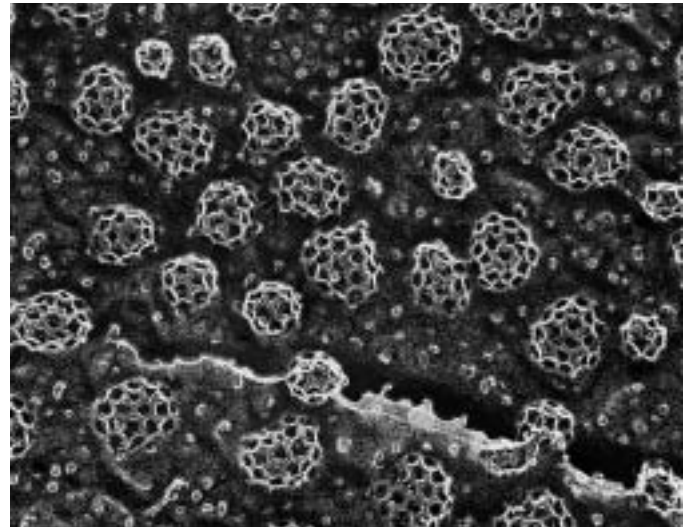


17

VESICULAR TRAFFIC, SECRETION, AND ENDOCYTOSIS



Electron micrograph of clathrin cages, like those that surround clathrin-coated transport vesicles, formed by the *in vitro* polymerization of clathrin heavy and light chains. [John Heuser, Washington University School of Medicine.]

In the previous chapter we explored how proteins are targeted to and translocated across the membranes of different intracellular organelles. In this chapter we turn our attention to the mechanisms that allow soluble and membrane proteins synthesized on the rough endoplasmic reticulum (ER) to move to their final destinations via the **secretory pathway**. A single unifying principle governs all protein trafficking in the secretory pathway: transport of membrane and soluble proteins from one membrane-bounded compartment to another is mediated by **transport vesicles** that collect “cargo” proteins in buds arising from the membrane of one compartment and then deliver these cargo proteins to the next compartment by fusing with the membrane of that compartment. Importantly, as transport vesicles bud from one membrane and fuse with the next, the same face of the membrane remains oriented toward the cytosol. Therefore once a protein has been inserted into the membrane or the lumen of the ER, the protein can be carried along the secretory pathway, moving from one organelle to the next without being translocated across another membrane or altering its orientation within the membrane.

