiment, transfer from the medial to the trans cisternae was inferred from the finding that G glycoprotein molecules synthesized in VSV-infected mutant cells that lacked the trans-Golgi enzyme galactosyltransferase were processed to the normal terminally glycosylated form after the infected cells were fused with wild-type cells. The unidirectionality of the transfer between the cisternae within a Golgi stack was demonstrated by the observation, in pulse-chase experiments, that if the cell fusion was carried out after the labeled protein was expected to have passed the appropriate subcompartment in the donor Golgi, processing by the wild-type Golgi apparatus enzyme did not occur.
Biochemical Dissection of Intra-Golgi Transport

The concept that vesicles mediate the intercisternal transfer of proteins in the Golgi apparatus has received its strongest support from experiments in which the transfer of the VSV G protein from the cis compartment of one Golgi stack to the medial compartment of another was achieved in a cell-free system. This allowed the isolation of the putative carrier vesicles and their extensive biochemical characterization, including a determination of the requirements for their formation and consumption. However, it has not yet been possible to show that the vesicles, once isolated, have the capacity to deliver their content to acceptor membranes.

The cell-free intra-Golgi transport system utilizes as a donor a Golgi fraction isolated from cells that lack GlcNAc transferase I and that were previously infected with VSV. The acceptor is a Golgi fraction obtained from uninfected wild-type cells. Arrival of the G glycoprotein to the acceptor compartment is detected by its acquisition of $[^{3}H]N$-acetylglucosamine residues. In this system, intercisternal transport occurs at physiological temperatures and requires ATP, a cytosolic fraction, and the integrity of proteins that are exposed on the surface of the acceptor membrane and are, therefore, sensitive to proteases.

The kinetics of in vitro intra-Golgi transport show a 7- to 10-min lag before the rate of acquisition of $[^{3}H]N$-acetylglucosamine becomes linear. EM reveals that during this period there is a marked increase in the number of “coated buds” and coated vesicles seen in close proximity to the donor Golgi membranes. Hence, the lag is thought to correspond to the time required for a “donor priming reaction” that leads to the generation of coated vesicles in the donor cisternae. Accordingly, the lag is markedly reduced when the donor Golgi fraction is preincubated with cytosol and ATP in the absence of the acceptor. The vesicles generated in this reaction appear to represent “bulk flow carriers,” in which the cargo molecules are not concentrated and, in fact, are not even necessary for vesicle formation. Thus, the concentration of G glycoprotein in the putative transport vesicles measured by immunoelectron microscopy was found to be very similar to that in the surrounding parental cisternal membranes. Moreover, the formation of the vesicles was not inhibited when the cells from which the donor Golgi was obtained were preincubated for a long time with the protein synthesis inhibitor, cycloheximide, which should clear the Golgi cisternae from protein molecules in transit through the organelle. However, primaquine, an agent that raises the pH within the Golgi cisterna, completely and irreversibly blocks the formation of vesicles but does not affect the targeting and fusion of the vesicles that were formed before the drug was added. Use of this inhibitor allowed the demonstration that the number of transport-competent vesicles that are present in the in vitro system reaches a maximum after approximately 15 min of incubation.

Before we discuss in detail the sequence of biochemical events that takes place during intercisternal transport, it will be useful to provide a general outline of this process. The formation of a transport vesicle in the donor membrane begins with the assembly of a coat from macromolecular complexes (600 to 700 kDa) called coatomers (for coat protomers) that are present in the cytosol. The coat is thought to serve as a mecanochemical device that induces the membrane curvature necessary to form a vesicle. The recruitment of coatomers to the Golgi membrane is triggered by the active, GTP-containing form of ARF, a small myristoylated GTP-binding protein that inserts into the membrane and is itself subsequently incorporated into the coat. The coated vesicle that is released from the donor membrane docks on the acceptor membrane and only then does the vesicle shed its coat, in a step that requires the hydrolysis of GTP bound to ARF. Uncoating is followed by fusion of the vesicle membrane to the acceptor membrane—a poorly understood process that requires the activity of NSF, a factor that normally appears to be incorporated into the transport vesicle during its formation at the donor membrane.
The purification of the coated vesicles and the biochemical characterization of their coat became possible after it was observed that addition of the nonhydrolyzable GTP analogue, GTP-γ-S, blocks transport in the cell-free system measured by the incorporation of $[^3H]\text{GlcNAc}$ into G glycoprotein and leads to the marked accumulation of coated vesicles ($\approx 110$ nm in diameter) on the acceptor membranes. This finding provided the first indication that GTP-binding proteins are involved in the transport process and that GTP hydrolysis may be required for vesicle uncoating. The accumulated coated vesicles could be removed from the Golgi stacks in a high-salt medium. It was thus possible to show that they contain a specific set of polypeptides, with prominent components of $M_r = 170, 110, 98, \text{ and } 61 \text{ kDa}$, which are exposed on the vesicle surface and were later called $\alpha$, $\beta$, $\gamma$, and $\delta$-coat proteins (COP), respectively. COP proteins have a peripheral association with the vesicle membrane. In fact, they are found predominantly in the cytosol within coatomer complexes that, when purified, were shown to contain two additional polypeptides of 36 and 20 kDa, now designated $\varepsilon$ and $\zeta$-COP, respectively.

$\beta$-COP was found to be identical to a 110-kDa peripheral membrane protein of the Golgi apparatus that was first identified by immunocytochemistry with a specific monoclonal antibody raised against a Golgi fraction. Immunelectron microscopy clearly established that $\beta$-COP is associated with forming buds in Golgi membranes and with the coated vesicles that accumulate during incubation of the Golgi fraction with GTP-γ-S. The sequence of $\beta$-COP, deduced from its cloned cDNA, revealed a limited but significant (17 percent) similarity between its N-terminal 450 residues and the corresponding portion of $\beta$-adaptin, a component of the coat of clathrin-coated vesicles (see below), suggesting that the two types of coat proteins are evolutionarily derived from a common precursor.

The coated vesicles that accumulate after GTP-γ-S treatment also contain two closely related small (21-kDa) GTP-binding proteins of the ARF family that play a critical role in coat assembly. The N-myristoylated proteins are present in stoichiometric amounts with respect to the COP polypeptides (three molecules of ARF per $\beta$-COP molecule) and, therefore, are also structural components of the coat. In their GDP-bound state, the ARF proteins—first discovered because of their role as cofactors in the cholera toxin-catalyzed ADP ribosylation of the $G_\alpha$ subunits of heterotrimeric G proteins—are found in the cytosol, but are not part of the coatomers. After acquiring GTP, the ARF proteins spontaneously insert through their myristate moiety into phospholipid bilayers. Within the cell, active ARF molecules are associated primarily with Golgi cisternae and Golgi-derived membrane vesicles. Apparently, this is determined by the presence of a specific guanine nucleotide exchange factor (GEF) in Golgi cisternae that locally activates ARF molecules and leads to their stable association with the membrane. This, in turn, promotes binding of the coatomers and their assembly into a coat (Fig. 16-27). In fact, the production of COP-coated vesicles from Golgi cisternae has been achieved in vitro in the absence of any other cytosolic proteins, by the simple addition of coatomers and a recombinant pure myristoylated ARF protein synthesized in E. coli. It is noteworthy that vesicle formation requires acyl CoA (supplied experimentally as palmitoyl CoA), and it is believed that this cofactor donates its fatty acid to an unknown acceptor that is activated by acylation.
Model for the ARF-mediated assembly of coatmer-coated vesicles in the Golgi apparatus. (1) A guanine nucleotide exchange factor (GEF) in the donor Golgi membrane catalyzes the exchange of GTP for GDP in cytosolic ARF molecules. In their active form these become associated with the membrane through the insertion of their N-linked myristate tails in the lipid bilayer. (2) Membrane-associated ARF molecules recruit (perhaps with the assistance ... 

Since the active ARF is a key structural component of the vesicle coat, it is expected that hydrolysis of its bound GTP, probably triggered by a GAP protein in the target membrane, will lead to disassembly of the coat. Thus, when ARF is charged with the nonhydrolyzable analogue, GTP-γ-S, transport is blocked and COP-coated vesicles accumulate on the acceptor membranes. It is noteworthy that proteins of the ARF family appear to also play critical roles in other vesicular transport steps, such as ER-to-Golgi
transport, and in the formation of clathrin-coated vesicles in the TGN. In fact, recombinant ARF1 stimulates both the binding of coatomers to Golgi membranes and the binding of AP1 adaptors, the proteins that promote the assembly of clathrin coats on the Golgi membrane.

A detailed understanding of the molecular interactions involved in the assembly of COP-coated vesicles on Golgi membranes owes much to studies using the drug BFA. In the in vitro system for intra-Golgi transport, BFA inhibited the production of COP-coated vesicles and also led to the formation of extensive tubular interconnections between Golgi cisternae and separate Golgi stacks. Because of these events, even though vesicular transport was interrupted, the processing of the VSV glycoprotein in donor membranes by enzymes in the acceptor membranes still took place due to the intermixing of Golgi components from adjacent stacks.

It is now clear that BFA acts by inhibiting the assembly of coatomers on Golgi membranes. This was first suggested by the observation that within 30 sec of applying the drug, while the Golgi stacks are still intact, β-COP dissociates from Golgi membranes, but rapidly rebinds to them on removal of the drug. Because this effect of BFA was prevented by AlF$_3$ or by GTP-γ-S (when this analogue was added to permeabilized cells to allow its entrance into the cytoplasm), and because in the absence of the drug these same agents enhanced the association of coatomers (i.e., β-COP) with Golgi membranes, it became obvious that the assembly of the coat is regulated by guanine nucleotide binding proteins and that BFA could act by interrupting the cyclic function of one such protein. In fact, the drug was also shown to cause the rapid and reversible dissociation of ARF from Golgi membranes. Moreover, the in vitro binding of ARF to these membranes is enhanced by GTP-γ-S and inhibited by BFA, when this is added before ARF was bound. Other studies have shown that BFA blocks (possibly indirectly) the GDP-GTP exchange on ARF that is catalyzed by an exchange factor in the Golgi membranes and is required for ARF binding. One can therefore conclude that the inhibition of coat assembly caused by the drug is the result of its blocking activation of ARF.

Two facts initially pointed to the possible participation of heterotrimeric G proteins as regulators of intra-Golgi vesicular transport. One is that ARF proteins serve as cofactors for cholera toxin in the ADP ribosylation of Gα subunits and, therefore, are likely to interact with Gα subunits. The other is that AlF$_{3-5}$ (a complex of Al and F) that inactivates Gα subunits but has no effect on low-molecular-weight GTP-binding proteins, such as ARF or rab proteins, inhibits transport and stimulates coat assembly. Although the specific G protein(s) involved in intra-Golgi transport have not been identified, it appears that both Gα stimulatory (Gsα) and inhibitory (Giα) subunits are involved in regulating this process. Thus, when added to an in vitro system, purified βγ subunits—which are expected to capture and block the activity of free α subunits—inhibited the binding of ARF and β-COP to the Golgi membranes that is promoted by the addition of GTP-γ-S. These findings suggested a role for a heterotrimeric G protein as a positive regulator of transport. In addition, a pertussis-toxin-sensitive G$_{13}$α inhibitory subunit that could exert a negative control on vesicular transport through the Golgi apparatus has been localized to this organelle in cultured epithelial cells. Overexpression of this subunit in transfected cells was found to retard the constitutive secretion of a basement membrane heparan sulfate proteoglycan, an effect that was suppressed by pretreating the cells with pertussis toxin. The activation of a pertussis-toxin-sensitive G protein by mastoparan has been shown to antagonize the effect of BFA on the β-COP association with Golgi membranes. This indicates that a Gi protein regulates the cycle of β-COP utilization, possibly by inhibiting the budding of vesicles from the donor membranes.
Molecular Machinery for Vesicle Targeting and Fusion

The specificity of vesicular transport between different membrane compartments must be based on precise interactions between molecular components characteristic of the donor and acceptor membranes. On the other hand, the universality of vesicle transport processes makes it likely that certain steps that are common to all membrane fission and fusion events are carried out by the same protein machinery, regardless of the specific membranes involved. The paradigm for a protein that participates in multiple targeting processes is NSF, a soluble factor that was first identified as necessary for the completion of an intracisternal transport event in the Golgi apparatus, but is now known to play a role in many other interorganelar transport events.

The observation that intra-Golgi transport was almost completely inhibited by treatment of the Golgi membranes with NEM was the basis for the identification and purification of NSF. This factor is normally associated with Golgi membranes, but can be released from them by incubation in buffers containing ATP, a nucleotide that is also required to stabilize the protein through the purification procedure. As mentioned above, NSF—which is equivalent to the yeast SEC18 gene product—is required for vesicular transport between different compartments of the endomembrane system. NSF is a homotetrameric molecule of 76-kDa subunits that contain two putative ATP-binding sites each. The factor manifests a low ATPase activity that appears to be essential for its function in mediating vesicle fusion.

NSF acts late in intra-Golgi transport, after vesicle docking and uncoating. In fact, when in vitro transport is carried out in the absence of NSF and with NEM-treated Golgi fractions, uncoated vesicles accumulate on the acceptor membranes. This is in contrast to the effect of the nonhydrolyzable analogue GTP-γ-S, which causes the accumulation of only coated vesicles. That the uncoated vesicles observed after NEM treatment are derived from coated ones, and that NSF acts after uncoating has taken place, was shown by the observation that when NEM-treated Golgi membranes are incubated in the in vitro system in the presence of the GTP-γ-S, only coated vesicles accumulate. Furthermore, the restoration of NSF to an NEM-treated system in which uncoated vesicles were allowed to accumulate led to a substantial completion of transport, even when GTP-γ-S was added. Although these observations indicate that NSF can function even when added late to the transport system, after vesicles have been formed and docked, the factor normally appears to be incorporated into the vesicles during their formation in the donor membrane.

As mentioned in the section on ER-to-Golgi transport, the binding of NSF to Golgi membranes is mediated by SNAPs, which themselves recognize receptors (SNAP receptors [SNAREs]) that are integral components of the membranes. Although SNAREs have not yet been purified from Golgi membranes, it has been possible to purify such proteins from a crude brain membrane fraction. It is now believed that NSF-SNAP-SNARE interactions are required to execute most, if not all, vesicular transport events within the endomembrane system (see refs. and ). There is indirect evidence for the existence of two types of SNAP receptors, those (U-SNAREs) which are found in the membranes of the donor organelle and in the vesicles derived from them and those (t-SNAREs) that are present in the acceptor or target membranes. It has been proposed that during the docking step that precedes membrane fusion complementary SNAREs interact in a process that is facilitated by SNAP and NSF molecules that hold the vesicle (donor) and acceptor (target) membranes together (Fig. 16-28). Fusion would then ensue by a not yet understood process.
Interactions between complementary SNAREs may determine the specificity of vesicle targeting to an acceptor membrane. SNAREs are integral membrane proteins that, in general, appear to be inserted in the membrane through their C-terminal ends and have most of their mass exposed on the cytoplasmic surface of the membrane. Complementary SNAREs are thought to be present in both the transport vesicles (v-SNARE), which acquire them during vesicle...

Three SNAP polypeptides, α-, β-, and γ-SNAPs (33, 34, and 36 kDa, respectively), were purified from bovine brain cytosol using as an assay their capacity to stimulate the binding of NSF to Golgi membranes. The first two SNAPs (α and β) are 83 percent identical in sequence and bind competitively in vitro to the same receptors, although β-SNAP is a protein specific to brain tissue. γ-SNAP binds to a different site and acts synergistically with α-SNAP in stimulating transport.

α-SNAP is the mammalian equivalent of the yeast Sec17 gene product required for ER-to-Golgi transport in yeast and it, but not β- or γ-SNAP, can restore the capacity of a sec17 yeast mutant cytosol to support intra-Golgi transport in a mammalian in vitro system.

NSF binds stably to SNAPs only after the latter are bound to their membrane receptors. NSF-SNAP-SNARE complexes formed on intact Golgi membranes can be isolated after detergent treatment but can also be formed when detergent extracts containing SNAREs are mixed with SNAPs and NSF. The complexes (which have a sedimentation coefficient of 20S) contain γ-SNAP and α- or β-SNAP as alternative subunits. NSF must contain bound ATP to assemble into these complexes. When ATP hydrolysis (which occurs in the presence of Mg$^{2+}$ even at 0°C) is allowed to take place, the particles disassemble and NSF is released. The 20S complexes, however, are stable in the absence of Mg$^{2+}$ or when the nonhydrolyzable ATP analogue ATP-γ-S is present. These properties, together with the availability of recombinant SNAPs and a functionally active form of NSF containing an epitope tag at its C-terminus, for which an antibody is available, have allowed the purification of the SNAP receptors from detergent extracts of crude brain membrane fractions. NSF-SNAP-SNARE complexes formed in the presence of ATP-γ-S were recovered through the binding of the epitope tag on NSF to an immobilized antibody. Subsequent addition of ATP-Mg$^{2+}$ led to the specific elution of the SNAP bound to their SNARE.
Four different brain SNARE polypeptides were obtained in this manner and recognized as previously known proteins on the basis of partial peptide sequence data. Two of these are the closely related proteins, syntaxins A and B, that are anchored to the plasma membrane through their hydrophobic C-terminal peptide segments and are thought to be involved in the docking of synaptic vesicles near the voltage-gated calcium channels in the presynaptic membrane (see ref. 360). Of the other two SNARE obtained, one (VAMP, also known as synaptobrevin-2) is an 18-kDa integral membrane protein of the synaptic vesicle that is also anchored to the surface of the vesicle by a C-terminal hydrophobic domain.504 The other is a protein named (coincidentally) SNAP25, (for synaptosome-associated protein of 25 kDa), whose exact location has not been determined, although it is found in the presynaptic terminal of neurons.

As previously noted in our discussion of ER-to-Golgi transport in yeast, the synaptic vesicle membrane protein VAMP/synaptobrevin shows significant sequence similarity to the yeast proteins Sly12pBet1p and Sly2p/Sec22p, which are required for transport between the ER and Golgi apparatus, with the latter protein actually becoming incorporated into the transport vesicles. Evidence has indicated that VAMP/synaptobrevin is even more closely related to a yeast gene (SNC2) product that is localized to post-Golgi vesicles and is required for Golgi-to-plasma-membrane transport.361 Syntaxins are related to three yeast proteins required for vesicular transport: Sec5p, a multicopy suppressor of the loss of the Erd2p, the carrier that retrieves ER proteins with the HDEL C-terminal signal; Pep12p, a protein required for targeting to the yeast vacuole; and the product of SSO1, a multicopy suppressor of a mutation in Sec1p, a protein required for transport to the plasma membrane (see ref. 361). These similarities suggest that the machinery responsible for the regulated secretion of neurotransmitter at the synaptic ending may have evolved from the primitive machinery that mediated constitutive vesicle delivery across the endomembrane system and to the plasma membrane.

SORTING OF PROTEINS THAT EXIT FROM THE GOLGI APPARATUS: SECRETORY PATHWAYS IN EUKARYOTIC CELLS56, 505–507

Proteins that traverse the Golgi apparatus and reach the TGN must be sorted into membrane-bounded carriers (vesicles or granules, see below) that transport them to their ultimate destination, which may be in the extracellular space, in a specific plasma membrane domain, or in endosomes or lysosomes. Many proteins destined for delivery to the plasma membrane or to be secreted from the cell are transported directly from the Golgi apparatus to the cell surface by a population of vesicles that continuously emerge from the TGN. On exocytosis, the membrane proteins of these vesicles become incorporated into the plasma membrane and the luminal content proteins are discharged into the extracellular medium. This is the basis for the so-called constitutive or nonregulated secretion of proteins. The continuous discharge that takes place during this process can be contrasted with the regulated secretion of proteins and other products (see below) that takes place in many specialized cells. Regulated secretion requires the prior concentration and storage of secretory products into secretory granules or vesicles that release their content into the extracellular space only when an appropriate stimulus leads to fusion of the granule membrane with the plasma membrane.

 Constitutive secretion is a generalized function of all cells. It represents the mechanism by which hepatocytes secrete a wide variety of serum proteins into the blood, fibroblasts secrete collagen and other components of the extracellular matrix, and plasma cells secrete immunoglobulins. The regulated secretory pathway, on the other hand, is utilized by many exocrine and endocrine cells, which must respond quickly to physiological stimulation with a burst of secretory activity. For example, digestive enzymes are stored within zymogen granules of pancreatic acinar cells and, on stimulation by a secretagogue, are released on the apical surfaces of the cells, which confront the acinar lumen, after which they are transported to the intestine. Similarly, distinct cell types in the anterior pituitary gland store
specific hormones that are released by exocytosis when the cells are stimulated by the appropriate hypothalamic releasing hormones. In addition, the extended processes of some neurosecretory cells, such as those that constitute the neurohypophysis, are packed with large amounts of secretory granules that are formed in the Golgi apparatus of hypothalamic neurons, and are transferred to the nerve terminals where exocytosis takes place. Many physiological mechanisms rely on unicellular glands—such as mast cells, blood granulocytes, and platelets—that also store their secretory products within granules and discharge their content at the cell surface after appropriate stimulation.

Release of neurotransmitters from neurons represents an important example of regulated secretion. In these cells, two types of secretory vesicles whose contents are released in a regulated fashion are produced. \(^{508}\) One consists of large dense core vesicles (LDCV), which contain peptide neurotransmitters derived from proteins synthesized in the ER. These vesicles are formed in the TGN and are equivalent to the secretory granules of other secretory cells. The other type consists of synaptic vesicles (SV), which are highly homogeneous in size (50 nm), lack a dense core, and secrete nonpeptide neurotransmitters on stimulation of the neuron. Synaptic vesicles are clustered near their release sites at nerve endings and undergo repeated cycles of exocytosis and endocytosis within the nerve terminal, where they are refilled with the neurotransmitters. \(^{507, 509}\)

The exocytotic event required for regulated secretion results from the activation of a signal-transducing mechanism that involves either the interaction of a ligand with a plasma membrane receptor or the arrival of a nerve stimulus that opens ion channels. By a variety of mechanisms, these events lead to a transient rise in the cytoplasmic concentration of a “secondary messenger,” such as \(\text{Ca}^{2+}\), cAMP, or a phosphoinositide (see ref. \(^{510}\)). In a process that is still not well understood, this leads to fusion of the membrane of the secretory vesicle or granule with the plasma membrane.