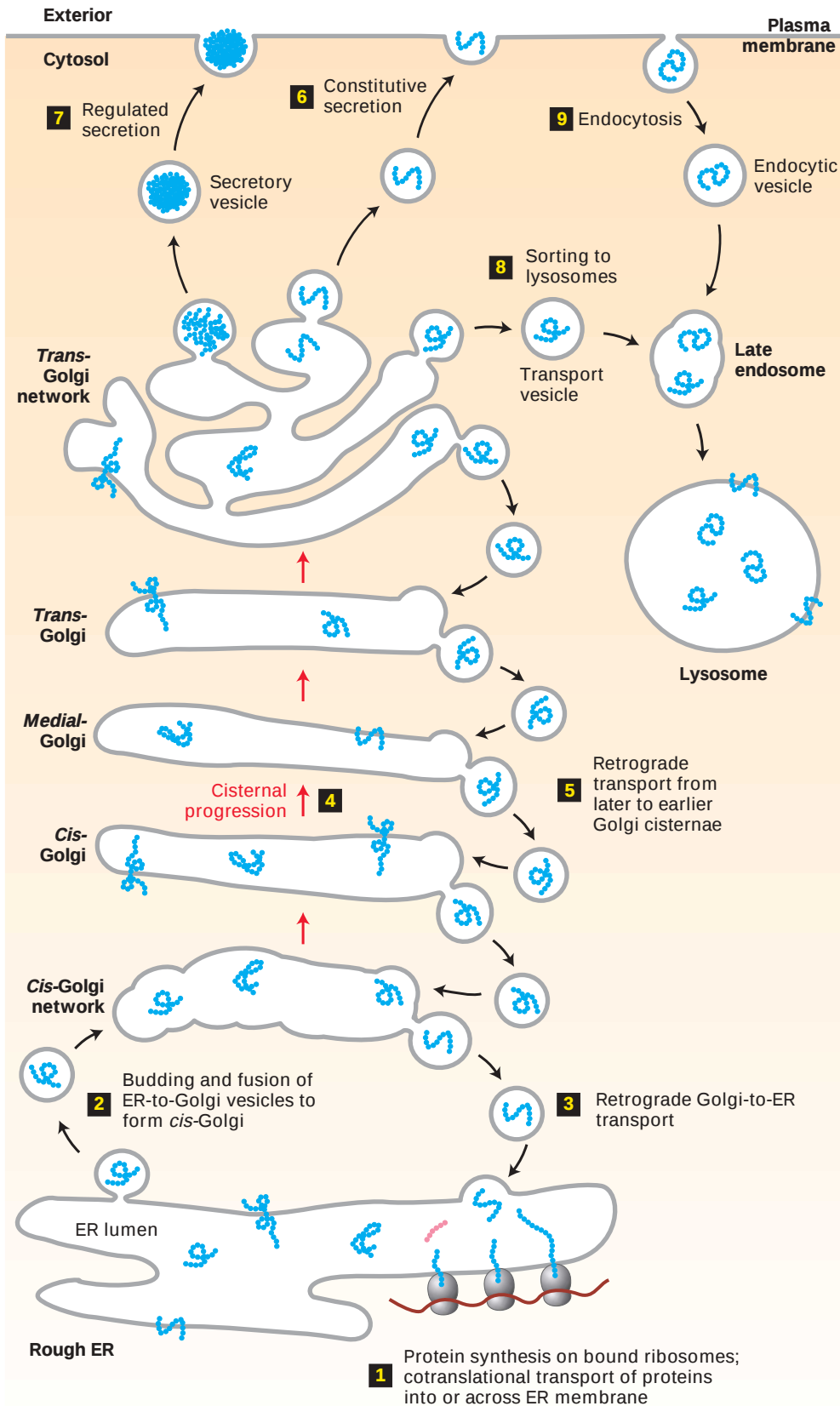
Figure 17-1 outlines the major routes for protein trafficking in the secretory pathway. Once newly synthesized proteins are incorporated into the ER lumen or membrane as discussed in Chapter 16, they can be packaged into *anterograde* (forward-moving) transport vesicles. These vesicles fuse with each other to form a flattened membrane-bounded compartment known as the *cis*-Golgi **cisterna**. Certain proteins, mainly ER-localized proteins, are retrieved from the *cis*-Golgi to the ER via a different set of *retrograde* (backward-moving) transport vesicles. A new *cis*-Golgi cisterna with its cargo of

proteins physically moves from the *cis* position (nearest the ER) to the *trans* position (farthest from the ER), successively becoming first a *medial*-Golgi cisterna and then a *trans*-Golgi cisterna. This process, known as cisternal progression, does not involve the budding off and fusion of anterograde transport vesicles. During cisternal progression, enzymes and other Golgi-resident proteins are constantly being retrieved from later to earlier Golgi cisternae by retrograde transport vesicles, thereby remaining localized to the *cis*-, *medial*-, or *trans*-Golgi cisternae.

Proteins in the secretory pathway that are destined for compartments other than the ER or Golgi eventually reach a complex network of membranes and vesicles termed the ***trans*-Golgi network (TGN)**. From this major branch point in the secretory pathway, a protein can be loaded into one

OUTLINE

- 17.1 Techniques for Studying the Secretory Pathway
- 17.2 Molecular Mechanisms of Vesicular Traffic
- 17.3 Vesicle Trafficking in the Early Stages of the Secretory Pathway
- 17.4 Protein Sorting and Processing in Later Stages of the Secretory Pathway
- 17.5 Receptor-Mediated Endocytosis and the Sorting of Internalized Proteins
- 17.6 Synaptic Vesicle Function and Formation