**Biogenesis of Secretory Granules** \(^{511-514a}\)

Secretory granules (SG) are membrane-bound structures that contain a dense core in which the secretory material is highly concentrated. In some granules, the dense core is a stable structure that remains intact even when the membrane is removed after isolation of the granules by cell fractionation. \(^{515, 516}\) The size and shape of a secretory granule is generally characteristic of the products that are stored in it. Frequently, different cell types can be identified by the morphologic characteristics of their granules, as in the pituitary, endocrine pancreas, and blood granulocyte populations.

Many cells capable of regulated secretion are specialized for the production of one major secretory protein (e.g., pituitary thyrotrophs) or of several polypeptides that are derived from a single precursor (e.g., proopiomelanocortin in pituitary corticotrophs). Other cells, such as those in the exocrine pancreas, store within a single type of granule a variety of independently produced secretory proteins \(^{517-521}\) that are released together on stimulation. However, some endocrine cells, such as the pituitary gonadotrophs, are able to store two different polypeptide hormones (i.e., LH and FSH) in separate granules, which may be easily identified by their different sizes. \(^{522}\) It is reasonable to assume that in gonadotrophs this segregation is important to allow for the differential release of each hormone at different periods of the menstrual cycle. In the somatomammotrophs of the bovine anterior pituitary, three major types of granules are formed: one contains primarily prolactin, another growth hormone, and a third granins (see below) together with luteinizing hormone (LH) and thyroid-stimulating hormone (TSH). \(^{523, 524}\) In human neutrophils, which also segregate different sets of secretory products into distinct granule types \(^{525-527}\) (e.g., azurophilic, specific, and secretory gelatinase-containing granules), different stimuli may lead to the differential release of the content of each type of granule. \(^{527}\)
Studies with pancreatic exocrine and endocrine cells and with cultured tumor cell lines have shown that both the constitutive and regulated pathways can operate in a single cell and that different products are channeled preferentially to each pathway. For example, AtT-20 cells (a line of rat pituitary origin) form secretory granules that contain ACTH and other derivatives of POMC. On stimulation with 8-Br-cAMP, they release their granule contents by exocytosis. Although these cells are used as a model to study secretion, the segregation of POMC to the secretory granules is not very efficient. More than two-thirds of the newly synthesized POMC is also steadily released by the constitutive pathway. On the other hand, laminin, a component of the extracellular matrix, is secreted from the same cells essentially exclusively by the constitutive route. A truncated secretory version of the VSV G glycoprotein, synthesized in AtT-20 cells transfected with the appropriate gene, is also excluded from the regulated pathway.

These observations suggest that proteins incorporated into secretory granules contain specific information that leads to their segregation from the constitutively secreted polypeptides which follow different pathways to the surface. In fact, other endocrine or exocrine products, such as proinsulin, growth hormone, and trypsinogen, when synthesized in transfected AtT-20 cells, are as effectively incorporated into granules as the endogenous derivatives of POMC. Similar findings have been made with GH₃ cells, a growth hormone-producing cell line, which after transfection with a gene encoding parathyroid hormone (PTH) incorporate this polypeptide in the same granules in which growth hormone is packaged. Since proteins such as these that normally follow the regulated pathway are secreted constitutively when expressed in cells that do not form granules, one can conclude that proteins packaged into granules have common features that can be decoded only by a sorting machinery specific to cells capable of regulated secretion. The notion that constitutively secreted proteins do not contain specific sorting information but specific signals are required for the incorporation of proteins into the granules is supported by the finding that a chimeric polypeptide consisting of the entire truncated secretory form of the VSV G protein (which by itself is secreted constitutively) linked to the C-terminus of human growth hormone is effectively incorporated into granules when synthesized in transfected AtT-20 cells.

As previously noted, many proteins that are stored in secretory granules are synthesized as larger precursors (proproteins) that are proteolytically processed intracellularly, either in the TGN or after sorting to immature secretory granules (ISG). Processing involves removal of an N-terminal (e.g., parathyroid hormone or prosomatostatin) or even interior (e.g., proinsulin) propeptide segment (see ref. 514). In some cases, such as that of prosomatostatin, the N-terminal propeptide sequence (82 amino acids) is sufficient to target a reporter polypeptide to storage granules. This is perhaps not surprising for prosomatostatin, since its propeptide constitutes the bulk of the precursor, the hormone consisting of only 16 amino acids. In contrast, the interior C peptide (34 amino acids long) of proinsulin that links the B and A chains can be replaced with an unrelated sequence without affecting the targeting of the prohormone. Similarly, the N-terminal propeptide of trypsinogen, which is normally removed from the protein after its secretion, is not required for packaging into granules.

Studies on the targeting of von Willebrand factor (vWF) to Weibel-Palade bodies, specialized secretory granules characteristic of endothelial cells, have been of particular interest because they suggest that this protein contains information that leads to its segregation, not only from constitutively secreted proteins, but also from other proteins that are packaged into granules. Weibel-Palade bodies are approximately 0.1 µm wide and 1 to 4 µm long and are characterized by the presence of a core structure consisting of a bundle of long narrow tubules that appear to be composed, at least in part, of the vWF itself. This is a very large protein (2050 amino acids) derived from a proprotein that dimerizes through disulfide bonds in the ER and undergoes removal of a large N-terminal propeptide segment (741 amino acids).
acids) at the time at which it also undergoes multimerization in the TGN and storage in the specialized granules. In cultured endothelial cells, only the large multimers of vWF (10 to 20 × 10^6 daltons) are stored in the Weibel-Palade bodies. Smaller ones are secreted constitutively.\(^5\) When the vWF is synthesized in transfected cells that carry out regulated secretion, such as the POMC-producing AtT-20 cells or the rat insulinoma RIN5F cell line, the factor is incorporated into newly formed Weibel-Palade body-like structures and not into the secretory granules characteristic of the host cell.\(^5\) Moreover, it was found that the vWF-containing Weibel-Palade-like granules did not form in transfected cell lines that do not carry out regulated secretion, such as Chinese hamster ovary (CHO), monkey kidney (COS), and 3T3 mouse fibroblast cells, even though some of these cells cleave the propeptide and form high-molecular-weight multimers.\(^\) This suggests that assembly of the storage granule characteristic of endothelial cells requires, in addition to vWF, one or more components that are involved in the formation of secretory granules in other cell types, but does not require components specific to the endothelial cells. In another study,\(^5\) however, Weibel-Palade-like bodies were found to form in transfected CV-1 cells—which are the parental line of COS cells and are not known to carry out regulated secretion. This led to the suggestion that a critical concentration of vWF in the TGN is the only factor required for granule formation and that components of a preexisting regulated secretion pathway are not necessary. Nevertheless, it should be noted that it has not been shown that some components of the regulated secretory machinery are not present in CV-1 cells.

It is interesting that the large propeptide of vWF, which is normally secreted as a noncovalently linked dimer together with the multimers of vWF, is required for storage of the factor in Weibel-Palade-like bodies formed \textit{de novo} in transfected AtT-20 and RIN cells. In itself, however, the propeptide is not sufficient to induce formation of the storage organelle, but its coexpression with the mature vWF (lacking its prosequence) leads to incorporation of both noncovalently-linked molecules into newly formed Weibel-Palade bodies.\(^5\) It can be concluded that the formation of a condensed multimeric form of vWF, for which the propeptide is required, is a key step in the biogenesis of the Weibel-Palade bodies. A study of the effects of specific mutations and deletions indicates, however, that the formation of disulfide bonds between vWF subunits is not necessary for packaging in the bodies.\(^5\),\(^5\) On the other hand, the acidic pH that prevails in the trans region of the Golgi apparatus is required, since Weibel-Palade bodies do not form in cultured endothelial cells when these are incubated with weak bases that dissipate the acidic pH of intracellular organelles.\(^5\)

**Mechanisms for the Sorting of Proteins into Secretory Granules**\(^5\)\(^1\)–\(^5\)\(^3\),\(^5\)\(^4\)\(^a\)

The sorting processes responsible for the formation of secretory granules begin to take place in the trans region of the Golgi apparatus and in the TGN. In some cell types that carry out regulated secretion, the condensation of secretory material that leads to the formation of the core of a secretory granule is first detected in the dilated rims of the trans-Golgi cisternae (see ref. \(^3\)\(^8\)\(^a\)). In other cell types they first appear in regions of the TGN. The large \textit{condensing vacuoles} that in pancreatic exocrine cells and in other cell types accumulate secretory material may also be specialized regions of the trans-Golgi network that ultimately bud off to form an immature secretory granule. In fact, immature granules and condensing vacuoles have many of the properties of the TGN, such as the presence of acid phosphatase, the capacity to carry out the sulfation of tyrosine residues and of oligosaccharides (see ref. \(^5\)\(^1\)), and the presence of patches of clathrin on their surfaces.\(^5\)\(^1\) After the dense core aggregate begins to form in the TGN, the process of granule formation continues, with its envelopment by a membrane and the subsequent budding of an \textit{immature secretory granule} (ISG). This undergoes a maturation process in which further condensation of the content occurs and excess membrane is removed. The cytoplasmic surface of the ISG membrane often possesses a patchy clathrin coat.\(^5\)\(^4\) It is not known if this is involved in the formation of the granule, perhaps by inducing its budding from the TGN, or if it is utilized to form coated vesicles that remove from the immature granule membrane and content proteins with other destinations. Indeed, the ISG appears to be a sorting compartment. In pancreatic islet β cells, it may be the site where
the C peptide cleaved from proinsulin is diverted into vesicles that bud off from the immature granule and release it constitutively at the cell surface. In some cells the maturation of immature granules involves their fusion with each other to form larger granules from which excess membrane must be removed. In other cell types, maturation involves solely condensation of the core and membrane removal and yields a smaller granule.

It was originally thought that the concentration of secretory material needed to form the core of immature granules in the TGN is effected by membrane-bound pH-sensitive receptors. These would ferry their ligands to regions of the Golgi complex that contain a proton pump capable of generating the acidic medium required to release the ligands. In this active sorting model (see ref. 511) receptors would then recycle to more proximal regions of the Golgi apparatus to bind additional ligand molecules. Much support has been obtained, however, for an alternative passive sorting model (Fig. 16-29) for the segregation of proteins in secretory granules. This model does not require the participation of receptors to effect the segregation, but simply involves the spontaneous aggregation of the protein molecules to be stored within the same granule. Such an aggregation would require a high concentration of the proteins and would be triggered by the conditions that prevail in the TGN and trans region of the Golgi apparatus (i.e., a mildly acidic pH and a high Ca\(^{2+}\) concentration). In some cells, the incorporation of proteins into aggregates may also require the presence of specific granule matrix components such as the granins. These (e.g., chromogranin A, chromogranin B/secretogranin I, and secretogranin II) are a family of acidic proteins that are present in secretory granules of many different types of endocrine cells and neurons, where they are stored and released together with cell type-specific peptide hormones and neuropeptides. In vitro, under the conditions that prevail in the TGN, the granins form aggregates that effectively exclude constitutively secreted proteins such as immunoglobulin. The Ca\(^{2+}\)-induced aggregation of granins is likely to be a consequence of their high content of acidic residues, which bind the divalent cation (see ref. 553).
Fig. 16-29:

Aggregation-mediated passive sorting of secretory and membrane proteins during the biogenesis of a secretory granule in the TGN. (1) The luminal contents of a TGN cisterna is depicted as consisting of a mixture of secretory proteins. One type (stars) is secreted constitutively, without being incorporated into secretory granules. (2) The two other types are capable of homophilic interactions and undergo self-aggregation in the milieu of the T...

The process that sorts regulated secretory proteins from constitutive proteins in neuroendocrine cells has been studied extensively using as a model PC12 cells, a line derived from a rat pheochromocytoma. In these cells, the granins undergo sulfation of tyrosine residues in the trans-Golgi, where constitutively secreted proteoglycans are sulfated in their carbohydrates. It is, therefore, possible to label selectively with $^{35}$S$^{2+}$ both types of proteins as they reach the TGN. This allowed the demonstration that the aggregation of the granins is determined by the environmental conditions in the TGN. Thus, the proteins could be extracted from detergent-permeabilized TGN-derived vesicles only when these were incubated in a medium of neutral pH that lacks Ca$^{2+}$, whereas the constitutively secreted free glycosaminoglycan molecules could be extracted even in Ca$^{2+}$-containing acidic mediums. In accordance with these findings, the packaging of secretogranin II into the granules of PC12 cells was markedly reduced when the cells were incubated with chloroquine or NH$_4$Cl. These agents, which are known to neutralize the pH of acidic intracellular organelles, led to the constitutive secretion of a large fraction of the newly synthesized granins.
The aggregation-mediated sorting model easily explains the formation of different secretory granule populations within the same cell, as a result of homophilic interactions (e.g., granules containing either prolactin or growth hormone in somatomammotrophs) or heterophilic interactions, (e.g., granules containing LH, TSH, and granins). The observation that different proteins may be segregated within the core of a single granule—as is sometimes the case with prolactin and growth hormone in the bovine somatomammotrophs and glucagon and glcycentin in the α granules of pancreatic islet cells—could be explained by the envelopment of separate aggregates into one immature granule in the TGN, or by the fusion of two different immature granules with each other. The cocondensation of many different proteins in pancreatic acinar cells required to form a zymogen granule would involve extensive heterophilic interactions.

After secretory protein aggregation, the next step in granule formation is the acquisition of a specific membrane. One mechanism by which this can occur is suggested by the finding that certain abundant granule content proteins, such as chromogranin B in PC12 cells, are also present in small amounts in a form tightly associated with the granule membrane. Through homophilic interactions with the luminal aggregate these membrane-associated forms would lead to the membrane envelopment of the developing core, largely excluding nonaggregating constitutive proteins (see Fig. 16-29). A similar homophilic membrane-core interaction may take place during the development of chromaffin granules, in which dopamine-β-hydroxylase exists not only as a content protein but also as a type II membrane protein, anchored to the membrane by its uncleaved signal sequence.

The sorting of membrane proteins to secretory granules has also become amenable to analysis with the identification of several specific proteins of granule membranes. P-selectin is a membrane protein specific to Weibel-Palade bodies of endothelial cells and α granules of platelets. This protein has a type I disposition and its 23 amino acid cytoplasmic C-terminal tail is necessary and sufficient for its sorting to the granules. However, the signal in this tail operates only in cells that assemble regulated secretory granules and the protein is constitutively transported to the plasma membrane in other cell types. The presence of a sorting signal in the cytoplasmic domain suggests that it is recognized by cytoplasmic components of a sorting machinery, such as the adaptor or coat proteins that mediate the assembly of clathrin-coated vesicles (see below). Peptidylglycine α-amidating monoxygenase (PAM) is another membrane protein whose cytoplasmic domain contains sorting information for secretory granules. Deletion of this portion of the protein abolishes its targeting to granules, although a truncated form that lacks both the transmembrane and cytoplasmic domains is sorted to secretory granules. It would seem, therefore, that the truncated soluble PAM is sorted by coaggregation with other endogenous regulated secreting proteins, but that this cannot take place when the protein is membrane-anchored. GP2 is a pancreatic zymogen granule membrane protein anchored in the membrane by a glycosylphosphatidylinositol moiety (GPI). Therefore, this protein lacks transmembrane and cytoplasmic domains, but like many luminal proteins, such as the granins, undergoes aggregation, which probably accounts for its sorting to the granule membrane. As will be mentioned below, GPI-linked proteins can be released from membranes by cleavage of their lipid anchor, and after detachment from the membrane, GP2 forms aggregates under TGN conditions.

Biochemical Requirements for the Formation of Constitutive and Regulated Secretory Vesicles in the TGN

The formation of constitutive and regulated secretory vesicles in the TGN resembles in its biochemical requirements the generation of vesicles in other compartments of the endomembrane system. Using a postnuclear supernatant fraction from PC12 cells pulse-labeled with $[^35]S\text{SO}_4^{2-}$, it has been possible to reproduce in vitro the formation of two distinct populations of vesicles from the TGN. One corresponds to constitutive secretory vesicles that contain heparan sulfate proteoglycan, and another to immature...
secretory granules containing secretogranin II. The two types of vesicles could be separated by centrifugation. Their production requires an energy supply and the hydrolysis of GTP within a GTP-binding protein, since it was inhibited to a significant extent by the GTP analogue GTP-\(\gamma\)-S.\(^{569}\) In addition, both in vivo and in vitro studies demonstrated that BFA also inhibits the formation of both types of vesicles in the TGN.\(^{570, 571}\) Since in other systems (see above) this drug blocks vesicle formation by inhibiting the ARF-dependent association of coat proteins with donor membranes, it seems very likely that a similar coat protein-membrane association takes place during the assembly of secretory vesicles in the TGN even though the biochemical nature of the coat remains to be elucidated.

The production of both types of secretory vesicles in the TGN is controlled by heterotrimeric G proteins of the Gi/Go and Gs classes. The involvement of an inhibitory (G\(\alpha_i\)) G protein became apparent from the findings that AIF\(_{3-5}\) suppressed vesicle formation in vitro, while highly purified \(\beta\gamma\) subunits, which should block the function of a G\(\alpha\), stimulated it.\(^{572}\) Moreover, treatment with mastoparan—the peptide that mimics an activated G protein-coupled receptor and preferentially activates G\(\alpha_i\)—inhibited cell-free vesicle formation in vitro. This effect was abolished by pertussis toxin pretreatment of the cells, which inactivates G\(\alpha_i\). As expected from these effects, treatment of the cells with the toxin alone increased the subsequent in vitro formation of both constitutive secretory vesicles and secretory granules. Evidence that a stimulatory G\(\alpha_s\) exerts a positive control in vesicle formation in the TGN came from the observation that pretreatment of the cells with cholera toxin, which activates G\(\alpha_s\) by inhibiting its GTPase activity, increases the production of vesicles and immature secretory granules in a subsequent in vitro incubation. Under these conditions, activation of the inhibitory Gi/Go subunits not affected by the toxin, by the addition of GTP-\(\gamma\)-S or AIF\(_{3-5}\), counteracted the stimulatory effect of cholera toxin.\(^{570}\) Based on these data, a hypothetical scheme has been proposed for the role of both inhibitory and stimulatory G proteins in the formation of secretory vesicles that is shown in Fig. 16-30.
Both stimulatory (Gsα) and inhibitory (Giα/Goα) heterotrimeric G proteins control the production of secretory vesicles in the TGN. This scheme (based on Bauerfeind and Huttner 513) depicts the formation of a secretory vesicle in the TGN as requiring the activated form of an ARF protein and the recruitment of soluble coat proteins to form a coated bud in the donor membrane (right side of the ...)

These findings indicate that a highly complex regulatory mechanism operates in the TGN to control vesicle formation. The identification of receptor-like molecules that presumably lead to GDP-GTP exchange in the Gα subunits and identification of the effectors controlled by these subunits is essential for understanding these processes. As previously mentioned, Gsα appears to promote COP-coated vesicle formation within the Golgi stacks by stimulating GDP-GTP exchange on ARF. It may have a similar role in the TGN, although the specific coat proteins have yet to be identified. The mechanism by which the Giα suppresses vesicle production in the TGN is unknown, but one possibility is that it acts to prevent fission of vesicles with a fully assembled coat. 513

**COVALENT LINKAGE OF LIPIDS TO PROTEINS** 39, 573–578

Many proteins synthesized in free and in bound polysomes contain lipid moieties covalently linked to their polypeptide backbones. In some cases, the lipid moiety serves as the sole membrane anchor for a polypeptide that lacks a hydrophobic segment. In others, a polypeptide bearing a permanent lipid anchor carries out a cyclic function that requires attachment and detachment from the membrane. In still other proteins, the lipid only reinforces a membrane anchorage mediated by a hydrophobic segment of the polypeptide. Finally, some proteins bearing lipids do not associate with membranes and remain in the cytoplasm or are secreted from the cell.
Lipid-modified proteins participate in a diverse array of cellular processes including signal transduction and growth regulation, intracellular vesicular transport and protein sorting, cell adhesion, and cytoskeletal organization.

**N-Myristoylation of Proteins**

Certain proteins synthesized in free polysomes have the 14-carbon saturated fatty acid chain, myristate, bound through an amide linkage to the α amino group of an N-terminal glycine. Examples include: (1) the catalytic subunit of the cAMP-dependent protein kinase, (2) NAD cytochrome-b5 reductase, (3) the phosphoprotein phosphatase calcineurin, (4) the Goα subunit of heterotrimeric G proteins, (5) the small GTP-binding ARF proteins, which play a critical role in vesicular transport, and (6) a number of oncogene products, including the transforming protein of the Rous sarcoma virus (p60<sup> v-src </sup>) and its corresponding cellular protooncogene (p60<sup> c-src </sup>). *N*-myristoylation of these proteins is a cotranslational process. Following proteolytic removal of the initiator methionine by an independent activity, the enzyme myristoyl CoA:protein N-myristoyltransferase (NMT) transfers a myristoyl group from myristoyl CoA to the glycine residue exposed at the new N-terminus of the nascent chain. The amino acid sequence following the myristoylated glycine varies significantly in different proteins, but it is clear that a short peptide sequence at the N-terminus determines the modification. In the case of p60<sup> v-src </sup>, the signal recognized by the NMT is wholly contained within the first 14 residues of the protein. A comprehensive analysis of the N-terminal sequences of chimeric polypeptides containing normal and mutated N-terminal portions of the vaccinia virus LIR protein indicated that the first 5 amino acid residues (GAAAS) represent a minimum sequence required for myristoylation of LIR. When the first 12 amino acids were included, myristoylation was as effective as in the natural protein.

In the case of the p60<sup> v-src </sup>, myristoylation leads to the association of the protein with the inner (cytoplasmic) face of the plasma membrane. This association is an absolute requirement for the transforming activity of the oncogene product. Similarly, the lipid-mediated association of NADH cytochrome b5 reductase with the cytoplasmic face of the ER membrane is required for the function of this enzyme in the electron transport chain that effects fatty acid desaturation in the ER. It is not yet clear what factors determine the association of different myristoylated proteins with different membranes. It seems likely that specific interactions with other membrane proteins facilitate the incorporation of the acylated polypeptide into a given organelle. In the case of the Src protein, targeting to the plasma membrane appears to be mediated by a specific receptor that binds to the myristoylated N-terminal region of the protein. It is clear that, at least in some cases, portions other than the myristoylated N-terminal region are required to maintain the association of a protein with the membrane. For example, the catalytic subunit of the cAMP-dependent protein kinase is found associated with the regulatory subunit on the cytoplasmic face of the plasma membrane, but is released into the cytoplasm when activated by cAMP. Similarly, the ARF protein—whose myristoylation is essential for its function in generating Golgi vesicles—binds to the Golgi membranes only when it contains bound GTP.

**C-Terminal Addition of Glycolipid Anchors to Plasma Membrane Proteins**

Many membrane proteins have been identified as being anchored to the outer leaflet of the plasma membrane lipid bilayer by a glycosylphosphatidylinositol phospholipid (GPI) moiety (also referred to as GPI or PIG) which is linked via a phosphorylethanolamine in an amide linkage to the C-terminus of the polypeptide (Fig. 16-31). These proteins can usually be released from the cell surface by treatment with a phospholipase C (PIPLC). GPI-linked proteins include the variant surface glycoprotein (VSG) of African trypanosomes, the placental and intestinal alkaline phosphatases, acetylcholinesterase (AChE), 5′ nucleotidase, the Thy-1 antigen of T lymphocytes, one form of the neural adhesion molecule N-CAM, and the decay-accelerating factor (DAF) that protects host red cells from...
complement-mediated lysis. The membrane anchor in the GIPL moiety is provided by the fatty acids of a diacylglycerol molecule that is part of the complex glycosylinositolphospholipid. The glycan group within GIPL contains a conserved linear core structure\(^{591,592}\) that includes three mannoses and a nonacetylated glucosamine (ethanolamine-\(\text{P-6Man}\alpha_1-2\text{Man}\alpha_1-6\text{Man}\alpha_1-4\text{GlcN}\alpha_1-6\)) linked to the inositol. Frequently other groups, such as an additional phosphorylethanolamine, mannose, \(N\)-acetylgalactosamine, or galactose residues, are linked to the core, and an additional fatty acid may be linked to the inositol.

**Fig. 16-31:**

Anchorage of proteins to membranes via a glycosyl phosphatidyl inositol (GIPL or GPI) moiety. A glycan that contains a core structure consisting of \(\text{Man}\alpha_1-2\text{Man}\alpha_1-6\text{Man}\alpha_1-4\text{GlcN}\) is linked by a phosphodiester bond from the 6-carbon of the first mannose residue to an ethanolamine, which is in turn linked in an amide bond to the C-terminal amino acid in the polypeptide. The glycan is also linked through the glucosamine re...
Proteins that acquire the glycosylphosphatidylinositol moiety (in a process also known as glypiation) are synthesized as transmembrane precursors. These are type I membrane proteins that are inserted in the ER by an N-terminal cleavable insertion signal and are anchored in the ER membrane by a short hydrophobic (8 to 20 amino acids) peptide segment at the C-terminus, so that few, if any, residues are exposed on the cytoplasmic surface of the membrane. GIPL addition takes place very soon after completion of translation and insertion of the protein in the ER membrane. The reaction can be considered a transeptidation in which the transmembrane protein precursor loses the C-terminal segment that served as peptide membrane anchor, while an amide linkage to the ethanolamine in a preformed GIPL anchor unit is established. The presence of preformed glycolipid anchors can be detected in both trypanosomes and murine lymphoma cells. Many of the details of the anchor synthesis have been worked out in these systems. The enzyme(s) that removes the hydrophobic peptide anchor from the precursor and replaces it with the lipid anchor has not been identified, and the fate of the cleaved peptide is unknown.

In the case of the DAF protein, in which addition of the GIPL anchor involves removal of the last 17 amino acids, the information that determines this modification has been shown to be contained within the last 37 amino acids of the primary translation product. Detailed mutagenesis studies have shown that, in addition to the C-terminal hydrophobic peptide segment, a critical feature for addition of the GIPL anchor is the presence at a position 10 to 12 residues N-terminal to the beginning of the hydrophobic domain of a pair of small amino acid residues that define the cleavage point in the precursor. Exhaustive mutagenesis of the amino acid residue in alkaline phosphatase to which the GIPL is added demonstrated that, in addition to the natural Asp at that site, only Gly, Ala, Cys, Asn, or Ser are compatible with normal processing of the precursor. These are the same residues that are naturally present at the sites of cleavage in the precursors of other GIPL proteins. The importance of the region that determines the cleavage site is highlighted by the fact that the sequences of the GIPL-linked and transmembrane forms of the chicken cell adhesion protein N-CAM (which are encoded in mRNA generated by alternative splicing of a primary transcript) diverge seven amino acids upstream (toward the N-terminus) of the site of GIPL addition. The requirement for the lack of a cytoplasmic C-terminal domain for GIPL-linkage is apparent from the fact that addition of such a domain to the precursor of alkaline phosphatase prevented its processing and resulted in a mature transmembrane protein. Moreover, the sequences of the GIPL-linked and transmembrane forms of the lymphocyte adhesion protein LFA-3 (whose mRNAs are also generated by alternative splicing of a primary transcript) diverge only at sequences beginning two amino acids from the C-terminal end of the transmembrane domain.

Several possible functions for the anchoring of proteins to the cell surface via the glycosylated phoshoinositides have been proposed. These include an enhanced mobility of the protein within the plane of the bilayer, the possibility of regulating the release of the protein by the action of an extracellular phospholipase C, or even generating the intracellular “messenger” diacylglycerol that could serve to activate a protein kinase C.

A defect in the pathway of GIPL addition is the basis for paroxysmal nocturnal hemoglobinuria (PNH), an acquired condition in which the GPI-linked proteins DAF and CD59 are absent from the surface of erythrocytes, which renders them susceptible to complement-mediated hemolysis. PNH is due to a somatic mutation in a pluripotent hematopoietic stem cell that affects a gene for an enzyme that functions in the GIPL synthesis pathway. On clonal expansion, the defect is manifested in a large, but variable, fraction of both erythrocytes and leukocytes, which lack GPI-linked proteins.
Genetic defects in the synthesis or addition of the GIPL anchor have also been found in murine lymphoma cell lines that do not express surface Thy-1 antigen.\textsuperscript{607} These fall in several complementation groups, some of which fail to synthesize the preassembled GIPL anchor due to blocks at different steps in the biosynthetic pathway. Some fail to synthesize dolichol-phosphate-mannose,\textsuperscript{578} while others secrete a soluble Thy-1, as expected if cleavage of the precursor protein takes place, but lipid addition does not occur. One of the complementation groups (PIG-A) corresponds to a mutation in the same gene that in humans is defective in PNH.\textsuperscript{602}

A diverse set of functions is associated with GPI-linked proteins, including enzymatic activities (e.g., acetylcholinesterase, placental and intestinal alkaline phosphatases, 5’ nucleotidase), a cell-adhesion role (N-CAM; LFA-3, and the carinoembryonic antigen, CEA), and a receptor function (for the Fc of IgG in the FcyRIII receptors). Surprisingly, despite the absence of cytoplasmic and transmembrane domains, several GPI-linked proteins can, on crosslinking with antibodies, transduce signals that lead to cell proliferation. This suggests that they are associated with other effector molecules in the membrane, such as tyrosine kinases.\textsuperscript{608}

A GIPL anchor may confer on a protein properties essential for its function. In trypanosomes, it permits the GIPL-linked VSG molecules to pack closely, forming the protective coat that covers the whole organism. Moreover, the blood stages of the parasites also express a glycosylphosphatidylinositol-specific phospholipase C, which may serve to release the surface glycoprotein from dying parasites, and thus facilitate the neutralization of the host immune response. In fact, the susceptibility of all GIPL-linked proteins to release by specific phospholipases may provide the cells with a means to down-regulate the surface levels of a particular protein. Under certain circumstances, this may cause its secretion (e.g., the GP2 pancreatic secretory granule membrane protein that appears in pancreatic juice).\textsuperscript{548a, 567}

Proteins with a GIPL anchor are able to diffuse in the plane of the membrane at rates approximately tenfold higher than proteins anchored by peptide domains and can attain rates comparable to those of lipids.\textsuperscript{576} Although the significance of this property is not clear, it might provide cells with a means to respond to stimuli by rapidly reorganizing their exposed surfaces (e.g., by forming or dissolving patches). It is noteworthy that GIPL-linked proteins are endocytosed very slowly. As first shown for Thy-1,\textsuperscript{609} they appear to be excluded from clathrin-coated pits formed at the plasma membrane. On the other hand, GIPL-linked proteins, such as the folate receptor, are closely packed within caveolae.\textsuperscript{610, 611}

One very important property of GIPL-linked proteins is that, when expressed in polarized epithelial cells, such as those that line the intestinal mucosa or kidney tubules, they are targeted to and accumulate predominantly in the apical plasma membrane domains of the cells.\textsuperscript{612, 613} In fact, the presence of a GIPL anchor (such as that of DAF or Thy-1) in a chimeric protein can redirect a basolateral plasma membrane protein (such as the VSV-G or herpes GDI glycoproteins) to the apical surface of the cells.\textsuperscript{614, 615} In a converse experiment, replacement of the GIPL anchor in alkaline phosphatase by the transmembrane and cytoplasmic domains of the VSV-G addressed the protein to the basolateral surface.\textsuperscript{615} Moreover, the GIPL-anchored and transmembrane forms of N-CAM, when expressed in the polarized Madin Darby Canine Kidney (MDCK) cell line of dog kidney origin, were differentially targeted to the apical and basolateral surfaces, respectively.\textsuperscript{616}

The sorting mechanism that recognizes GIPL anchors and leads to the apical targeting of proteins that bear them, appears to involve the formation of mixed patches of the anchors with sphingoglycolipids in the TGN. Sphingoglycolipids are synthesized in the Golgi apparatus and, themselves, are concentrated in the outer leaflet of the apical membrane to which they are vectorially delivered from the TGN (see refs.\textsuperscript{617}}
and \(^\text{618}\). Evidence has been presented that many GIPL-linked proteins do indeed cluster with glycolipids in the Golgi apparatus to form aggregates that are not extractable with the neutral detergent Triton X-100 at low temperature. \(^\text{619}\) This is in accordance with an earlier suggestion that such clusters would be formed in the TGN and be selectively incorporated into vesicles routed to the apical surface. \(^\text{620}\) It seems plausible that the sorting of such aggregates involves their recognition by a transmembrane protein that in its cytoplasmic domain would interact with components of the sorting machinery.

**C-Terminal Prenylation of Proteins**\(^\text{39, 577, 621, 622}\)

Two types of prenyl groups, the 15-carbon farnesyl and the 20-carbon geranylgeranyl, are found linked by thioether linkages to cysteine residues at or near the C-terminal end of certain proteins synthesized in free ribosomes. The farnesyl moiety consists of three isoprene units and is transferred to the protein by a farnesyltransferase from farnesylpyrophosphate (FPP), an intermediate in cholesterol biosynthesis. The geranylgeranyl moiety consists of four isoprene units and is delivered by transferases from geranylgeranyl pyrophosphate (GGPP), an intermediate whose only known function is in protein prenylation. Proteins containing the geranylgeranyl group are much more abundant than those containing the farnesyl group. The most prominent of the latter are the ras proteins and the nuclear envelope lamins. Among the geranylgeranylated proteins are the low-molecular-weight GTP-binding proteins of the rab family, which participate in vesicular transport, and the γ subunits of heterotrimeric G proteins. Prenylated proteins with a C-terminal CAAX box (where C is cysteine, A an aliphatic, and X any amino acid) are farnesylated when X is serine or methionine (and perhaps to a limited extent when it is cysteine, alanine, or glutamine) and are geranylgeranylated when X is leucine or phenylalanine. \(^\text{351, 623, 624}\) These prenylation reactions are carried out by heterodimeric farnesyl (FTase) and geranylgeranyl (GGTase I) transferases that share a common α subunit. \(^\text{623}\) The specific prenylation is determined solely by the sequence of the extreme C-terminal tetrapeptide, which when transferred to another protein leads to the expected modification. \(^\text{625}\) In fact, appropriate tetrapeptides can serve as specific substrates in vitro for these two prenyl transferases. \(^\text{351, 624}\)

Prenylation of proteins containing the CAAX motif is followed by two additional modifications: proteolytic removal of the three C-terminal residues, followed by carboxymethylation of the new C-terminal cysteine. \(^\text{625}\) In some cases, a palmitate residue is added via a thioester linkage to another cysteine residue just upstream of the CAAX motif. \(^\text{625}\) The rab proteins contain C-terminal dicysteine motifs in various arrangements (CC, CXC, CCX, CCXX, CCXXX), which are modified by prenylation. A distinct geranylgeranyl transferase (GGTase II) has been identified and purified \(^\text{626}\) that adds geranylgeranyl groups to rab proteins with the CXC (rab3a) and CC (rab1a) motifs. Some of these proteins (like rab3a) may be geranylgeranylated on both cysteine residues at the C-terminus, although it is not clear if the same enzyme carries out both modifications, and may also undergo carboxymethylation at the C-terminal cysteine. \(^\text{627}\) GGTase II does not simply recognize the C-terminal tetrapeptide but also an as yet unidentified sequence in the rab proteins that is far from the C-terminus. \(^\text{628}\) The capacity of the enzyme to recognize features other than the C-terminus of the substrate can be attributed to the fact that, in addition to α and β subunits, similar but not identical to those of the other transferases, GGTase II also contains an additional subunit (95 kDa) designated component A, or rab escort protein (REP). REP binds the unprenylated rab, presents it to the catalytic α,β-subunit complex (component B), and remains bound to the substrate after the reaction, until this is taken up by another protein that interacts with the rab. \(^\text{629, 630}\) REP appears to be encoded by the gene affected in choroideremia (CHM), an X-linked form of retinal degeneration, since the human gene product is very similar (90 percent) to the rat REP. \(^\text{630}\) Moreover, lymphoblasts from patients with the disease show a reduced ability to modify rab3a and rab1a in vitro, and this defect can be compensated for by the addition of purified rat REP. \(^\text{631}\) Strikingly, the CM gene product had been noted to be related to the GDI for rab3a. \(^\text{632}\) This relationship can now be understood since both REP and GDI must recognize a rab protein. The gene for another human protein, CHML, \(^\text{633}\) that is 76 percent identical to that encoded in the CHM gene and 70 percent identical to REP, has also been
identified. It was suggested that that protein represents another REP subunit that confers a different rab protein specificity to GGTase.

The prenyl modification has been shown to be critical for the function of many proteins and necessary for their insertion into membranes (see ref. 577). Mutant ras proteins that do not undergo prenylation no longer associate with the plasma membrane and are not transforming. However, substitution of their farnesyl group with a geranylgeranyl (by altering the C-terminal residue in the primary translation product) does not eliminate the plasma membrane targeting and transforming activity of the v-ras oncogene product.634, 635 On the other hand, replacement of the farnesyl group with geranylgeranyl on the normal cellular ras protein leads to the potent growth inhibition of mouse NIH 3T3 cells.635 Since proteins with the same C-terminal prenylation have different subcellular distributions (e.g., the various rab proteins, or the lamins and ras), targeting information for specific membranes must be encoded in other parts of the polypeptide. For the ras protein the presence of palmitoyl residues just upstream of the farnesylated C-terminus plays a role in targeting to the plasma membrane. Each rab protein has a characteristic distribution in the cell and, in some cases, sequences 30 to 40 residues upstream from the C-terminus have been shown to determine their organellar localization, probably by specific protein-protein interactions.37 Thus, whereas rab2 is normally located in the intermediate compartment between the ER and Golgi, replacement of its C-terminus with a short segment (39 residues) of rab5 or rab7 led to its relocation to the early or late endosome compartments, the respective natural habitats of the latter two proteins.37

**Acylation of Proteins through Ester and Thioester Linkages**574, 575, 636–638

Many transmembrane polypeptides, such as the envelope glycoproteins of a variety of viruses (e.g., G of VSV, HA of influenza, and E2 of α viruses), and cellular membrane proteins, such as the myelin proteolipid protein, the transferrin, insulin, and β-adrenergic receptors, and the HLA-B histocompatibility antigen contain one or more palmitate moieties linked in a thioester bond to a cysteine residue located within the cytoplasmic segment of the protein, near its membrane-anchoring domain. For the viral glycoproteins, acylation is not required for transport to the cell surface but is necessary for viral assembly and budding.539

Palmitoylation is a posttranslational event that, for the viral envelope glycoproteins, has been shown to occur approximately 20 min after synthesis of the polypeptide is completed,640 and probably takes place in the late ER or intermediate compartment just before transport to the Golgi apparatus, and before the high-mannose N-linked oligosaccharides are converted into the complex chains.641 The incorporation of labeled palmitate into some membrane proteins, such as the transferrin receptor, has been shown to continue even several hours after synthesis of the polypeptide is completed.642 but this may only represent the turnover of preexisting polypeptide-bound palmitate moieties, which turn over three to four times faster than the protein itself.642 The β-adrenergic receptor (and probably other G protein-coupled receptors with seven membrane-spanning domains) is also palmitoylated on a conserved cysteine. This modification is believed to be necessary to hold the receptor in a conformation that allows its coupling to the G protein.643

The enzyme(s) responsible for the acylation of the membrane polypeptides are not strictly specific for palmitic acid and in some cases it may add myristate, stearate, or oleate residues. Because of the location of the modified amino acid residue, the enzyme must be located on the cytoplasmic side of the membrane and could be the same enzyme that catalyzes the palmitoylation of some cytoplasmic proteins, described above.
Some secretory proteins, such as the gastric mucous glycoproteins and immunoglobulin heavy and light chains, have also been shown to be acylated, the first with palmitate, stearate, or oleate, and the latter with myristate moieties that are probably bound in amide linkages to lysine side chains. Similar linkages also appear to be present in the luminal domains of the \( \alpha \) and \( \beta \) subunits of the insulin receptor, which also contains palmitate, probably linked in a thioester bond. The enzymes responsible for the fatty acylation of secretory proteins and luminal domains of membrane proteins have not been characterized, but they must be distinct from the enzyme(s) that add fatty acids to cytosolic proteins and to cytoplasmically exposed cysteine residues of membrane proteins.

Many proteins synthesized in free polysomes, such as the H-ras oncogene product, which is also prenylated, carry a palmitic acid moiety linked to a cysteine near the C-terminus of the protein. The presence of the fatty acid is required for the membrane association and full transforming activity of the ras protein, which functions in signal transduction from tyrosine kinase receptors at the plasma membrane. Ankyrin, a red-cell cytoskeleton protein, also carries palmitate residues, but the association of this polypeptide with the membrane is a peripheral one and is mediated by a high-affinity binding to the anion transporter (band III) in the membrane.

ENDOCYTOSIS AND THE BIOGENESIS OF LYSOSOMES

Lysosomes are membrane-bound cytoplasmic organelles that contain a wide variety of hydrolytic enzymes that function at an acidic pH and together are capable of digesting essentially all types of biologic macromolecules. Both extracellular materials that are taken into the cell by endocytosis and intracellular components that undergo autophagy are broken down within lysosomes to their elementary constituents. These may then be transferred across the organellar membrane to the cytosol for further degradation or for reutilization in the synthesis of new macromolecules. A cell may contain several hundred lysosomes, and these may be quite variable in their size, shape, and morphologic appearance. This heterogeneity reflects the character of the material being digested within the lysosomes, as well as the various stages in the process of digestion that may be taking place. Some lysosomes may contain recently ingested materials whose origin is easily recognizable, while others, known as residual bodies, may contain only undigested remnants.

*Lysosomes have been defined as membrane-bound vacuoles rich in lysosomal hydrolases. The term primary lysosome was used to designate lysosomes that have not yet acquired the substrate for digestion, and the term secondary lysosome for those that have received these substrates subsequent to endocytosis or autophagy. Primary lysosomes are prominent in polymorphonuclear leukocytes, where they are represented by the azurophilic granules. In most other cells (see below), the term primary lysosomes can at best only be applied to the small vesicles containing mannose 6-phosphate receptor molecules (see below) that carry newly synthesized hydrolases from the Golgi apparatus (or the TGN) to endosomes that are undergoing transformation into lysosomes.

The term endocytosis refers to a variety of cellular processes that lead to the interiorization of extracellular material. The essential feature of endocytosis is that extracellular fluid or solid particles become surrounded by a portion of the cell plasma membrane that ultimately pinches off from the cell surface to form a membrane-bound cytoplasmic compartment.
The term *phagocytosis* is reserved for the internalization of large particles, such as bacteria, protozoa, cellular debris, carbon or silica grains, etc. In general, this process involves the formation of cytoplasmic extensions or pseudopodia that completely surround the particle being ingested to produce a *phagosome*, which later acquires lysosomal enzymes to become a *phagolysosome* in which digestion takes place. The process of phagocytosis requires the reorganization of a network of actin microfilaments located immediately beneath the plasma membrane and, therefore, can be inhibited by the drug cytochalasin D, which interferes with microfilament function. In multicellular organisms, phagocytosis is an activity reserved to "professional phagocytes," such as macrophages and polymorphonuclear leukocytes. Specific proteins in the plasma membrane of these cells serve as receptors which recognize ligands on the surface of the particle being taken in. For example, a receptor in the plasma membrane of macrophages mediates the phagocytic uptake of opsonized bacteria by binding to the Fc portion of specific antibodies that recognize bacterial surface antigens.

*Receptor-mediated endocytosis* (see ref. 654) is an important mechanism for the efficient uptake of extracellular substances—such as hormones, growth factors, and nutrient-carrier proteins—capable of binding to specific cell surface receptors that mediate their internalization (Fig. 16-32). The receptor-ligand complexes are concentrated in small invaginations of the plasma membrane known as *coated pits*, because of their appearance on EM. The coat covering the region of the pit is composed primarily of clathrin, a protein that consists of heavy (approximately 190 kDa) and light (23 to 27 kDa) chains, and a set of *adaptor* or *assembly proteins* (see below). The adaptor proteins facilitate the formation of a clathrin lattice on the cytoplasmic surface of the membrane, and this brings about the clustering of receptors in the coated pits. The pinching off of the pits into the cytoplasm generates *coated vesicles* that contain the receptors and their associated ligands and are completely surrounded by a cage composed of clathrin and its associated proteins (see ref. 657).
Receptor-mediated endocytosis, membrane recycling, and biogenesis of lysosomes. After binding to its
ligand at the cell surface, a plasma membrane receptor is concentrated in a clathrin-coated pit (CP), which pinches off into the cytoplasm to form a coated vesicle (CV). The coated vesicle becomes at least partially uncoated (not shown) and fuses with an early endosome (E. endo) and, at the low pH in this compartment, the receptor ...

In receptor-mediated endocytosis, interaction of the ligands with their specific receptors markedly increases the efficiency of their uptake, since they are actually concentrated on the surface of the cell before being taken in. This process is responsible for the cellular uptake of some nutrients, such as vitamin B\textsubscript{12} bound to transcobalamin II, cholesterol incorporated in LDL particles, and iron within transferrin molecules. After they bind to the cell-surface receptors that mediate their function, polypeptide hormones and growth factors, such as insulin or EGF, are also interiorized in coated vesicles. This leads to the removal of the hormones and factors from the extracellular fluid. It also provides a means to modulate the capacity of the cell to respond to these agents that control cellular metabolism and proliferation, since it reduces the number of receptors available at the cell surface (i.e., down-regulation). Some receptors internalized by endocytosis, such as the EGF receptor, are normally distributed over the surface of the cell\textsuperscript{668, 669} and move to, or from, coated pits only after binding their ligands. Others, such as the LDL receptor, are continuously concentrated in coated pits, and internalized whether or not they contain a bound ligand.\textsuperscript{670}

During the course of receptor-mediated endocytosis, solutes, such as albumin or horseradish peroxidase, that are present in the extracellular fluid but are not bound to specific receptors are also taken in and remain dissolved in the lumen of the endocytic vesicles. The intake of extracellular fluid, and of substances dissolved in it, which takes place continuously in this manner and occurs in all cells, is known as fluid-phase endocytosis or pinocytosis. Kinetically, this is characterized by a strictly linear dependency on the concentration of the solute taken in. In contrast, receptor-mediated endocytosis can be saturated and shows typical Michaelis-Menten kinetics. Pinocytosis may also be carried out, independent of receptor-mediated endocytosis, by vesicles that are not coated by clathrin (see below).

**Signals for Endocytosis via Clathrin-Coated Vesicles\textsuperscript{671, 672}**

Immunocytochemical studies first showed that while certain plasma membrane proteins are selectively incorporated into clathrin-coated pits, others are excluded from them.\textsuperscript{609} The recognition that a mutation in the cytoplasmic tail of the human LDL receptor eliminated its capacity to cluster in coated pits and, hence, to undergo endocytosis\textsuperscript{673, 674} not only provided evidence for the role of the cytoplasmic domain of the receptor in determining its endocytic behavior, but also pointed to the role of a specific tyrosine residue in this domain as a critical component of an endocytic signal (or interiorization signal) required for the clustering of receptors in clathrin-coated pits.

Studies on the effects of specific mutations on the endocytic capacity of the LDL,\textsuperscript{675} MPR300,\textsuperscript{676} MPR46 kDa,\textsuperscript{677} and transferrin receptors,\textsuperscript{678} as well as of acid phosphatase,\textsuperscript{679, 679a} a lysosomal membrane protein that reaches the plasma membrane, have shown that endocytic signals consist of short peptide segments (four to six amino acids in length) that contain at least one aromatic residue and are located near, but not immediately adjacent to, the membrane. In the human LDL receptor, the endocytic signal consists of the sequence FDNPVY,
serine; T, threonine; V, valine; W, tryptophan; and Y, tyrosine; X corresponds to any amino acid.

but comparative studies with other species and mutagenesis studies have shown that the motif NPXY contains sufficient information for clustering this receptor in a coated pit. In the transferrin receptor, which is a type II membrane protein and therefore has an N-terminal cytoplasmic domain, the endocytic signal has the sequence YXRF. However, this could be replaced by the endocytic signal from various other receptors, such as the poly Ig and mannose 6-phosphate/insulin-like growth factor II (Man-6-P/IGFII) receptors, both of which are type I membrane proteins. The most characteristic feature of an endocytic signal appears to be its capacity to self-determine the formation of a tight turn within the three-dimensional conformation of the polypeptide. MRI determinations of the solution structure of peptides encompassing the endocytic signals in the LDL receptor and the lysosomal acid phosphatase have shown that the peptides by themselves adopt the tight-turn configuration.

The cyclic function of the cation-independent 300-kDa Man-6-P/IGFII receptor (MPR300), a protein that reaches the plasma membrane, requires that it be incorporated into two types of clathrin-coated vesicles, first in those that form in the TGN and second in those that mediate its interiorization at the plasma membrane. This requires the presence in the cytoplasmic domain of the MPR300 of two clustering signals, which have been identified by mutagenesis studies. The one functioning at the plasma membrane has the sequence YKYSKV and is located 24 to 29 residues from the membrane, whereas the one functioning in the TGN includes the C-terminal four amino acids (LLHV), as well as a sequence that at least partly overlaps with the internalization signal that functions at the plasma membrane.

The identification of the sequences that serve as internalization signals in the cytoplasmic domains of proteins incorporated into clathrin-coated vesicles has made it possible to demonstrate that these signals are recognized by the adaptor proteins that mediate the assembly of the clathrin coat (Fig. 16-33). Coat proteins can be released from purified preparations of clathrin-coated vesicles and separated chromatographically into clathrin “triskelions” and two additional distinct tetrameric protein complexes, HA1 (or AP1) and HA2 (or AP2). The latter were originally designated assembly proteins because of their capacity to promote clathrin assembly into cage structures in vitro. The triskelions, which are three-legged hexameric complexes consisting of three heavy and three light clathrin chains, serve as subunits that undergo higher-order assembly into the clathrin lattice that forms on the cytoplasmic face of the plasma membrane (Fig. 16-34). Each of the adaptors (Fig. 16-35) contains two specific polypeptides of approximately 100 kDa, called adaptins (α and β in HA2 and γ and β in HA1), and two other smaller polypeptides (AP50 and AP17 in HA2, and AP47 and AP19 in HA1). Immunocytochemical studies with antibodies specific for the α and γ subunits of the different adaptor complexes indicate that the HA1 adaptor, which in clathrin-coated vesicles from brain is much less abundant than the HA2, is a component of the coat of vesicles that form in the TGN. The HA2 adaptor is derived from the coat of the endocytic vesicles that form in the plasma membrane. EM analysis indicates that, when assembled in vitro, the adaptors form an inner layer beneath the clathrin cage, as expected if in native coated vesicles they interact directly with the membrane. Adaptor proteins interact specifically with the endocytic signals in the cytoplasmic tails of receptor polypeptides and also with the clathrin molecules (see Fig. 16-33). Thus, the adaptors cause the clustering of receptors in coated pits and facilitate the formation of the clathrin lattice. The capacity of the adaptors to bind specifically to endocytic signals was demonstrated in affinity chromatography experiments that employed as immobilized ligands fusion proteins containing the cytoplasmic tails of the LDL receptor or the MPR300. These experiments showed that HA1 complexes bind only to the MPR300, whereas HA2 bind to both. In addition, competition studies showed that replacement of the two tyrosine residues within the cytoplasmic domain of the MPR300 eliminated its capacity to compete for binding of HA1 to the wild-type MPR300 receptor, but not for the binding of HA2 to that receptor. Questions now being investigated relate to the targeting and recycling of the adaptors.
does the HA2 adaptor released after the uncoating of an endocytic vesicle not bind to the MPR in the TGN? Conversely, what prevents the binding of the HA1 adaptor to the plasma membrane? The development of cell-free systems in which coated vesicles are formed from immobilized plasma membrane fragments should help in the identification of the protein factors and small molecules, such as GTP, that regulate the endocytic process and confer specificity and directionality to it.
Fig. 16-33:

1. Plasma membrane
2. Coated pit formation
3. Clathrin coated vesicle
4. Uncoating
5. Docking
6. Early endosome

- Plasma membrane receptor
- Plasma membrane protein excluded from coated pits
- Docking protein in the acceptor membrane
- Extracellular ligand
- Clathrin
- Adaptor
Formation of a clathrin-coated vesicle at the plasma membrane: the role of adaptors in receptor clustering and clathrin coat assembly. (1) Plasma membrane molecules (which may be receptors bearing a ligand in their extracellular domains) that contain endocytic signals (solid semicircles) in their cytoplasmic tails interact via these signals with AP2 adaptor molecules that in turn bind the clathrin triskelions that assemble into the lattice.

Structure of a clathrin triskelion and of the hexagonal lattice into which it assembles. A clathrin triskelion is a three-legged hexameric complex consisting of three heavy and three light chains. Each heavy chain has a proximal C-terminal segment that interacts with the light chain, a middle region, and a compact globular N-terminal domain that is flexible and bends toward the membrane. The light chains are oriented with their C-terminal en...
Schematic representation of the oligomeric structures of adaptors. The HA1 and HA2 adaptors that mediate clathrin-coat assembly in the trans-Golgi apparatus or the plasma membrane, respectively, are both tetrameric proteins. Freeze-etch EM of the HA2 adaptor shows that it resembles a head with two small protruding ears derived from the C-terminal domains of the adaptins, as depicted. Proteolytic digestion removes the ears as 30-kDa C-termina...

**Endosomal Compartment**

Soon after pinching off from the plasma membrane, coated vesicles lose their clathrin coat, as they fuse with membrane-bound tubulovesicular organelles known as endosomes.

The term *receptosome* has also been proposed to designate this structure (see ref. 652).

Biochemical studies indicate that endosomes contain a distinct set of membrane proteins and, therefore, do not simply result from the fusion of coated vesicles with each other. The biochemical individuality of endosomes must reflect their biogenetic derivation, at least in part, from other intracellular, presumably Golgi-derived vesicles (see Fig. 16-32). Endosomes contain a membrane-associated ATPase that concentrates protons and acidifies the lumen of the organelle. Soon after entering endosomes located near the plasma membrane (designated early endosomes), many ligands are...
dissociated from their receptors because of the acidic pH and are released into the endosomal lumen. The receptors are then segregated to a tubular region of the endosomal membrane from which vesicles pinch off that return the receptors to the cell surface to participate in further rounds of endocytosis (see Fig. 16-32). An endosome with these features has been referred to as CURL, for compartment of uncoupling of receptors and ligands. The ligands released in the endosome and, in some cases, nondissociated receptor-ligand complexes, can subsequently undergo intralysosomal digestion. The early endosome is, therefore, a functionally complex compartment in which sorting steps control the flow of material into deeper endosomes and lysosomes and its return to the cell surface. When endocytosis is allowed to proceed in cells incubated at temperatures between 16 and 20°C, transfer of the endocytosed material to deeper, late endosomes and lysosomes is inhibited. Temperature-shift experiments proved useful to study the various elements that compose the endosomal compartment and the pathway by which endocytosed material reaches the lysosome.

The two main classes of endosomes (early endosomes and late endosomes) differ not only in their morphologic appearance, cellular location, and time at which they acquire the endocytosed marker, but also in their biochemical composition and density (which has allowed their separation by centrifugation in Percoll gradients). They differ also in their relative acidity, which is higher in deeper endosomes. Two main discordant views are currently held with respect to the functional and biogenetic relationships between the two kinds of endosomes (see ref. 651). One postulates that both types of endosomes are stable structures, i.e., distinct organelles (see ref. 702). In this view, the early or sorting endosome would generate two types of vesicles, one that returns receptors to the plasma membrane and another that delivers the endocytosed material to the late endosome, from which it would reach the lysosome. The other view (see ref. 703) is that early endosomes, after returning receptors to the plasma membrane, undergo a “maturation process” in which they are gradually transformed into late endosomes. Subsequently, these would be transformed into lysosomes by acquiring the necessary complement of lysosomal hydrolases and membrane proteins brought to them by clathrin-coated vesicles (see Fig. 16-32) that originate from the trans Golgi region or TGN. 651, 704, 705 The presence of lysosomal hydrolases in multivesicular bodies, which are endosomes that contain invaginated membrane tubules or vesicles, suggests that these represent a transition stage in the conversion of an early endosome into a lysosome (see Fig. 16-32). However, according to the view that early and late endosomes are preexisting stable compartments, the multivesicular bodies would be vehicles that transport endocytosed material from the early to the late endosomal compartment.

It has been argued that the protein compositional differences that have been detected between early and late endosomes represent evidence for the stable character of the two endosomal compartments. Such differences, however, could simply reflect the presence of passenger proteins transiently associated with these structures. Nevertheless, the functional distinctiveness of the two endosomal compartments is clear. In vitro studies (see ref. 706) have shown that early endosomes can fuse with each other, but not with late endosomes. This fusion appears to be regulated by the low-molecular-weight GTP-binding protein, rab5, which is exclusively associated with early endosomes and does not associate with late endosomes even in cells overexpressing it. Conversely, another GTP-binding protein, rab7, appears to be exclusively associated with late endosomes (see ref. 27).

Biosynthesis of Lysosomal Enzymes 653, 655, 656, 662, 709–711a

The biogenesis of lysosomes is a complex process that requires that specific sets of soluble hydrolases and membrane proteins, which are synthesized in the ER, be segregated from proteins with other subcellular destinations and be transferred to developing or preexisting lysosomes. Much progress has been made toward an understanding of the mechanism by which the newly synthesized hydrolases are sorted from secretory proteins. Clues as to the nature of the signals that ensure the incorporation of
specific proteins into the lysosomal membrane have also begun to emerge.

With rare exceptions, lysosomal enzymes are glycoproteins that contain \( N \)-linked and sometimes \( O \)-linked oligosaccharide chains. Like secretory glycoproteins, lysosomal enzymes are synthesized by membrane-bound ribosomes in the ER, and their nascent ribosome-associated chains contain cleavable \( N \)-terminal insertion signals that lead to the cotranslational passage of the polypeptides into the lumen of the ER.\(^{712-714}\) Translocation through the ER membrane is accompanied by the acquisition of \( N \)-linked high-mannose oligosaccharide chains and cleavage of the signal sequence. These processes are indistinguishable from those that take place during the early stages of the biosynthesis of secretory glycoproteins.\(^{712, 713, 715}\) Subsequently, however, the lysosomal polypeptides undergo modifications that confer on some of their oligosaccharide chains the \textit{lysosomal marker}, a mannose 6-phosphate (Man-6-P) residue. The Man-6-P distinguishes lysosomal from secretory glycoproteins and is responsible for addressing the former to their lysosomal destination (see below). The incorporation of a phosphate group at the C-6 position of some mannose residues is the result of two sequential reactions (see Fig. 16-26) that are catalyzed by enzymes that appear to be concentrated in the \textit{cis} region of the Golgi apparatus.\(^{413}\) The first step is the transfer by a \textit{phosphotransferase} of \( N \)-acytyleglucosaminyl-1-phosphate from UDP-\( N \)-acytyleglucosamine to the C-6 hydroxyl group of a mannose residue in a partially trimmed high-mannose oligosaccharide. This results in the formation of a phosphodiester bond linking the mannose to the \( N \)-acytyleglucosamine.\(^{716, 717}\) In the second reaction, a specific \textit{phosphodiesterase} removes the \( N \)-acytyleglucosamine residue and thus uncovers the phosphate on the modified mannose residue.\(^{718-720}\) A defect in the first enzyme in this modification pathway occurs in patients with I-cell disease (mucolipidosis II)\(^{721-727}\) or pseudo-Hurler polydystrophy.\(^{722-726, 728}\) It is responsible for the secretion of nonphosphorylated hydrolases from cultured fibroblasts derived from these patients, and for the high levels of hydrolases in the patients' sera.

The specificity of the \textit{UDPGlCNac:lysosomal enzyme N-acetyl-glucosamine-1-phosphotransferase} for some features of the lysosomal polypeptide is the key factor responsible for addition of the lysosomal marker only to those polypeptides destined for lysosomes. Partially purified transferase preparations modify the C-6 position of mannose residues present in lysosomal enzymes much more efficiently (lower \( K_m \)) than those in the isolated high-mannose oligosaccharide chains or in the monomeric sugar \( \alpha \)-methylmannoside.\(^{716, 717}\) Mannose residues in nonlysosomal glycoproteins are phosphorylated no more effectively than \( \alpha \)-methylmannoside.\(^{716, 717}\) The transferase recognizes a specific feature of the lysosomal polypeptide and positions the acceptor oligosaccharide chain in proximity to the enzyme's active site that modifies the mannose residue. Deglycosylated hydrolases are specific inhibitors of the transferase when other lysosomal enzymes are used as acceptors but not when \( \alpha \)-methylmannoside is the acceptor.\(^{729}\) The amino acid sequences of many lysosomal hydrolases have been determined from the nucleotide sequences of cDNA clones, but no significant similarity between the primary sequences can be recognized (e.g., refs. \(^{730}\) to \(^{737}\)).

Because most lysosomal hydrolases contain several oligosaccharide chains, and mannose phosphorylation takes place on more than one of these chains, the transferase appears to recognize a global structural feature of each protein rather than a localized domain around each oligosaccharide chain. In some cases of mucolipidosis III, the phosphotransferase appears to be defective only in its ability to recognize the lysosomal enzymes as substrates at physiological concentrations (\( \mu \)M), but is unaffected in its capacity to phosphorylate the \( \alpha \)-methylmannoside at concentrations near its \( K_m \) (200 mM).\(^{728}\)
The phosphorylation of mannose residues in some of the oligosaccharide chains in lysosomal hydrolases does not prevent the processing of other chains, or branches of the same chain, by the set of trimming enzymes and glycosyltransferases that are present in the Golgi apparatus. Thus, the nonphosphorylated chain can become complex oligosaccharides containing N-acetylglucosaminyl, galactosyl, and sialyl residues that are characteristic of many secretory proteins.\textsuperscript{738–741}

The analysis of chimeric proteins formed between different portions of two highly homologous aspartyl proteases, the lysosomal enzyme cathepsin and the secretory protein pepsinogen, has thrown light on the structural features that constitute the signal for mannose phosphorylation in cathepsin D. The three-dimensional structure of pepsinogen has been determined and it indicates that the N- and C-terminal halves of these proteins are contained in two distinct lobes within each molecule. It was shown that two noncontinuous segments in the C-terminal lobe of cathepsin D (amino acids 188 to 230, and in particular Lys 203, and amino acids 265 to 292) are necessary and sufficient for the phosphorylation of the oligosaccharides in this protein.\textsuperscript{742–744} The three-dimensional structure of pepsinogen indicates that the two polypeptide segments that form the phosphotransferase recognition site are closely apposed on the surface of cathepsin D and hence constitute a single site. Nevertheless, the presence of several other segments within the N-terminal lobe of cathepsin D enhanced the phosphorylation of the oligosaccharide in pepsinogen-cathepsin D chimeras and may be part of an extended recognition signal.\textsuperscript{744–746} Experiments with chimeras containing artificial glycosylation sites demonstrated that recognition of a lysosomal hydrolase by the phosphotransferase can lead to the modification of oligosaccharides linked at many different residues within the molecule, although those closest to the recognition determinant are preferentially phosphorylated.\textsuperscript{745}

Studies with cultured cells indicate that a fraction of newly synthesized phosphorylated hydrolases is normally secreted into the medium (see refs. \textsuperscript{713} and \textsuperscript{747}). The presence of some lysosomal hydrolases in serum and urine also suggests that some cells in the intact organism normally secrete lysosomal hydrolases.\textsuperscript{748} Some of the secreted molecules contain the Man-6-P marker, as well as complex oligosaccharide chains. Some hydrolases that reach the lysosome also acquire terminal sugars during their passage through the trans-Golgi cisterna, but sugars are later lost within the lysosome. The phosphate group itself is also removed from mature enzymes by lysosomal phosphatases.

Lysosomal hydrolases also undergo a maturation process that involves one or more proteolytic cleavage steps. These cleavages either reduce the molecular weight by loss of a C-terminal segment, as is the case with β-glucuronidase, or generate, by two successive cleavages, two polypeptides from a single precursor, as is the case with cathepsin D.\textsuperscript{713, 749, 750} Lysosomal enzymes secreted when the lysosomal marker is not added, as in I-cell disease, or in normal cells in which glycosylation is prevented by treatment of cells with tunicamycin, do not undergo proteolytic processing.\textsuperscript{713, 751} Most of the proteolytic modifications of lysosomal enzymes take place in the lysosome itself.\textsuperscript{752}

The Man-6-P Marker and the Targeting of Lysosomal Hydrolases to Lysosomes

The study of I-cell disease fibroblasts played an important role in the discovery of the Man-6-P lysosomal marker and of the receptors that recognize it and effect the segregation of newly synthesized hydrolases to the lysosomes. It was originally observed that cultured fibroblasts from patients with I-cell disease are deficient in lysosomal hydrolases and that the medium in which these cells are cultured contains higher levels of the hydrolase activities than the mediums from cultures of normal fibroblasts.\textsuperscript{753} It was subsequently demonstrated that lysosomal hydrolases secreted by normal fibroblasts can be taken up by both normal cells and cells from patients with I-cell disease, whereas the enzymes secreted by the defective cells could not be taken up by either cell type.\textsuperscript{747} The uptake of normal enzymes was shown to
be mediated by a saturable receptor that recognizes these proteins. These observations showed that in I-cell disease, the enzymes themselves are defective—not the cellular apparatus that leads to their incorporation into lysosomes. It was, therefore, proposed\textsuperscript{747} that the defect in I-cell disease is in the inability of the cells to equip newly synthesized enzymes with the marker necessary for their interiorization by receptor-mediated endocytosis. The crucial marker present in the oligosaccharide chains of secreted enzymes recognized by the receptor was later shown to be the Man-6-P residue.\textsuperscript{754}

The ability of certain cells to take up exogenous lysosomal hydrolases reflects the presence of the Man-6-P receptor in their plasma membrane. However, it is now well established that most of the receptor molecules are located in intracellular membranes and that, in fact, only a small proportion of the cellular complement of receptors is present at the cell surface.\textsuperscript{755–757}

Although in cultured fibroblasts the Man-6-P marker is necessary for the targeting of many, if not all, newly synthesized lysosomal hydrolases to lysosomes, alternative mechanisms must operate in other cell types. Thus, hepatocytes, Kupffer cells, and leukocytes from patients with I-cell disease contain nearly normal levels of some lysosomal enzymes, despite their deficiency in phosphotransferase activity.\textsuperscript{758} In addition, some hydrolases, such as acid phosphatase and β-glucocerebrosidase, are present at normal levels in fibroblasts of patients with I-cell disease.\textsuperscript{759} Acid phosphatase is synthesized as a transmembrane protein and is released from the membrane by proteolytic cleavage after it reaches the lysosome.\textsuperscript{679, 760} Glucocerebrosidase lacks a Man-6-P marker. In human hepatoma cells, HepG2, its precursor becomes membrane-associated by an unknown mechanism soon after it leaves the ER.\textsuperscript{761} A similar membrane association that is independent of the Man-6-P marker was found for the precursor of cathepsin D, which normally contains the marker. Thus, membrane association may represent an important step in an alternative pathway for the delivery of these enzymes to lysosomes.\textsuperscript{761}

A sorting signal within the polypeptide rather than in an oligosaccharide chain\textsuperscript{762, 763} operates in yeast to address digestive enzymes, such as procarboxypeptidase Y, to the vacuole, an organelle analogous to the lysosome. Mutational studies on chimeric proteins indicated that only the first 10 amino acids of procarboxypeptidase Y are required to determine its vacuolar localization.\textsuperscript{764} In recent years more than 40 genes (vacuolar protein sorting genes, VPS) have been identified (see refs. \textsuperscript{765} and \textsuperscript{766}) that play essential roles in targeting proteins to the yeast vacuole. The properties of some of these gene products indicate that vacuolar targeting in yeast may be regulated by protein phosphorylation and phosphatidylinositol signaling events (see ref. \textsuperscript{767}).

**Man-6-P Receptors**\textsuperscript{662, 711a, 768}

Two different types of Man-6-P receptor (MPR) molecules have been purified by affinity chromatography of solubilized cellular membranes on matrices containing immobilized ligands. They differ in their binding properties and divalent cation requirements for ligand binding. The first receptor that was identified (referred to as MPRCI, MPR300, or Man-6-P/IGFII receptor) is a 275- to 300-kDa glycoprotein that does not require cations to bind ligand.\textsuperscript{769} Subsequently, it was discovered to be identical with the receptor for insulin-like growth factor II (IGFII), although the two ligands bind at different sites (e.g., ref. \textsuperscript{770}). Specific antibodies to the Man-6-P/IGFII receptor have allowed studies of its subcellular distribution and of the pathway it follows in sorting and transport.\textsuperscript{701, 704, 771–778} The amino acid sequence of this receptor, deduced from the cDNA clone,\textsuperscript{779} revealed that it is a type I transmembrane glycoprotein that has a small segment of 17 kDa exposed on the cytoplasmic side of the membrane.\textsuperscript{780} The luminal domain of the receptor, where the ligand binding site(s) is found, is composed of 15 homologous segments, each of approximately 145 amino acids in length. The amount of Man-6-P/IGFII receptor present on the cell surface varies with the cell type, but most of this receptor is found in intracellular membranes. By
immunocytochemistry, the receptor has been shown to be present in coated vesicles, endosomes, and Golgi membranes, but not in mature lysosomes (see below). The lysosomal sorting role of the MPR300 has been directly demonstrated by gene transfer experiments. Expression of the transfected receptor cDNA in certain cell lines that lack it and normally secrete large amounts of lysosomal enzymes restored the efficient delivery of these enzymes to lysosomes and also conferred on the cells the capacity to take up exogenous hydrolases containing the Man-6-P marker.\textsuperscript{781, 782}

The second Man-6-P receptor (MPRCD or MPR46) is a 46-kDa glycoprotein that in bovine and murine (but not human tissues) requires divalent cations, particularly Mn\textsuperscript{2+}. It requires a somewhat more acidic pH (6.3) for ligand binding than the larger receptor, which has high affinity for the phosphorylated ligand at neutral pH.\textsuperscript{783, 784} The amino acid sequence of the 46-kDa receptor, derived from a cDNA clone,\textsuperscript{785–786a} shows that it is also a type I transmembrane protein, with a cytoplasmic domain of 69 residues and a luminal domain that shows homology to the repeated segments within the luminal domain of the Man-6-P/IGFII receptor. In particular, significant similarity is found within a pentadecapeptide, limited by cysteine residues, that is also present in all the repeating domains of the large receptor. The discovery of the 46-kDa MPR resulted from the observation that the cell lines that lack the large MPR receptor\textsuperscript{787} are still capable of sorting some of their lysosomal hydrolases (with approximately 40 percent efficiency). These cells were found to be incapable of taking up the exogenous lysosomal hydrolases when these were administered under the conditions usually employed, which do not satisfy the cation and pH requirements for binding to the MPR46. Moreover, expression of high levels of MPR46 in these cells, after transfection with the appropriate cDNA, only slightly increased their capacity to take up exogenous hydrolases in neutral medium.\textsuperscript{788, 789} The MPR46 may well play a role in mediating secretion of lysosomal enzymes, since overexpression of this receptor in cells that contain the MPR300 induces the appearance of large amounts of newly synthesized hydrolases in the medium.\textsuperscript{790}

The critical function of the receptors that allows them to selectively transfer lysosomal enzymes bearing the Man-6-P marker to developing lysosomes is the pH dependence of their affinity for the ligands. At neutral or slightly acidic pH, the receptors bind strongly to phosphomannose-containing oligosaccharides or lysosomal hydrolases bearing the marker. However, they release these ligands quantitatively at the strong acid pH characteristic of endosomes or lysosomes.\textsuperscript{741, 784, 789} To explain the fact that the MPR300 is highly efficient in targeting hydrolases to lysosomes, whereas MPR46 leads to secretion of its ligands, it was suggested that the former releases its ligands in deeper endosomal compartments, whereas the latter releases them in early endosomes from which the enzymes can more easily exit from the cell.\textsuperscript{790}

Binding of hydrolases to the receptors requires removal of the GlcNac group that initially covers the phosphate group on the C-6 of the mannose residue,\textsuperscript{741, 789} and high-affinity binding requires the presence of at least two Man-6-P residues in the same molecule, although not necessarily on the same oligosaccharide chain.\textsuperscript{784, 791, 792} Lysosomal hydrolases of the slime mold \textit{Dictyostelium discoideum} bind effectively to their receptor, even though their Man-6-P residues are covered by methyl groups.\textsuperscript{793} However, only the MPR300 is capable of recognizing Man-6-P residues bearing this modification.\textsuperscript{787}

**Delivery of Hydrolases to Incipient Lysosomes**

As previously noted, the enzymes that synthesize the Man-6-P marker are located in the \textit{cis} region of the Golgi apparatus and, in principle, the hydrolases could be engaged by the MPR as soon as they are modified. Indeed, in some cell types, such as pancreatic, hepatic, and epididymal, the MPR300 is most concentrated in \textit{cis}-Golgi cisternae.\textsuperscript{772, 794} In other cell types, however, it is most abundant in the \textit{trans}-Golgi region, TGN, and endosomes.\textsuperscript{795} The steady state distribution of MPR46 also varies with the cell type,\textsuperscript{796, 797} but in all cases studied to date the MPR receptors are absent from mature
There is now a consensus that the occupied receptors emerge from the Golgi apparatus in the TGN (Figs. 16-32 and 16-36), where HA1 adaptors must recognize targeting signals in the cytoplasmic domain of the receptors, and mediate their incorporation into clathrin-coated vesicles in which lysosomal enzyme precursors have been found. After uncoating, these vesicles then must fuse with endosomal structures that represent intermediates in the formation of lysosomes. As a result of the low pH in the endosomal compartment, the ligand would then dissociate from the receptor, which would recycle to the TGN and occasionally reach the cell surface.

Immunocytochemical studies on the distribution of the MPR300, lysosomal hydrolases, and membrane proteins, and of endocytosed markers at different times after internalization, have indicated that various types of endosomes may serve as acceptors for the vesicles that deliver the hydrolases during the biogenesis of a lysosome. Some investigators reported that the vast majority of MPR300 molecules are located in a deep endosome compartment that contains lysosomal membrane proteins and hydrolases, but is inaccessible to endocytic markers during incubation at 20°C. This prelyosome or intermediate compartment, therefore, has the properties of a deep endosome from which a lysosome could be generated by continued delivery of hydrolases and membrane proteins, followed by retrieval of all MPR molecules. Other investigators have found substantial amounts of MPR and some lysosomal enzymes even in early endosomes, which, at 37°C, rapidly acquire endocytic markers, but can even be labeled with them after longer incubations at 18.5°C. These observations are consistent with the maturation model for lysosome biogenesis that involves the progressive transformation of early endosomes to late endosomes and finally into lysosomes.

There is some controversy over whether movement of the receptor from the Golgi apparatus to the receiving endosome takes place only if the receptor is occupied by a ligand, or whether the receptor moves constitutively. One group has reported that receptors accumulate in Golgi membranes and that endosomes are depleted of receptors when cells are treated with tunicamycin, a drug that blocks core glycosylation in the ER and therefore prevents the acquisition of the Man-6-P marker by the newly synthesized hydrolases. The same group also observed that when dissociation of the receptors from their ligands is prevented by administration of lysosomotropic drugs, such as chloroquine or NH₄Cl, that concentrate in the lumen of acidic compartments and raise their pH, the receptors accumulate in endosomes. These authors showed that, under these conditions, the failure of the receptor to return to the Golgi apparatus results from its inability to release the ligand and not from the altered pH of the endosome. When cells treated with chloroquine were incubated with Man-6-P, which enters the endosome compartment by fluid-phase pinocytosis, this competing ligand led to dissociation of the complex, which in turn was followed by reappearance of the receptor in Golgi membranes. However, other investigators have concluded that the receptor recycles constitutively, i.e., even in the absence of the ligand. It was observed that the level of receptor in the endosome was not significantly reduced when the synthesis of new lysosomal hydrolases was blocked by the protein synthesis inhibitor cycloheximide. The failure of the Man-6-P receptor-ligand complexes to dissociate at the altered pH of the endosomes in cells treated with lysosomotropic drugs accounts for the original finding that secretion of lysosomal enzymes is increased under these conditions, since all the receptors remain occupied and, therefore, inaccessible to the newly synthesized hydrolases.

In addition to undergoing recycling between the Golgi apparatus and prelyosomal endosomes, receptor molecules also recycle through the plasma membrane, where the presence of variable but relatively small amounts (approximately 10 percent) of receptor has been demonstrated in many cell types (see ref. 699). Experiments in which surface receptors were desialylated by treatment with neuraminidase, to follow their rate of resialylation by the TGN sialyl transferase, indicate that after internalization by endocytosis, surface MPR receptors reach the TGN, with a half-time of approximately 3 h. The presence of surface
receptors and their recycling, as well as the significant levels of lysosomal hydrolases found in normal serum, suggest that secretion and uptake of lysosomal enzymes is a physiological process. Indeed, there are conditions in which the secretion of enzymes from some cells and their uptake by others correct a genetic defect. Thus, in female carriers of Hunter disease, an X-linked lysosomal disorder that is characterized by a deficiency in iduronate sulfatase, cells that cannot synthesize the normal enzyme because of inactivation of the normal X chromosome appear to be phenotypically normal. Presumably this is the result of cross-feeding by cells in which only the affected X chromosome was inactivated.807

Lysosomal Membrane

The lysosomal membrane serves as a selective permeability barrier between the lysosomal lumen and the cytoplasm. It is equipped with carriers and transport systems that control the passage of substances between both compartments and with a proton pump that creates the acidic environment necessary for intralysosomal digestion. The lysosomal membrane prevents the egress of macromolecules brought into the lysosome by endocytosis or autophagy and allows the escape of only the end products of their digestion. The amino acids, dipeptides, nucleosides, small monomeric sugars, phosphate or sulfate ions, and other molecules released from lysosomes by active or passive transport may be utilized for biosynthetic reactions in the cytoplasm. Certain nutrients such as cholesterol, released from cholesterol esters brought into lysosomes by endocytosis of LDL particles,274, 810 and cobalamin (vitamin B12), taken up complexed to transcobalamin II,811, 812 are delivered to the cytoplasm by transport through the lysosomal membrane.

Several carrier-mediated transport systems specific for cystine,813–818 cationic amino acids,819, 820 small neutral amino acids,821 or tyrosine and other bulky neutral amino acids822 have been identified in the lysosomal membrane. In the recessively inherited disease nephropathic cystinosis, a defect in the carrier that mediates the transport of cystine leads to the accumulation of large amounts of this disulfide amino acid within the lysosome.813–815, 817 Although cystine accumulates within the lysosomal compartment, it could not exist in the cytoplasm, where strongly reducing conditions prevail. The therapeutic administration of the aminothiol cysteamine reduces the accumulation of cystine through the formation within the lysosome of the mixed disulfide of cysteine and cysteamine, which behaves as a lysine analogue and is recognized by the system that transports cationic amino acids.814, 823 Certain compounds such as the acidotropic amines, chloroquine and primaquine, as well as amino acid esters, dipeptides, and oligopeptides (particularly if rich in hydrophobic amino acids) traverse the lysosomal membrane from the cytoplasm to the luminal side. The protonated amines, as well as the free amino acids generated by hydrolysis of the ester and peptide bonds, accumulate within the lysosomes and exit only slowly through the lysosomal membrane. Monosaccharides seem to cross the lysosomal membrane by facilitated diffusion, and a carrier has been identified that mediates the transfer of sialic acid. This carrier appears to be defective in Salla disease,824, 825 in which an intralysosomal accumulation of sialic acid occurs.

A membrane-associated enzyme, acetyl CoA: α-glycosaminide N-acetyltransferase, transfers acetyl groups from acetyl CoA in the cytoplasm to acceptor glucosamine moieties linked in terminal α linkages to heparan sulfate molecules within the lysosome.826 The acetylation on heparan sulfate appears to be necessary for its degradation. A deficiency in the transferase produces the Sanfilippo C syndrome,827 resulting from intralysosomal accumulation of heparan sulfate.

There is a proton pump in the lysosomal membrane that creates and maintains an acid environment (pH 5) within the lysosomal lumen (see refs. 828 and 829). The pump utilizes cytoplasmic ATP, is believed to function in an electrogenic manner,830–832a and operates most effectively in the presence of chloride ions, which also accumulate in the lysosomal lumen. In its sensitivity to inhibitors,830, 831 the lysosomal pump...
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strongly resembles other ATPases (known as vacuolar ATPases) that have been identified in the ER,\textsuperscript{832a} the Golgi apparatus,\textsuperscript{417} and endocytic and secretory vesicles (see refs. \textsuperscript{829} and \textsuperscript{833}). These proton ATPases are responsible for the progressive acidification of the lumen of the organelles that constitute the endomembrane system and are selectively inhibited by the macroline antibiotic \textit{bafilomycin A}$_1$.\textsuperscript{834} The vacuolar pumps are insensitive to oligomycin, azide, N,N'-dicyclohexylcarbodiimide (DCCC) and other inhibitors that block the function of the mitochondrial F$_0$F$_1$ ATPase, and to vanadate, which inhibits the E$_1$E$_2$ phosphoenzyme-type ATPases (such as the Na$^+$/K$^+$-ATPase of the plasma membrane). On the other hand, levels of the sulfhydryl reagent NEM that inhibit the function of vacuolar ATPases do not affect the other ion-motive enzymes (the F$_0$F$_1$ and E$_1$E$_2$ ATPases).

The luminal surface of the lysosomal membrane is thought to be protected from the attack of lysosome hydrolases by complex oligosaccharides rich in poly-$N$-acetyllactosamines bearing sialic acid, which appear to be characteristic of lysosomal membrane glycoproteins (see below). Acting as immobilized polyanions, the sialic acid moieties may also play an important role in establishing a Donnan potential for protons that contributes to the internal low pH of lysosomes.\textsuperscript{835}

Very little is known of the cytoplasmic surface of the lysosomal membrane, although it must possess receptors that mediate the fusion of lysosomes with other lysosomes or with phagosomes or endosomes to which lysosomal enzymes must be made available. This surface must also interact with the cytoskeletal elements responsible for lysosomal movement.\textsuperscript{836, 837} A lysosomal membrane protein has been purified that binds to preassembled microtubules and may participate in this process.\textsuperscript{838}

Through protein purification\textsuperscript{839–841} and the use of polyclonal and monoclonal antibodies prepared against lysosomal membranes,\textsuperscript{842–847} several lysosomal membrane proteins have been identified. These have received various names, such as lysosomal associated membrane proteins (\textit{lamp}), lysosomal integral membrane proteins (\textit{limp}), lysosomal glycoproteins (\textit{lgp}), and lysosomal endosomal plasma membrane proteins (\textit{lep 100} and \textit{endolin}), corresponding to their organellar derivation. Immunocytochemical methods indicate that these proteins colocalize with cytoplasmic structures identified as lysosomes because of their content of lysosomal hydrolases and their capacity to accumulate the dye acridine orange.\textsuperscript{848} Although their function remains unknown, these proteins have become useful models to study the biosynthesis of the lysosomal membrane. Some of the proteins identified appear to represent equivalent gene products in different species (e.g., human lamp 1 = mouse limp 3 = rat lgp 120 = chicken lep 100; human lamp 2 = mouse limp 4 = rat lgp 110; lamp 3 = CD63). All of them are glycoproteins rich in $N$-linked oligosaccharides, most of which are of the complex type and are, therefore, initially synthesized in the ER and modified in the Golgi apparatus. The oligosaccharides represent up to 65 percent of the mass of the individual proteins, and the lamp molecules bear the bulk of the cellular complement of poly-$N$-acetyllactosamines $[(Galβ1-4GlcNAcβ1-3)_n]$, which are composed of $N$-acetyllactosamine repeats in the side chains of the $N$-linked oligosaccharides. These oligosaccharides usually carry added carbohydrate structures, such as the blood group antigen sialyl Le$^x$ (NeuNAcc$α2-3Galβ1-4, (Fuc$α1-3$)GlcNac$α$).\textsuperscript{849} Some lysosomal membrane proteins are found in significant amounts only in mature or developing lysosomes and are absent from endosomes or the plasma membrane,\textsuperscript{842–844} whereas others appear to undergo a constant circulation through the three compartments.\textsuperscript{846}

The complex oligosaccharide chains in lysosomal membrane proteins are likely to play a role in protecting the exposed portions of the polypeptides from the attack of lysosomal proteases, since in tunicamycin-treated cells the proteins have shorter-than-normal half-lives.\textsuperscript{847} Lamp 1 and lamp 2 are so abundant that their oligosaccharides may completely coat the luminal side of the membrane and thus
serve as an effective barrier to the hydrolases.

It has been suggested that in cells capable of the exocytic discharge of lysosome-like granules (such as cytotoxic lymphocytes, platelets, and phagocytes) the lysosomal membrane proteins incorporated into the plasma membrane serve to protect the cell from the noxious effect of the released content enzymes. It has been suggested that in cells capable of the exocytic discharge of lysosome-like granules (such as cytotoxic lymphocytes, platelets, and phagocytes) the lysosomal membrane proteins incorporated into the plasma membrane serve to protect the cell from the noxious effect of the released content enzymes. 809 The lamp molecules that appear on the surface of granulocytes and monocytes that discharge their granules in inflammatory sites also may provide exposed sialyl Le\(^x\) oligosaccharides that serve as ligands recognized by cell-adhesion molecules such as ELAM-1 and GMP-140 (P selectin), of endothelial cells and platelets, respectively (see ref. 809). In fact, since a small proportion of lysosomal membrane proteins are normally present in the plasma membrane (see below) their adhesive properties may contribute to normal cell-cell interactions.

**Transport of Membrane Proteins to the Lysosome**

Evidence indicates that unlike the situation with the lysosomal hydrolases, the sorting of lysosomal membrane proteins to the lysosome is not mediated by an oligosaccharide bearing the Man-6-P marker. Thus, the membrane-associated enzymes β-glucocerebrosidase and acetyl-CoA α-D-glycosaminide N-acetyltransferase are present at normal levels in I-cell disease fibroblasts. The former enzyme has been directly shown not to be phosphorylated in normal cells. Moreover, the oligosaccharides of some of the previously mentioned purified lysosomal membrane proteins identified with antibodies to the lysosomal membrane do not appear to contain phosphate groups. In pulse labeling and cell fractionation experiments with tunicamycin-treated cultured cells, some of the newly synthesized membrane proteins are, nevertheless, transported to the lysosome with rapid kinetics. Under the same conditions, the lysosomal hydrolases lacking N-linked oligosaccharides are secreted. Therefore, the transport of membrane proteins to lysosomes does not seem to be coupled obligatorily to the transport of lysosomal hydrolases, which takes place via the Man-6-P receptor and is interrupted after tunicamycin treatment.

Several studies in which the fate of altered lysosomal membrane proteins or chimeric polypeptides containing segments of lysosomal membrane proteins were examined in transfected cells have demonstrated that the signals that target those proteins to the lysosome are contained within their short cytoplasmic domains. Human lamp 1 and the equivalent rat lgp 120 contain a glycine\(^7\), tyrosine\(^8\) (G\(^7\)Y\(^8\)) motif in their 11 amino acid cytoplasmic tails. This motif is also present in lamp 2 and 3, as well as—in an almost equivalent position—in the precursor of the lysosomal acid phosphatase (LAP), a transmembrane protein with a slightly longer cytoplasmic tail (19 amino acids). These proteins are normally present at low levels in the plasma membrane, from which they are removed by rapid endocytosis. Mutation of the single tyrosine within the G\(^7\)Y\(^8\) motif led to the accumulation of the proteins in the plasma membrane, as expected from the abolition of the tyrosine-containing endocytic signal, which is similar to that present in the transferrin and LDL receptors. These observations are consistent with a pathway for delivery of membrane proteins to lysosomes that involves two steps: first, their direct transfer from the TGN to the plasma membrane, and second, their internalization into endosomes and appearance in lysosomes. Indeed, several analyses of the kinetics of delivery of lysosomal membrane proteins to lysosomes and determinations of their transient appearance at the plasma membrane have provided evidence for this indirect pathway. This mechanism of delivery would require that the interiorization signals that trigger endocytosis of the lysosomal membrane protein have the added feature of favoring transfer of those proteins from early endosomes to deeper endosomes and lysosomes, although a certain fraction of the interiorized molecules would constantly recycle from endosomes to the cell surface. The studies on the effect of mutations within the cytoplasmic domain of lysosomal membrane proteins, and of appropriate chimeric transmembrane proteins, established that this domain contains a motif that is necessary and sufficient for targeting the proteins to
An alternative view for the pathway of delivery of lysosomal membrane proteins to lysosomes is that, like the lysosomal hydrolases, the bulk of the membrane proteins are sorted in the TGN from proteins with other destinations and transferred directly to the developing lysosome. Support for this view was obtained from labeling and cell fractionation experiments showing that newly synthesized membrane proteins appear in lysosomes with kinetics so rapid as to make it unlikely that a major fraction of such proteins first reaches the cell surface to then undergo interiorization, a step which proceeds with relatively slow kinetics. It currently appears that both the direct and indirect routes may be utilized with different efficiencies in different cell types and, possibly, at different steps of cell differentiation.

One study indicates that the machinery that effects the direct delivery of membrane proteins from the TGN to lysosomes can be easily saturated. The membrane proteins that are synthesized in excess of its capacity take the indirect route. It was observed that mutation of either glycine or tyrosine within the cytoplasmic domain of lgp 120 abolished direct transfer of the protein from the TGN to lysosomes and led to the delivery of the mutants to the cell surface. However, whereas the mutant lacking tyrosine was unable to reach the lysosome by either the direct or indirect route and therefore accumulated at the cell surface, the mutant lacking glycine lost only the capacity to follow the direct route. This mutant, therefore, appeared only transiently at the cell surface and followed the indirect route to reach the lysosome.

**Potocytosis**

Recently, the existence of a process, termed *potocytosis*, has been recognized that may effect the uptake of small molecules and ions into cells within flask-shaped plasma membrane invaginations called caveolae. These structures are prominent in fibroblasts and endothelial cells but are also found in many other cell types. Although in endothelial cells they are involved in transcytosis, perhaps through the fusion of caveolae emerging from opposite sides of the cell, it is not yet clear if caveolae actually pinch off from the plasma membrane or always remain connected to the cell surface.

Caveolae appear to function as specialized sites for the concentration, uptake, and perhaps storage of small molecules that serve as ligands for GIPL-linked plasma membrane receptors. Indeed, it has been proposed that small molecules and ions are brought to caveolae by specific plasma membrane receptors (e.g., the folate receptor), carrier proteins, or enzymes that, like 5'-nucleotidase, are GIPL-linked (see below). The release of the ligands within the caveolae would create a high local concentration of small molecules and ions, sufficient to drive their transport into the cytoplasm. It is possible, for example, that in this manner potocytosis is responsible for the uptake of adenosine, which would be released into caveolae by the action of 5'-nucleotidase on AMP, a nucleotide that cannot traverse the plasma membrane. Potocytosis may also be important in the transport across the capillary endothelium of the thyroid hormone, thyroxine, which in serum is bound to the carrier protein transthyretin. This carrier has been found to enter the caveolae of endothelial cells, where it would release its ligand without ever reaching the pericapillary space. Free thyroxine, however, would diffuse into the cytoplasm of the endothelium and across its plasma membrane to the perivascular space.

The most striking structural feature of caveolae is that they contain clusters of GIPL-anchored proteins, such as the receptor for folate and the enzymes 5'-nucleotidase and alkaline phosphatase. More than 80 percent of the plasma membrane content of these proteins appears to be immobilized in clusters within caveolae, and studies with GIPL-linked proteins indicate that, although the unclustered and clustered fractions appear to be in dynamic equilibrium, only the mobile molecules can be interiorized in clathrin-coated vesicles, probably because they associate with other transmembrane receptors. The formation of the clusters of GIPL-linked proteins and the integrity of the caveolae appear to require the
presence of cholesterol in the membrane, since manipulations that decrease the concentration of this sterol disperse the GIPL-linked proteins, disassemble the caveolae, and inhibit the uptake of folate that is mediated by its lipid-anchored receptor.\textsuperscript{868}

The cytoplasmic face of caveolae is covered by a distinct striated coat that was shown to contain a 22-kDa integral membrane protein, now named caveolin,\textsuperscript{865} previously identified as a substrate of the v-src tyrosine kinase in virally transformed cells. In this regard, it is striking that GPI-linked proteins in the plasma membrane seem to be associated in some way with tyrosine kinases, such as p56lck and p59fyn, and that antibodies to the GPI-linked proteins are able to trigger T-cell activation.

Caveolin is also identical to VIP-21,\textsuperscript{869} which was found to be an integral membrane component of TGN-derived vesicles thought to be involved in transport of proteins to the apical surface of polarized epithelial cells.\textsuperscript{870} As previously mentioned, in these cells GIPL-linked proteins and sphingolipids cluster together in the TGN to be delivered to the apical surface, where GIPL-linked proteins are known to accumulate preferentially. It is therefore conceivable that there is a mechanistic relationship between the association of caveolin with GIPL-linked proteins in caveolae and in the apically targeted vesicles.

**MITOCHONDRIAL BIOGENESIS\textsuperscript{870a–880}**

Mitochondria are cytoplasmic organelles that carry out cellular respiration and generate most of the ATP that fuels the activities of the cell. They contain a vast array of enzymes that may vary with the cell type, reflecting their central role in various aspects of metabolism. Mitochondria are also capable of storing and releasing calcium ions, and it is thought that this enables them to serve, together with the ER, as regulators of cytoplasmic calcium levels.

Structurally, mitochondria are characterized by the presence of two concentric lipoprotein membranes and two internal compartments (Fig. 16-37). The outer mitochondrial membrane (OMM) completely surrounds the organelle, and all molecules entering or leaving the mitochondrion must pass through it. The inner mitochondrial membrane (IMM) is separated from the outer by an *intermembrane space* and encloses the major intramitochondrial compartment, known as the *matrix* space or stroma. The surface area of the inner membrane is greatly increased by the presence of numerous invfoldings, known as *cristae*, that vary in number and configuration depending on the type of cell and its physiological state. Regions of close contact between the inner and outer mitochondrial membranes are frequently observed, and importation of proteins into the organelle is known to take place at these “contact” sites.\textsuperscript{881–887}
Mitochondrial subcompartments. A mitochondrion contains two concentric membranes, an inner (IM) and an outer one (OM), separated by an intermembrane space. The matrix represents the space completely surrounded by the inner membrane. Contact sites (CS) between the two membranes (marked by arrows) appear to be the sites for the incorporation into the organelle of polypeptides synthesized in the cytoplasm. The cristae are represented by platel...

Specific mitochondrial proteins are found in each one of the four submitochondrial compartments (i.e., the two membranes and the intermembrane and matrix spaces). The outer membrane has a relatively low protein content and is characterized by large amounts of the transmembrane protein “porin,” which forms channels through which ions and small molecules can pass freely. It also contains receptors for protein import (see below), monoamine oxidase, and, in some cell types such as hepatocytes, cytochrome b5 and cytochrome b5 reductase, proteins that have corresponding, but not identical, counterparts in the ER.

In contrast to the OMM, the IMM is impermeable to small molecules and contains specialized transport systems, such as the ATP-ADP carrier or translocator and transporters for phosphate, carnitine esters, pyruvate, malate, and glycerol phosphate. Most importantly, this membrane contains the components of the electron transport chain that receives electrons generated by dehydrogenation of the citric acid cycle substrates. It also contains the ATP synthase (F1F0-ATPase) that carries out oxidative phosphorylation (see ref. 870a).

The intermembrane space contains enzymes, such as myokinase (adenylate kinase), which functions to equilibrate ATP and AMP with ADP. These enzymes can be released relatively easily from isolated mitochondria by osmotic shock or sonication. The mitochondrial matrix houses enzymes of the citric acid cycle, the pyruvate dehydrogenase complex, and the enzymatic system that carries out the β-oxidation of fatty acids. In the hepatocyte, the matrix is also the site of some of the enzymes of the urea cycle.

The mitochondrial membranes contain both peripheral and integral proteins, and each membrane polypeptide has a characteristic disposition relative to the phospholipid bilayer. For example, cytochrome oxidase, a protein that consists of several subunits and serves as the terminal member of the electron transport chain that carries out the reduction of oxygen, is an integral component of the inner membrane and is exposed on both sides of this membrane. On the other hand, cytochrome c, which delivers electrons to cytochrome oxidase, is a peripheral membrane protein reversibly bound to a portion of cytochrome oxidase exposed on the outer face of the inner membrane. The ATP synthase that is driven
by a proton gradient to effect ATP synthesis is also a complex protein that in yeast contains nine subunits. Four of these form the $F_0$, or stalk portion of the complex, which is embedded in the inner membrane. The other subunits ($\alpha, \beta, \gamma, \delta, \epsilon$) form a round particle, the $F_1$ ATPase (with the composition $\alpha_3\beta_3\gamma\delta\epsilon$), which protrudes into the matrix space and is held onto the membrane by the stalk (see ref. 870a, for organization of components of the electron transport chain).

Mitochondria are the only organelles of mammalian cells that possess a separate genome and the enzymatic machinery necessary for its replication and expression. 871 The mitochondrial genes are contained in a circular double-stranded DNA molecule that in the human consists of 16,569 nucleotide pairs whose sequence has been determined889 (see Chaps. 104 and 105).

The mitochondrial DNA encodes the ribosomal RNA of the mitochondrial ribosomes, as well as 22 different transfer RNA molecules. Although mitochondria contain at least several hundred different polypeptides, only 13 of these are encoded in the organellar genome and are synthesized within the mitochondria. These include some, but not all, of the subunits of the cytochrome oxidase and of the ATPase, as well as subunits of the coenzyme QH$_2$-cytochrome c reductase, another member of the electron transfer chain. The mitochondrially synthesized polypeptides contain hydrophobic segments and are, in general, components of the inner membrane. Almost all other mitochondrial proteins, including the remaining subunits of the ATPase and cytochrome oxidase, are encoded in nuclear genes and are synthesized outside the mitochondria. They are subsequently taken up into the organelle and sorted into one of the four submitochondrial compartments.

**Incorporation of Proteins into Mitochondria (Fig. 16-38)**

Much has been learned about this process from studies carried out with yeast and Neurospora. These unicellular eukaryotic organisms are suitable for both genetic analysis and in vivo pulse-chase experiments in which the precursors of polypeptides destined to mitochondria can be identified. The study of protein transfer into mitochondria has also made rapid progress because it has been possible to reproduce this phenomenon in vitro by adding polypeptides synthesized in cell-free systems to isolated mitochondria obtained from either unicellular organisms or cells of higher animals.
Pathways for the incorporation of polypeptides into mitochondria. A. Incorporation of matrix proteins. A targeting signal located within a cleavable presequence at the N-terminus of the polypeptide (1) leads first to the association of the polypeptide first with the outer membrane (2) and then, in the presence of a transmembrane potential (3), to the passage of the polypeptide into the matrix through a point of contact between the two membra...
Mitochondrial proteins encoded in the nuclear genome are synthesized in polyribosomes that are not bound to ER membranes. Most, but not all, proteins destined to the interior of the mitochondrion are synthesized as larger precursors containing N-terminal extensions or presequences (20 to 80 amino acids long) that are removed by intramitochondrial proteases, whose function is essential for normal mitochondrial biogenesis. Each presequence contains an addressing or targeting signal that is responsible for the incorporation of the polypeptide into the organelle. The presequence may also contain a sorting or localization signal downstream from the targeting signal that determines the intramitochondrial location of the polypeptide. In the absence of a localization signal, the targeting signal within the presequence directs the polypeptide across both the inner and outer mitochondrial membranes into the matrix. The localization signal, which in some cases is present within the sequence of the mature protein, may arrest translocation of the polypeptide through the outer or inner membrane or, after the protein reaches the matrix, may direct its re-export through the inner membrane toward the intermembrane space.

Proteins of the outer mitochondrial membrane are synthesized without cleavable presequences and, therefore, must contain permanent targeting signals. These include porin, the MAS70 protein that functions as an import receptor for a subclass of mitochondrial proteins, some proteins of the interior mitochondrial subcompartments (such as cytochrome c of the intermembrane space), and the ADP-ATP translocator of the inner membrane.

The insertion of outer membrane polypeptides into the membrane and the passage of apocytochrome c into the intermembrane space are processes that appear to occur spontaneously, without the requirement of energy. The uptake of other polypeptides into the inner mitochondrial compartments (i.e., matrix, inner membrane, and in some cases, the intermembrane space), is an energy-dependent process that requires not only the availability of ATP, but also the existence of an electrochemical potential across the inner membrane (i.e., an energized inner membrane). Thus, uptake of such polypeptides is blocked by the addition of respiratory inhibitors, uncouplers of oxidative phosphorylation, or the ionophore valinomycin, which eliminates the membrane potential by allowing for the equilibration of the K⁺ ion concentrations on both sides of the inner membrane.

**Signals for Targeting of Proteins to Mitochondria**

The cleavable presequences within precursors of mitochondrial proteins generally contain at or near the N-terminal region a segment that is rich in basic and hydroxylated amino acids and usually lack acidic amino acids and extensive stretches of hydrophobic residues. In mediums of low polarity, or on insertion into lipid bilayers, these segments appear to be able to form amphipathic helices, which contain positively charged or polar residues on one side of the helix and hydrophobic residues on the other. The structure of the mitochondrial presequences is, therefore, fundamentally different from that of the signal sequences characteristic of polypeptides synthesized in the ER.

The presence within the presequences of targeting signals for incorporation of the polypeptides into mitochondria was established by experiments that employed recombinant DNA methods to link the presequences, or portions thereof, to segments of other polypeptides that are not normally targeted to the mitochondria. Thus, when gene segments encoding the presequences of the yeast cytochrome oxidase subunit IV, alcohol dehydrogenase III, or rat liver ornithine transcarbamylase (OTC) were fused with a DNA sequence encoding dihydrofolate reductase (DHFR), a cytosolic enzyme, the resulting chimeric polypeptides were incorporated into mitochondria, both in vivo and in vitro. Experiments of this type showed that, although the presequence (i.e., the cleaved segment) of the yeast cytochrome oxidase subunit IV is 25 residues in length, only its first 12 amino acids serve as the targeting signal and are
required for incorporation of the chimera into the mitochondrial matrix. Proteolytic removal of the shortened presequence, however, did not take place. The efficacy of shortened presequences of only 9 to 12 amino acids in length has also been demonstrated for other mitochondrial proteins, such as δ-aminolevulinate synthase. The crucial role of presequences in determining the uptake of mitochondrial proteins is highlighted by the existence of a natural mutation in a patient with methylmalonic acidemia in which a short, apparently N-terminal, deletion within the precursor of methylmalonyl CoA mutase prevents the incorporation of the enzyme into the mitochondria.

The targeting sequences need not always be located at the extreme N-terminus of the precursor polypeptide. In OTC, which contains a 32-residue cleavable presequence, residues 8 to 23 were found to be essential for uptake. However, the function of a targeting signal requires that it remain exposed when the polypeptide folds in the cytoplasm after being discharged from the ribosome. Thus, the cytosolic protein DHFR contains a cryptic targeting signal located between residues 26 to 85, but this signal does not normally operate to bring the polypeptide into the mitochondria, apparently because it is “masked” in the folded protein. However, when the first 85 residues of DHFR are linked to the N-terminus of an intact DHFR sequence, or to subunit IV of cytochrome oxidase lacking its own targeting sequence, the normally cryptic signal becomes exposed and the chimeric polypeptides are efficiently incorporated into mitochondria.

**Receptors for the Uptake of Proteins into Mitochondria (Fig. 16-39)**

With the exception of cytochrome c, polypeptides to be incorporated into mitochondria are recognized by receptors present in the OMM. Under appropriate experimental conditions (e.g., inhibition of polypeptide chain elongation) polysomes synthesizing certain mitochondrial proteins are recovered selectively with sedimentable mitochondria. This suggests that interaction of precursors of mitochondrial proteins with their receptors within the cell may take place before their synthesis is completed. The presence of proteinaceous receptors on the mitochondrial surface for precursors of mitochondrial proteins was first inferred from the observation that the in vitro binding and uptake of precursors is nearly completely abolished by brief treatment of intact mitochondria with low concentrations of proteases. Since several hundred different polypeptides must be imported into the mitochondria, separate receptors for each mitochondrial polypeptide seemed highly improbable. In fact, studies have shown that there are two major types of receptors on the mitochondrial surface, which recognize different sets of proteins. The existence of more than one type of receptor was initially suggested by observations that the addition of a synthetic presequence peptide or of a purified unfolded mitochondrial protein to an in vitro uptake system, or mild protease treatment of the mitochondria, selectively blocked the incorporation of some precursors but not others into the organelle.
Organization of the translocation machineries in the outer and inner mitochondrial membranes. The scheme (based on Pfanner et al. 878) represents a region of a mitochondrion where translocation of a polypeptide from the cytosol into the matrix is taking place. This region is shown as flanked by putative adhesion sites that bring the outer (OM) and inner (IM) membranes close to each other. Polypeptides to be inco...
that contains noncleavable internal targeting signals). Antibodies to this receptor could coprecipitate, from detergent-treated mitochondria, ATP-ADP carrier molecules that had been bound to the organelle before lysis together with the antigen.\textsuperscript{932} MAS70, however, is not essential for mitochondrial import. A null mutation in the corresponding yeast gene was not lethal\textsuperscript{933} although it decreased the rate of import of many mitochondrial precursor proteins in vivo.\textsuperscript{898} 

Antibodies to the second receptor, MOM19, a 19-kDa outer membrane protein of Neurospora,\textsuperscript{934} selectively impaired the uptake of precursors that contain cleavable N-terminal targeting sequences, a process that was not inhibited by antibodies to MOM72. MOM19 appears to be a master receptor of broad specificity. It appears to be capable of mediating the uptake of all precursors containing cleavable presequences, regardless of the mitochondrial subcompartment to which they are destined.\textsuperscript{934} This receptor is also capable of mediating the uptake of the ATP-ADP carrier when MOM72 is absent or incapacitated, albeit at low efficiency.\textsuperscript{899} Surprisingly, different parts of MOM19 may interact with different precursor proteins. Treatment of mitochondria with elastase, a protease that impairs the uptake of many precursors and converts MOM19 to a 17-kDa membrane-associated fragment,\textsuperscript{934} did not abolish the import into the matrix of the precursor of the β subunit of the F\textsubscript{1}-F\textsubscript{0} ATPase,\textsuperscript{925, 931} a protein that contains an N-terminal cleavable signal.

A putative third import receptor is a 32-kDa protein with affinity for the targeting signals in mitochondrial presequences. It was identified in yeast mitochondria from its binding to anti-idiotypic antibodies prepared using a synthetic presequence peptide.\textsuperscript{935} As in the case of MOM72, antibodies against this membrane protein inhibited the uptake of several mitochondrial precursors and were capable of precipitating from detergent-treated mitochondria complexes containing the antigen associated with precursors that had been previously bound to the organellar surface. A null mutation that eliminated the 32-kDa protein was not lethal, indicating that it too, like the MOM72 receptor, is functionally redundant. The unexpected finding that the 32-kDa protein is identical in amino acid sequence to the phosphate carrier, a protein that functions in the inner mitochondrial membrane, has raised questions about its physiological role in mitochondrial protein import.\textsuperscript{936}

Immunolabeling studies indicate that both the MOM19 and MOM72 receptors are distributed throughout the mitochondrial surface, although MOM19 is slightly more concentrated, and MOM72 much more so, at regions of contact between the IMM and OMM.\textsuperscript{932, 934} This suggests that the import receptors may accept precursor proteins throughout the organellar surface but subsequently carry them by lateral displacement to the sites of contact between the two membranes, where translocation takes place.

**Common Import Sites Effect the Translocation of Many Proteins across the Outer Membrane**

Following their binding to the surface receptors, whether MOM19 or MOM72/MAS70, precursors of mitochondrial proteins are transferred to common translocation sites, variously called channels, pores, or general insertion protein (GIP), which effect their insertion into the outer membrane, rendering them resistant to the attack of exogenous proteases. The existence of these common import sites is strikingly demonstrated by the fact that porin—a major outer membrane protein that does not compete with the ATP-ADP carrier for binding to a surface receptor—does, however, compete with it in an assay in which, after the precursors are bound to mitochondria at low temperature, their importation is allowed to take place during a subsequent incubation at 25°C.\textsuperscript{931}
Considerable insight into the process of import was obtained after it became possible to interrupt and arrest the translocation of precursors before its completion. This could be achieved by lowering the temperature or the ATP levels in the system or by sterically blocking traversal of the outer membrane, either by preventing the unfolding of the C-terminal domain of a translocating protein, or by binding antibodies to its C-terminal region. Under the latter conditions, blocked translocation intermediates were detected that spanned both membranes, as indicated by the fact that their N-terminal targeting signals had already been cleaved by the matrix peptidase, while the C-termini remained exposed on the surface of the mitochondria, accessible to exogenous proteases and secondary antibodies.

Translocation intermediates trapped in the state in which they traverse both membranes appear to be contained within hydrophilic proteinaceous channels, since they can be extracted from mitochondria by treatment with protein denaturants, such as solutions of urea or of alkaline pH, that do not remove integral membrane proteins from phospholipid bilayers. A yeast outer membrane protein, designated ISP42 (import site protein of 42 kDa), is a likely component of the importation site in the outer membrane since it could be photochemically crosslinked to a modified trapped precursor. ISP42, whose sequence was derived from the cloned gene, is essential for cell viability since disruption of the gene was lethal in haploid yeast strains. Moreover, a reduced expression of this protein was found to lead to the accumulation of mitochondrial precursors in the cytoplasm.

The Neurospora homologue of ISP42, called MOM38, was identified as a component of a macromolecular complex that can be recovered from digitonin-treated mitochondria by immunoprecipitation with anti-MOM19 antibodies. Strikingly, this complex also contains MOM72, as well as several other membrane proteins. In fact, it was also possible to demonstrate that the ATP-ADP carrier, which on the mitochondrial surface is recognized by MOM72, is also recovered in the complex immunoprecipitated with anti-MOM19 antibodies when trapped at the general insertion site. That MOM38 is a major component of the general insertion site is supported by the finding that in intact mitochondria this protein, and no other component of the complex immunoprecipitable with MOM19 antibodies, has the same sensitivity to exogenous proteases as the general insertion site measured in a functional assay.

**Organization of the Translocation Machinery (see Fig. 16-39)**

It appears that mitochondria contain equimolar amounts of the receptors, MOM19 and MOM72, and of the import site protein, MOM38, and that all MOM19 molecules are complexed with MOM38, whereas only a fraction (approximately 50 percent) of MOM72 molecules are in such complexes. The stoichiometry suggests that precursor proteins recognized by MOM19 are directly transferred to the site of translocation, whereas those, like the ATP-ADP carrier, that bind to MOM72 would have to move laterally in the membrane to become associated with the MOM38/ISP42 component of the general insertion site.

Insertion of a translocating polypeptide into the IMM has been found to require the presence of a potential across this membrane. However, once insertion into the inner membrane has taken place, completion of translocation through it can proceed without maintenance of the transmembrane potential, requiring only ATP within the matrix to support the activity of molecular chaperones (see below) that apparently pull the translocating polypeptide inward.

Given the positively charged nature of the N-terminal targeting signal present in many protein precursors and the inside negative character of the transmembrane potential, it seems possible that insertion into the inner membrane is the result of an electrophoretic effect that leads to the correct positioning of the positively charged signal with respect to the membrane, or activates a charged transmembrane component that effects the insertion. In any case, insertion into the inner membrane is thought to lead...
to the coupling of independent distinct passageways in the two membranes, which become linked by the translocating polypeptide (see Fig. 16-39). 

Immunoelectron microscopy revealed that trapped translocating polypeptides containing a blocked C-terminus primarily accumulate at translocation contact sites. It has been estimated, from biochemical measurements of the number of trapped polypeptides at saturation, that there are a few thousand active translocation sites in each mitochondrion.

The components of the inner membrane that contribute to the passageway have not yet been identified. Still, it is clear that a separate functional passageway exists in that membrane that is independent of its coupling to the outer membrane (see Fig. 16-39). Thus, when translocation sites of intact mitochondria are jammed by arrested translocating polypeptides, or are destroyed with proteases, removal of the outer membrane to produce mitoplasts leads to the exposure of new importation sites. These sites in the inner membrane are capable of accepting mitochondrial precursors in a process that is dependent on the membrane potential and on ATP hydrolysis, but is not inhibited by antibodies to the outer membrane receptors.

The translocation machinery in the outer membrane can also function independently, when not dynamically coupled to the inner membrane. Thus, in the absence of the transmembrane potential, cytochrome c heme lyase can be completely translocated across the outer membrane to reach its destination in the intermembrane space, where it is inaccessible to exogenous proteases, unless the outer membrane is removed. Similarly, when its import is carried out in the absence of a transmembrane potential, the ATP-ADP carrier reaches the intermembrane space, even though it is not inserted in the inner membrane.

**Role of Molecular Chaperones in the Import of Proteins into Mitochondria** (Fig. 16-40)

The import into mitochondria of precursor polypeptides released from free polysomes into the cytosol requires that they be kept in a “transport competent” state in which their targeting signals can be recognized by the receptors in the mitochondrial surface. In addition, for most precursor proteins, spontaneous folding into the “tight conformation” of the mature protein must be prevented. To be able to cross the mitochondrial membranes, the precursor must be capable of undergoing unfolding. Maintaining the unfolded state is the function of molecular chaperones that bind the newly synthesized mitochondria polypeptides in the cytosol (Fig. 16-40).
Role of molecular chaperones in the importation of polypeptides into the mitochondrial matrix. Recently synthesized cytoplasmic polypeptides are kept in a loosely folded conformation by cytosolic Hsp70 molecules. In this scheme the polypeptide is represented in the course of translocation through both membranes, and components of the translocation and signal processing machinery other than the chaperones are not shown. As the polypeptide eme...

The need to prevent folding of a protein to allow its import into mitochondria first became apparent from the observation that a fusion protein containing a mitochondrial targeting signal attached to the cytosolic protein DHFR could not be taken up into the mitochondria in the presence of methotrexate, a ligand that binds to DHFR and stabilizes it in a tightly folded conformation. The methotrexate does not mask the presequence, since the protein still binds to the mitochondrial surface in the presence of this analogue but it is not translocated across the membrane. The block in import reflects the inability of the mitochondrial import apparatus to unfold the tightly folded DHFR on the mitochondrial surface. DHFR has been a convenient probe to follow the state of folding of a protein during its translocation into the mitochondrial matrix since the folded polypeptide is resistant to proteases, which digest the unfolded protein. This
property of DHFR is manifested even in chimeras containing DHFR linked to the presequence of a mitochondrial protein, such as subunit IV of cytochrome oxidase, or cytochrome $b_2$. This has allowed the demonstration that during the uptake of such chimeras (in the absence of the ligand), DHFR undergoes unfolding on the mitochondrial surface and refolds after entering the matrix.937

Cytosolic factors with “antifolding” activity, that also prevent the improper aggregation of incompletely folded polypeptides, were in fact identified originally by their capacity to stimulate mitochondrial uptake of precursors in in vitro systems. One group of such antifolding factors comprises the molecular chaperones known as ct-hsp70 (cytosolic heat-shock protein of 70 kDa)—a subset of the heat-shock family of proteins. The expression of many proteins in this family is induced by stress conditions, such as heat shock or glucose starvation, that lead to the partial denaturation (or misfolding) of many proteins within the cell. The ct-hsp70 proteins, however, are constitutively expressed at substantial levels in normal cells, in which they bind to newly synthesized polypeptides and play a role in ensuring their proper folding and oligomerization. Ct-hsp70 proteins are required for the viability of yeast cells, and their role in the uptake of mitochondrial proteins is shown by the fact that, when their levels are decreased by genetic manipulation, the uncleaved precursors of those proteins accumulate in the cytosol.139 Release of the ct-hsp70 chaperones from the polypeptide precursor to which they bind appears to occur concomitantly with its translocation and requires ATP hydrolysis (see Fig. 16-40), which explains the necessity of extramitochondrial ATP for mitochondrial protein import.937, 954–958 Thus, mitochondrial protein precursors that are first unfolded by treatment with denaturants can be subsequently imported into mitochondria without the need of extramitochondrial ATP.958, 959

Other cytosolic proteins, not members of the heat-shock family, have been identified that also stimulate the in vitro uptake of mitochondrial polypeptides. One of these isolated from reticulocyte lysate, and named presequence binding factor (PBF), was shown to bind to the presequence of OTC and may serve as a chaperone maintaining its translocation competence.960 A different factor isolated from the reticulocyte lysate that binds to the presequence of ornithine aminotransferase (OAT) is believed to play a role in targeting. Antibodies to this factor blocked the binding to the mitochondrial surface and uptake into the organelle of two different precursor polypeptides.961

The insertion of a polypeptide into the inner membrane requires a transmembrane potential ($\Delta \psi$). Its subsequent translocation into the matrix is dependent on the function of another molecular chaperone, mt-hsp70, a mitochondrial 70-kDa heat-shock protein (analogous to the bacterial Dnak protein) in the matrix that binds to the entering polypeptide (see Fig. 16-40).946 A yeast temperature-sensitive mt-hsp70 has been obtained (SSC1) in which, at the nonpermissive temperature, the passage of precursor proteins into the mitochondrial matrix is arrested at translocation contact sites.944 It appears that during normal translocation several mt-hsp70 molecules within the matrix bind successively to a single emerging translocating polypeptide (see Fig. 16-40). The energy of this interaction is thought to pull the polypeptide into the matrix, causing the unfolding of C-terminal portions that may remain exposed on the mitochondrial surface.17 That mt-hsp70 plays a role in the unfolding of precursors is indicated by the fact that mitochondria containing a defective mt-hsp70 are still able to take up precursors in vitro if these precursors are previously unfolded by urea denaturation.944 However, such precursors do not refold properly within the mutant mitochondria, indicating that mt-hsp70 also functions in the refolding of polypeptides after their importation. As is the case with ct-hsp70, the release of mt-hsp70 molecules from the translocating polypeptide requires the hydrolysis of ATP. In the absence of this nucleotide, the chaperonin molecules in the matrix remain bound to the polypeptides, which do not fold into the mature conformations.
Imported polypeptides released from mt-hsp70 are transferred to a second heat-shock protein within the matrix, the chaperonin hsp60 (see Fig. 16-40), which is analogous to a bacterial chaperonin, GroEL, that functions in the assembly of bacteriophage capsids. Like GroEL, hsp60 is a large oligomer that consists of 14 60-kDa subunits arranged in two superimposed seven-member rings. Hsp60 is not required for translocation itself, but carries out the ATP-dependent folding of matrix polypeptides and, when necessary, their oligomeric assembly. Therefore, it is necessary for yeast viability. The capacity of mt-hsp60 to promote the assembly of oligomeric proteins is markedly stimulated by a matrix cochaperonin, hsp10, that is analogous to the bacterial protein GroES and promotes the ATPase activity of hsp60. The precursors of both mitochondrial chaperonins mt-hsp70 and hsp60 contain matrix targeting signals that are removed by the matrix peptidase. It is interesting to note that assembly of the hsp60 tetradecamers themselves requires the function of hsp60 molecules already present in the matrix.

Proteolytic Removal of Matrix Targeting Sequences

A matrix processing peptidase (MPP) whose activity depends on divalent cations such as Mn$^{2+}$ and Zn$^{2+}$ removes the matrix targeting signals within the presequences of mitochondrial precursors. This enzyme usually cleaves the precursor at a site located two amino acids downstream of an arginine residue. The matrix peptidase consists of two components. One (approximately 55 kDa; known as MPP in Neurospora crassa and MAS2/MIF2 in Saccharomyces cerevisiae), now designated MPPα, has intrinsic peptidase activity and the other, MPPβ (52 kDa, originally called processing enhancing protein or PEP in N. crassa and MAS1/MIF1 in S. cerevisiae), binds directly to the presequence, markedly enhancing the activity of MPPα. Both components are essential for viability in yeast. It has been shown in Neurospora that they too are synthesized as precursors, whose presequences are removed in the matrix by the action of the mature MPP. The two components of the matrix peptidase are members of the same gene family, and MPPβ is identical (in Neurospora) or highly homologous (in yeast) to subunit 1 of the bc1 complex (complex III or ubiquinone-cytochrome c reductase) of the respiratory chain. In Neurospora, therefore, MPPβ is a bifunctional protein bound to the matrix face of the inner membrane.

Many matrix targeting sequences undergo two proteolytic cleavages within the matrix. Examples include the precursors of OTC, malate dehydrogenase, cytochrome oxidase subunit IV, and the Fe/S subunit of the bc1 complex. In these cases, the first cleavage, effected by the MPP, creates an intermediate form of the precursor from which a second peptide, generally eight amino acids in length, is subsequently removed by a second specific matrix enzyme, the mitochondrial intermediate peptidase (MIP), that has not yet been fully characterized. It has been suggested that the existence in the precursors of these proteins of an octapeptide segment between the two cleavage sites allows for the presence in the mature proteins of N-terminal sequences that would have been incompatible with efficient cleavage by the matrix processing protease. Presequences, such as those in cytochrome peroxidase and cytochromes b$_2$ and c$_1$, that in addition to a matrix targeting signal contain a sorting or mitochondrial localization signal that directs the protein to the intermembrane space (see below), undergo removal of these signals by a third mitochondrial peptidase, which is located on the outer face of the inner membrane (see below).

Mechanisms for Submitochondrial Localization of Proteins
Insertion of Proteins into the Matrix.

A current view of the pathway followed by mitochondrial polypeptides containing targeting signals to reach the matrix is schematically represented in Fig. 16-38A. The N-terminal targeting signal within the presequence first binds to a receptor on the mitochondrial surface (not represented in the figure). This recognition step may occur anywhere on the surface of the organelle, but subsequent steps in import take place only at contact sites, where the inner and outer membranes are closely apposed. At these sites the targeting signal inserts in the inner membrane in a process which requires a transmembrane potential ($\Delta \psi$) and leads to the translocation of the signal across this membrane. Cleavage of the signal by a matrix metalloprotease then takes place, but this step is not required for the completion of translocation.883–887 Intermediate stages in the uptake process, in which the polypeptide is exposed both within the matrix and on the surface of the mitochondria, have been observed in experiments in which the completion of translocation of the $\beta$ subunit of the Neurospora F$_1$ ATPase was interrupted by carrying out the import at low temperatures.883 These experiments have also shown that after insertion into the inner membrane has been initiated, completion of translocation does not require the transmembrane potential.

Insertion of Proteins into the Outer Mitochondrial Membrane (Fig. 16-38B).

Proteins destined to the OMM are synthesized without cleavable presequences and, as expected, their uptake does not require an energized inner membrane. It appears that a variety of mechanisms are used to effect the incorporation of different proteins into the OMM. The MOM19 receptor, which is anchored in the outer membrane by an N-terminal hydrophobic sequence, is targeted to the mitochondria independently of protease-accessible surface receptors. This receptor protein appears to assemble directly with the general insertion protein MOM38 since antibodies to MOM38 block its incorporation into mitochondria. MOM38 itself contains no obvious hydrophobic segment that could serve as a transmembrane domain.941 The mechanism for its association with the outer membrane remains obscure. On the other hand, newly synthesized porin molecules of Neurospora bind to the MOM19 master receptor, from which they must be transferred to the general insertion site (MOM38) before entering the phospholipid bilayer of the outer membrane.

The MAS70 outer membrane receptor of yeast mitochondria contains an N-terminal, noncleavable, targeting signal. The first 12 residues of this protein, when linked to another polypeptide, were capable of targeting the chimera to the mitochondrial matrix.893 The outer membrane localization of MAS70, however, is determined by a hydrophobic segment that immediately follows the targeting signal and exerts its function by halting transfer of the polypeptide across the outer membrane, preventing its insertion into the inner membrane, as depicted in Fig. 16-38B. When the hydrophobic segment was shortened or deleted, the resulting modified polypeptide was translocated into the matrix.897, 976

An unusual mechanism seems to explain the incorporation of bovine monoamine oxidase B into the outer mitochondrial membrane. For insertion, this protein must first be coupled in an ATP-dependent reaction to ubiquitin, a 76-amino-acid polypeptide that is generally linked to lysine residues in proteins to mark the proteins for degradation.978

Import of Proteins into the Intermembrane Space (Fig. 16-38C to 16-38F).

Several different routes are used to transport newly synthesized polypeptides to the intermembrane space. The import of apocytochrome c occurs by a unique pathway.979 It does not require a protease-sensitive surface receptor, ATP, or a transmembrane potential. This protein, which lacks a cleavable signal, inserts directly into the phospholipid bilayer of the outer membrane. Its sequestration in the intermembrane space is a consequence of its covalent linkage to heme, catalyzed by
cytochrome c heme lyase.\textsuperscript{924, 981, 982} When heme addition is blocked, translocation of apocytochrome c is arrested with the polypeptide spanning the outer membrane so that a portion remains exposed to proteases on the mitochondrial surface, while another is tightly bound to the heme lyase in the intermembrane space.\textsuperscript{926} The heme lyase itself, which also lacks a cleavable signal, follows a second import pathway that requires neither ATP hydrolysis nor an energized inner membrane, but involves the general import machinery of the outer membrane that includes the MOM19–MOM38 receptor complex.\textsuperscript{951}

An indirect third route to reach the intermembrane space is followed by some proteins of the inner membrane, such as the iron/sulfur protein of the cytochrome bc\textsubscript{1} complex\textsuperscript{983} and, probably, subunit IV of cytochrome oxidase.\textsuperscript{914} The precursors of these proteins are first transported to the mitochondrial matrix, where their matrix targeting presequences are removed by the two-step cleavage process that involves two different matrix proteases. The presequences do not contain distinct submitochondrial localization signals, and the mature proteins do not become integrated into the phospholipid bilayer of the inner membrane, but rather assemble directly into the multisubunit complexes of that membrane in which they function. In an unknown manner, this assembly leads to their traversal of the inner membrane and their appearance in the intermembrane space. When cleavage of their presequences is inhibited by chelating agents, these proteins accumulate in the matrix.\textsuperscript{983}

Other proteins that are free in the intermembrane space (such as cytochrome c peroxidase\textsuperscript{984} and cytochrome b\textsubscript{2}\textsuperscript{985, 986}) or are attached to the outer face of the inner membrane (such as cytochrome c\textsubscript{1}\textsuperscript{987}) are synthesized with long bipartite cleavable presequences that contain distinct targeting and localization signals.\textsuperscript{894} These presequences are cleaved off in two steps.\textsuperscript{988–990} The first is carried out by the matrix metalloprotease, which removes the N-terminal matrix targeting signal. The second cleavage, which removes the localization signal, is carried out by an enzyme (called inner membrane protease 1) that is located on the outer face of the inner membrane and, in contrast to the matrix protease, is resistant to chelating agents.\textsuperscript{989, 991, 992} The localization signals within the presequences of these proteins consist of approximately 20-amino-acid-long hydrophobic segments flanked by basic residues.\textsuperscript{985–987}

Two alternative possibilities (Fig. 16-38C to 16-38F) for the mode of action of the localization signals in cytochromes b\textsubscript{2} and c have been proposed. Supporting evidence, often directly contradictory, has been provided for both mechanisms.\textsuperscript{877, 880} In one view (Fig. 16-38D and 16-38F), the hydrophobic segment in the localization signal acts as a cleavable halt-transfer signal that stops translocation of the polypeptide as it traverses the inner membrane.\textsuperscript{892, 895} In the other view (Fig. 16-38C and 16-38E), after the polypeptide is translocated into the matrix and the matrix targeting signal is removed, the localization signal serves as a cleavable insertion signal (equivalent to the signal sequence of eukaryotic secretory proteins or to the leader peptide of bacterial secretory proteins) that initiates re-export of the polypeptide from the matrix to the intermembrane space.\textsuperscript{993} The latter mechanism has been designated a "conservative sorting mechanism"\textsuperscript{994} because the re-export step is analogous to the export of proteins from bacteria, which are believed to be the evolutionary ancestors of mitochondria. The two alternative mechanisms are both consistent with the observation that the incorporation of cytochrome b\textsubscript{2} into the intermembrane space takes place in two stages.\textsuperscript{988, 989} The first, leading to cleavage of the targeting signal by the matrix protease, requires an electrochemical gradient across the inner membrane. The second, which leads to discharge into the intermembrane space and proteolytic removal of the remainder of the presequences on the outer face of the inner membrane, does not require an energized inner membrane. Proponents of both views agree that during the uptake of cytochromes b\textsubscript{2} and c\textsubscript{1} into mitochondria, intermediate precursors are generated that are anchored in the inner membrane by the hydrophobic segments within the localization signals, but are largely exposed on the intermembrane space.\textsuperscript{988, 989} From these precursors, the released mature proteins are generated by the second cleavage. In the case of cytochrome b\textsubscript{2} (Fig.
16-38C and 16-38D) and the cytochrome c peroxidase, the mature proteins remain free in the intermembrane space. On the other hand, cytochrome c \(_1\) (Fig. 16-38E and 16-38F) becomes associated with the outer face of the inner membrane, probably via the insertion of a second hydrophobic segment near its C-terminus.\(^{987, 995}\)

The conflicting evidence supporting both models relates to whether (1) the intermediate forms of cytochromes \(b_2\) and \(c_1\) that still contain their localization signals but have lost their matrix targeting signals are at any time found soluble within the mitochondrial matrix; (2) the hsp60 matrix chaperonin that binds to polypeptides that enter the matrix is required for the proteins to achieve their normal disposition in the intermembrane space, as judged by the cleavage of their localization signals; and (3) cleavage of the localization signal can still take place in the absence of intramitochondrial ATP, which would be expected to prevent translocation of the polypeptide into the matrix.

**Sorting of Proteins to the Inner Mitochondrial Membrane.**

The inner mitochondrial membrane contains several large heterooligomeric protein complexes that carry out electron transport (complexes I to IV) and ATP synthesis (complex V or ATP synthase, or \(F_1 F_0\) ATPase), as well as an ATP-ADP translocator. Some of the polypeptide subunits of these complexes are encoded in the mitochondrial genome. For example, in human cells, 7 of the 39 polypeptides of complex I (NADH: ubiquinone oxidoreductase), 1 (cytochrome \(b\)) of the 10 in complex III, 3 of the 13 in complex IV (cytochrome oxidase), and 2 of the 12 polypeptides of the ATP synthase are synthesized by ribosomes in the mitochondrial matrix and directly incorporated into the membrane, where they assemble with the other subunits. There is at least one species difference. Subunit 9 of the ATPase is encoded in the mitochondrial genome in yeast, but is encoded in the nucleus in Neurospora and human cells.

From a biogenetic perspective, proteins peripherally associated with the inner face of the inner mitochondrial membrane, such as the \(\beta\) subunit of the \(F_1 F_0\) ATPase, can be regarded as matrix proteins. After undergoing cleavage of their presequences, these polypeptides become part of multimeric complexes that include subunits that are integral components of the inner membrane. Some of these integral membrane polypeptides are synthesized in mitochondrial ribosomes and are directly inserted into the membrane, in which they are anchored by their hydrophobic segments. However, several integral membrane polypeptides of the inner membrane that contain transmembrane hydrophobic segments are synthesized in cytoplasmic ribosomes. This is the case with the ADP-ATP translocator\(^{902}\) and with subunit 9 of the \(F_0\) ATPase.\(^{996, 997}\) In Neurospora the latter protein undergoes complete translocation into the matrix, before insertion into the inner membrane as an integral membrane protein.\(^{994}\) On the other hand, the ADP-ATP translocator—which is the major polypeptide of the inner membrane, in which it is anchored by three membrane-spanning helixes\(^{998}\)—is synthesized without a cleavable presequence,\(^{902}\) and is never discharged into the matrix. Rather, after binding to the MOM72/MAS70 outer membrane receptor and being transferred to the general import site, the ATP-ADP translocator appears to enter the phospholipid bilayer of the inner membrane directly. This polypeptide is directed to the mitochondria by a segment that is not at its extreme N-terminus. Thus, chimeric polypeptides containing the first 111 (but not just the first 72) residues of the yeast translocator linked to \(\beta\)-galactosidase bind to the outer membrane MAS70 receptor and are incorporated into the organelle in an ATP-dependent process, becoming resistant to exogenous proteases.\(^{902, 999}\) The chimeric polypeptide with the first 111 (N-terminal) residues of the translocator precursor, however, does not become incorporated to the inner membrane in a form that is resistant to alkali extraction. This requires the presence of downstream helical portions of the protein and occurs only in the presence of an energized inner membrane.\(^{956, 999, 1000}\) As expected for insertion of a protein that does not enter the matrix, intramitochondrial ATP and the hsp60 chaperonin are not required for the insertion of the ATP-ADP carrier into the inner membrane.
BIOGENESIS OF PEROXISOMES

Peroxisomes are small membrane-bound organelles that are present in all eukaryotic cells (see Chap. 129). They carry out oxidative reactions that generate hydrogen peroxide and contain the hemoprotein enzyme catalase, which breaks down this reactive product (see ref. 1001). The set of oxidative enzymes present in mammalian peroxisomes varies with the species and the tissue source. In addition to the enzymes that carry out β-oxidation of fatty acids (see below), they include D-amino acid oxidase, urate oxidase, oxalate oxidase, L-α-hydroxyacid oxidases, and other oxidases with specific substrates. Peroxisomal catalase may catalyze the direct decomposition of H₂O₂, to generate H₂O and O₂ from two molecules of H₂O₂, or act peroxidatically, if appropriate substrates for oxidation, such as methanol, ethanol, formate, and nitrites are available. It has been estimated 1001 that approximately 20 percent of the oxygen consumed by rat liver is used for peroxisomal respiration.

Peroxisomes were first recognized on EM as small dense bodies (microbodies) surrounded by a single membrane and containing a dense granular matrix. They are easily identified histochemically by virtue of the peroxidative activity of catalase which, during incubation with diaminobenzidine, generates an electron-dense, polymerized product. Histochemical use of this reaction demonstrated that all cells contain small peroxisomes (0.1 to 0.2 μm; micropEROXISOMES) and that only some cell types, such as hepatocytes and kidney tubule cells, contain large peroxisomes. In many species, peroxisomes are also characterized morphologically by a crystalline core that is composed of the enzyme urate oxidase (uricase). This enzyme, which functions in the purine degradative pathway to oxidize uric acid to allantoin, is not present in humans and in other higher primates.

Although peroxisomes appear as round, ovoid, pear-, or dumbbell-shaped individual bodies in thin tissue sections examined by transmission EM, three-dimensional reconstructions from serial sections indicate that many peroxisomes are interconnected to form an extended network. The variable appearance of this peroxisomal reticulum suggests that it is a dynamic structure that undergoes constant remodeling involving membrane fusion and fission events.

Peroxisomes and lysosomes have similar sedimentation properties. In early cell fractionation studies, they were recovered together in what was designated a “light mitochondrial fraction.” In later work it was possible to separate the two organelles by injecting the animals shortly before sacrifice with the detergent Triton WX-1339. This is taken into lysosomes, which become significantly lighter and can be separated from peroxisomes by isopycnic centrifugation. More recently, methods using sedimentation in metrizamide gradients have yielded highly purified peroxisomes from the livers of untreated animals. Such fractions have allowed rigorous biochemical characterization of the organelle, as well as in vitro studies on its biogenesis.

Both mitochondria and peroxisomes play important roles in the metabolism of fatty acids that are taken into the cell or released from the triglycerides that are stored in cytoplasmic fat droplets. The degradation of fatty acids takes place by β-oxidation, which requires their prior activation by an acyl CoA synthetase and involves the successive removal of acetyl groups from their carboxyl ends. It is believed that the peroxisomal β-oxidation system is responsible for the oxidation of very long chain fatty acids (VLCFA) and that the shortened products generated from VLCFA in the peroxisome may be efficiently degraded in mitochondria. Acyl CoA synthetases that accept long chain fatty acids are associated with the cytoplasmic face of mitochondria, the ER, and peroxisomes. Enzymes that utilize VLCFA, such as C24:0 and C26:0, are also present in peroxisomes and in the ER, but have not been found in mitochondria. The acyl CoA synthetase that activates VLCFA in peroxisomes, but not that in the ER, has been found to be deficient in X-linked adrenoleukodystrophy, a condition in which VLCFA accumulate in tissues and
plasma (see Chap. 131). The mechanism by which acyl CoA esters are transported across the peroxisomal membrane has not been elucidated. High levels of the peroxisomal β-oxidation system are induced by various hypolipidemic agents, such as clofibrate, that also lead to an increase in the number of peroxisomes.

The mitochondrial and peroxisomal β-oxidation systems carry out similar biochemical reactions but employ different enzymatic components. The peroxisomal system consists of three enzymes that carry out four distinct reactions (see refs. and ). The first reaction is catalyzed by acyl CoA oxidase, an FAD-containing enzyme that uses molecular oxygen and generates H\textsubscript{2}O\textsubscript{2} as a product. The second and third reactions in peroxisomal β-oxidation are carried out by a single bifunctional protein that has enoyl CoA hydratase and β-hydroxyacyl CoA dehydrogenase activities. The final reaction involves the cleavage of 3-ketoacyl CoA by 3-ketoacyl CoA thiolase to yield acetyl CoA and a saturated acyl CoA that has two fewer carbons than the original substrate.

Rat liver peroxisomes appear to contain two acyl CoA oxidases and two 3-ketoacyl CoA thiolases, and only one of each of these is inducible by clofibrate. The two acyl oxidases are apparently encoded in mRNAs generated by differential splicing from the primary transcript of the same gene, but it has been shown that the two thiolases are derived from two different genes. A carnitine acyltransferase is contained in peroxisomes that may generate carnitine esters from the acetyl, propionyl, and other acyl CoA products of β-oxidation that are to be transferred to the mitochondria.

Important steps in the synthesis of bile acids also take place in peroxisomes (see ref. ). These organelles carry out the conversion, by β-oxidation, of 3-α, 7-α, 12-α-trihydroxy-5β-cholestanoic acid (THCA) into cholic acid and of 3-α, 7-α, dihydroxy-5β-cholestanoic acid into chenodeoxycholic acid. The role of peroxisomal enzymes in bile acid synthesis accounts for the accumulation of THCA and various of its polar metabolites in the serum and bile of patients with Zellweger syndrome (see below), a genetic disease in which morphologically distinct peroxisomes are absent in hepatocytes (see Chap. 129).

Peroxisomes also contain enzymes of the acyl-dihydroxyacetone phosphate (DHAP) pathway (DHAP acyltransferase, alkyl-DHAP synthase, and acyl/alkyl-DHAP oxidoreductase) that catalyze the initial steps in the biosynthesis of glycerol-ether lipids (plasmalogens) (see refs. and ). Both the β-oxidation enzymes and the glycerol-ether lipid biosynthetic enzymes are markedly diminished in patients with the Zellweger syndrome, consistent with the notion that peroxisomes represent the primary site of plasmalogen synthesis.

The enzyme alanine:glyoxylate aminotransferase (AGT), that catalyzes the conversion of glyoxylate into glycine, which can then be utilized in gluconeogenesis, is present in human liver only within peroxisomes. The absence of this peroxisomal enzyme leads to the overproduction of oxalate from glyoxylate that characterizes primary hyperoxaluria type (see Chap. 133). In certain tissues of other animals AGT is present exclusively in mitochondria, or in both mitochondria and peroxisomes. It is striking that in rat liver the mitochondrial and peroxisomal forms of AGT are encoded by mRNA molecules transcribed from different initiation sites of the same gene, and that in humans a mutation in the AGT gene has been identified that leads to the mistargeting of the enzyme to mitochondria. It was proposed that a polymorphism for this gene normally exists in the human population so that in some individuals the mitochondrial targeting signal is present in the protein but is not functional because it is overridden by the presence of the peroxisomal targeting signal in the same polypeptide. A mutation that abolishes the latter signal would then lead to mistargeting to the mitochondria.
The intriguing finding has been made by immunoelectron microscopy and cell fractionation that HMG-CoA reductase, the key enzyme of the cholesterol biosynthetic pathway whose major site of function is in the ER membrane, is also present, albeit in an unglycosylated state, in the peroxisomal matrix. Moreover, it has been reported that the specific activity of HMG-CoA reductase in the peroxisomal fraction increases markedly after the administration of cholestyramine, a treatment that also raises the levels of the microsomal enzyme, albeit to a lesser extent. A mechanism that addresses some HMG-CoA molecules to peroxisomes would require either elimination of their signal for cotranslational insertion in the ER or an abrogation of its function.

**Synthesis of Peroxisomal Proteins and Their Incorporation into the Organelle**

It is now well established that both matrix proteins and integral proteins of the peroxisomal membrane are synthesized in free polysomes, discharged into the cytosol, and incorporated posttranslationally into the organelle. Early in vivo studies on the biosynthesis of rat liver catalase first demonstrated that, immediately after synthesis, catalase polypeptides appear in the cytosol and are only subsequently taken up into the peroxisomes, without passage through the ER. Incorporation of the heme and tetramerization of the polypeptide into the mature form of the hemoprotein were shown to take place after uptake into the organelle. Subsequently, it was demonstrated directly that several enzymes of the peroxisomal matrix, including urate oxidase, catalase, and the three proteins involved in \( \beta \)-oxidation are synthesized on free polysomes (see Chap. 129).

The posttranslational incorporation into isolated peroxisomes of catalase and fatty acyl CoA oxidase synthesized in mRNA-dependent cell-free systems has been reconstituted in vitro, and found not to proceed at 0°C and to require cytosol and ATP hydrolysis. A peroxisomal integral membrane protein (22 kDa) of unknown function has also been synthesized in vitro and shown to assemble into the peroxisomal membrane posttranslationally, as indicated by its pattern of resistance to exogenous proteases. The uptake of the matrix proteins into mammalian peroxisomes could be dissected into two stages: binding of the protein to the surface of the organelle, which takes place at 0°C and does not consume energy; and transport across the membrane, which occurs at 37°C and requires ATP. The translocation step did not appear to be driven by a transmembrane potential, since it proceeded in the presence of proton ionophores, such as CCCP. On the other hand, studies with the methylotropic yeast *Candida boidinii* (see below) suggested that in this organism the translocation step requires a transmembrane potential, since it was inhibited by CCCP. This ionophore, however, is known to cause a depletion of cellular ATP through its action on mitochondria.

It appears that two main mechanisms, involving two different types of peroxisomal targeting signals (PTS), effect the uptake of proteins into the peroxisomal matrix. With few exceptions (see below) the import of peroxisomal enzymes into the organelle is not accompanied by the removal of cleavable presequences. Indeed, the major mechanism for uptake into peroxisomes of the polypeptides released from free polysomes involves the recognition of a C-terminal tripeptide, which can serve as a PTS in organisms as evolutionarily diverse as fungi, plants, insects, and mammals. It was first shown that luciferase, the firefly enzyme which is normally present in the lantern organ of the insect, is incorporated into peroxisomes when synthesized in transfected cultured monkey kidney cells. This demonstrated the extraordinary degree of evolutionary conservation of the mechanism that effects the sorting of peroxisomal proteins. A study of the distribution of chimeric constructs containing portions of the luciferase polypeptide linked to reporter polypeptides revealed that a C-terminal segment of luciferase, comprising the last 12 amino acids, was sufficient to ensure targeting to peroxisomes. Similarly, C-terminal segments of human catalase, \( \Theta \)-amino acid oxidase, rat acyl CoA oxidase, and the bifunctional rat enoyl CoA-hydratase-\( \beta \)-OH-acyl CoA dehydrogenase served as peroxisomal targeting signals when linked to the C-termini of nonperoxisomal reporter proteins. All these proteins contain the
conserved C-terminal tripeptide sequences SKL or SHL, and site-directed mutagenesis studies demonstrated that the consensus tripeptide motif (Ser/Ala/Cys)-(Lys/Arg/His)-Leu could function as a targeting signal. It appears that this PTS tripeptide must be present at the extreme C-terminus of the polypeptide, since the addition of even one or two C-terminal residues to a polypeptide containing it eliminated its activity. On the other hand, in human catalase a C-terminal 27-amino-acid segment that contains an internal SHL sequence could serve as a targeting signal when linked to a reporter protein.

Immunoblot analysis suggests that many peroxisomal proteins contain the SKL sequence. A specific antibody for this tripeptide recognized a substantial fraction of the protein bands representing the set of peroxisomal proteins. The sorting capacity of the exposed SKL signal has been dramatically demonstrated by the observation that serum albumin, when crosslinked to a short synthetic peptide containing the SKL sequence and microinjected into cells, localizes to peroxisomes. Moreover, it was found that co-injection of a synthetic peptide containing the SKL signal with luciferase suppresses the incorporation of this protein into peroxisomes, suggesting that the import system is saturable.

The second mechanism for peroxisomal import involves the recognition of an N-terminal signal that is cleaved off within the organelle. Signals of this type are present in the two very closely related forms of the peroxisomal 3-ketoacyl CoA thiolase. The newly synthesized thiolases contain either 36-(thiolase A) or 26-(thiolase B) amino-acid-long cleavable segments, which differ only in the presence of 10 extra amino acids at the N-terminus of thiolase A. In the case of thiolase B, the first 11 amino acids were shown to be sufficient for peroxisomal targeting. It seems likely that the same segment serves as the targeting signal in thiolase A, even though it is not present at the extreme N-terminus. As will be discussed below, analysis of the import of peroxisomal proteins in cells from patients with generalized peroxisomal defects, as well as with a variety of yeast mutants, has established that the two types of signals present in peroxisomal proteins are decoded by different molecular machineries. Further studies with Zellweger fibroblasts indicate that cleavage of the N-terminal signals in the thiolase is not required for peroxisomal uptake of this enzyme.

Another peroxisomal matrix protein, the nonspecific lipid transport protein (nsLTP), also known as sterol carrier protein 2, or SCP-2, is also synthesized with a cleavable N-terminal sequence, but this segment of the polypeptide (20 amino acids) may not be necessary for import into the organelle since the protein also contains a C-terminal targeting tripeptide. The acyl CoA oxidase that carries out the first step in β-oxidation also undergoes cleavage within the peroxisome, but the two segments produced remain associated in the mature enzyme.

More than 10 proteins have been identified as specific components of the peroxisomal membrane in several species (see refs. and ). These include the acyl CoA synthetase (76-kDa) exposed on the cytoplasmic face of the membrane, a 69-/70-kDa ATP-binding protein that may be involved in transport across the membrane, and a pore-forming protein (22 kDa). The sequences of five peroxisomal membrane proteins have been determined from cDNA clones and only one of them, which is a methanol-induced protein (ppm20) of Candida boidinii, contains the C-terminal peroxisomal targeting signal. None of the membrane proteins so far identified is glycosylated. Direct evidence has been obtained that three of the major membrane proteins are synthesized in free polysomes and do not undergo posttranslational proteolytic processing.

Several genetic diseases have been recognized in which the biogenesis of the peroxisome is impaired (see Chap. 129). In some conditions, such as the Zellweger cerebrohepatorenal syndrome, neonatal adrenoleukodystrophy, infantile Refsum disease, and hyperpipecolic acidemia, known as generalized
peroxisomal disorders (see refs. 1065 and 1066), normal peroxisomes appear to be absent or markedly reduced in number, and the patients show metabolic defects in all the major biosynthetic peroxisomal pathways. In particular, they fail to synthesize ether lipids, do not β-oxidize very long chain fatty acids, and accumulate bile acid precursors. The generalized disorders are likely to be manifestations of single defects in peroxisomal components that are essential for normal assembly of the organelle. Thus, fibroblasts from patients with Zellweger syndrome, or with the infantile form of Refsum disease, appear to synthesize peroxisomal enzymes at normal rates, but many of them undergo rapid degradation. Some matrix enzymes, such as catalase, D-amino acid oxidase, L-α-hydroxyacid oxidase, and alanine:glyoxylate amino transferase (AGT), have been found to be localized in the cytosol of Zellweger cells rather than in membrane-bound structures. 1034

Immunocytochemical and immunoblot analysis using antibodies against several different proteins of the peroxisomal membrane have shown that fibroblasts from Zellweger patients contain abnormal membrane structures of low isopycnic density, which were designated “peroxisomal ghosts.” 1067–1069 The ghosts contain uncleaved precursors of the 3-keto-acyl CoA thiolase 1035, 1070, 1071 and acyl CoA oxidase, as well as residual DHAP acyltransferase activity (DHAPAT). 1071 Thus, Zellweger fibroblasts appear to be capable of assembling a peroxisomal membrane but to be defective in a component of the machinery that effects the import of some, but not all, proteins of the peroxisomal matrix. As a result, the development of the organelle is incomplete. Since the uptake of the thiolase precursor into the peroxisome is mediated by a mechanism that recognizes an N-terminal signal in the protein and not by the more common one that recognizes a C-terminal SKL tripeptide, one can surmise that the Zellweger defect is in a specific component required only to import proteins with the C-terminal targeting signal. This conclusion is supported by the observation that contrary to the case with normal fibroblasts, albumin molecules crosslinked to the tripeptide signal, when microinjected into Zellweger fibroblasts, fail to localize to peroxisomes. 1057b The fact that in Zellweger fibroblasts the thiolase and the acyl CoA oxidase were taken up but not cleaved 1071 further suggests that the processing protease(s) is itself incorporated into the peroxisome by the mechanism that recognizes the tripeptide C-terminal signal. Further evidence that the import machineries that recognize C- or N-terminal signals operate independently of each other was provided by the isolation of yeast mutants in which, of all tested peroxisomal enzymes, only the thiolase was not imported and by the finding that this defect could not be corrected by introducing into the cells the thiolase wild-type gene. 1072

Extensive complementation analysis carried out by pairwise fusion of cultured fibroblasts from Zellweger, infantile Refsum disease, or neonatal adrenoleukodystrophy patients followed by measurements of the reappearance of peroxisomal functions has defined eight complementation groups, 1073, 1074 demonstrating that mutations in as many different genes can all result in generalized peroxisomal defects. It was also shown that in the heterokaryons resulting from the fusions, previously mistargeted matrix enzymes were rapidly incorporated into preexisting peroxisomal ghosts.

A somatic-cell genetics approach has also been used to generate, identify, and characterize mutants defective in peroxisomal assembly with characteristics similar to Zellweger fibroblasts from permanent Chinese hamster ovary cell (CHO) lines. 1075, 1076 The gene affected in one mutant was identified after transfection with a normal rat cDNA expression library, and the cDNA that corrected the defect found to encode a 35-kDa polypeptide of the peroxisomal membrane that was designated peroxisomal assembly factor 1 (PAFT). 1077 This cDNA, as well as its human homologue, were shown to be also capable of correcting the peroxisomal biogenesis defect in fibroblasts from a particular Zellweger patient. 1078
In other peroxisomal defects, such as acatalasemia, X-linked adrenoleukodystrophy, and hyperoxaluria type I, specific mutations lead to deficiencies in single enzymatic activities, but peroxisomes are present and appear normal in other functions. Although the pseudo-Zellweger or Zellweger-like syndrome displays some of the biochemical defects of Zellweger syndrome, this condition appears to result from a deficiency in 3-ketoacyl thiolase. In these patients, plasmalogen synthesis is normal but both long chain fatty acids and intermediates in bile acid synthesis accumulate, indicating that a single metabolic step involving the thiolase is common to both pathways.

Studies on peroxisome biogenesis in yeast, in which the synthesis of peroxisomal enzymes can be induced under conditions that also markedly increase the number of peroxisomes, have been particularly informative. Thus, in C. boidinii, peroxisomes are hardly detectable when the cells are grown on glucose. However, within 15 h after they are transferred to a medium containing methanol they occupy 20 to 80 percent of the cytoplasmic volume. This induction leads to a marked accumulation of peroxisomal enzymes and particularly of two enzymes involved in the early steps of methanol metabolism—alcohol oxidase, a homo-octamer flavin-containing enzyme, and dihydroxyacetone synthase. Peroxisomal function is also required for growth of S. cerevisae on fatty acids which induce peroxisomal proliferation, such as oleate. Screening for S. cerevisae mutants unable to grow on oleate led to the isolation of mutants defective in peroxisomal assembly (pas mutants). The mutants fall into 18 complementation groups. Of the many PAS genes cloned, one (PAS1) encodes a hydrophobic protein that contains two consensus sequences for ATP binding and shows striking sequence similarity to the Sec18 gene product, which is the yeast homologue of the mammalian NSF, a protein required for many vesicle fusion events that take place during vesicular transport along the endomembrane pathway (see above). Of the other PAS genes, PAS2 appears to be related to the ubiquitin conjugating enzymes, and PAS3 appears to be an integral membrane protein of undetermined subcellular distribution. The complete characterization of the PAS mutants and the identification of the corresponding mammalian orthologous genes is bound to provide a much better understanding of the complex process of peroxisome biogenesis.

Future studies on peroxisome biogenesis are likely to focus on the mechanism for the uptake of proteins into the organelle and on the identification of receptors, transporters, and soluble factors, including chaperonins, that participate in this process.

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Chapter 16: The Biogenesis of Membranes and Organelles


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