◀ **FIGURE 17-1 Overview of the secretory and endocytic pathways of protein sorting.**

Secretory pathway: Synthesis of proteins bearing an ER signal sequence is completed on the rough ER (1), and the newly made polypeptide chains are inserted into the ER membrane or cross it into the lumen (Chapter 16). Some proteins (e.g., ER enzymes or structural proteins) remain within the ER. The remainder are packaged into transport vesicles (2) that bud from the ER and fuse together to form new *cis*-Golgi cisternae. Missorted ER-resident proteins and vesicle membrane proteins that need to be reused are retrieved to the ER by vesicles (3) that bud from the *cis*-Golgi and fuse with the ER. Each *cis*-Golgi cisterna, with its protein content, physically moves from the *cis* to the *trans* face of the Golgi complex (4) by a nonvesicular process called cisternal progression. Retrograde transport vesicles (5) move Golgi-resident proteins to the proper Golgi compartment. In all cells, certain soluble proteins move to the cell surface in transport vesicles (6) and are secreted continuously (constitutive secretion). In certain cell types, some soluble proteins are stored in secretory vesicles (7) and are released only after the cell receives an appropriate neural or hormonal signal (regulated secretion). Lysosome-destined membrane and soluble proteins, which are transported in vesicles that bud from the *trans*-Golgi (8), first move to the late endosome and then to the lysosome.

Endocytic pathway: Membrane and soluble extracellular proteins taken up in vesicles that bud from the plasma membrane (9) also can move to the lysosome via the endosome.

of at least three different kinds of vesicles. After budding from the *trans*-Golgi network, the first type of vesicle immediately moves to and fuses with the plasma membrane, releasing its contents by **exocytosis**. In all cell types, at least some proteins are loaded into such vesicles and secreted continuously in this manner. Examples of proteins released by such constitutive (or continuous) secretion include collagen by fibroblasts, serum proteins by hepatocytes, and antibodies by activated B lymphocytes. The second type of vesicle to bud from the *trans*-Golgi network, known as **secretory vesicles**, are stored inside the cell until a signal for exocytosis causes release of their contents at the plasma membrane. Among the proteins released by such regulated secretion are peptide hormones (e.g., insulin, glucagon, ACTH) from various endocrine cells, precursors of digestive enzymes from pancreatic acinar cells, milk proteins from the mammary gland, and neurotransmitters from neurons.

The third type of vesicle that buds from the *trans*-Golgi network is directed to the **lysosome**, an organelle responsible for the intracellular degradation of macromolecules, and to lysosome-like storage organelles in certain cells. Secretory proteins destined for lysosomes first are transported by vesicles from the *trans*-Golgi network to a compartment usually called the **late endosome**; proteins then are transferred to the lysosome by a mechanism that is not well understood but may involve direct fusion of the endosome with the lysosomal membrane. Soluble proteins delivered by this pathway include lysosomal digestive enzymes (e.g., proteases, glycosidases, and phosphatases) and membrane proteins (e.g., V-class proton pump) that pump H^+ from the cytosol into the acidic lumen of the endosome and lysosome. As we will see, some of the specific protein-processing and -sorting events that take place within these organelles depend on their low luminal pH.

The endosome also functions in the *endocytic pathway* in which vesicles bud from the plasma membrane bringing membrane proteins and their bound **ligands** into the cell (see Figure 17-1). After being internalized by **endocytosis**, some proteins are transported to lysosomes, while others are recycled back to the cell surface. Endocytosis is a way for cells to take up nutrients that are in macromolecular form—for example, cholesterol in the form of lipoprotein particles and iron complexed with the serum protein transferrin. Endocytosis also can function as a regulatory mechanism to decrease signaling activity by withdrawing receptors for a particular signaling molecule from the cell surface.

17.1 Techniques for Studying the Secretory Pathway

The key to understanding how proteins are transported through the organelles of the secretory pathway has been to develop a basic description of the function of transport vesicles. Many components required for the formation and fu-

sion of transport vesicles have been identified in the past decade by a remarkable convergence of the genetic and biochemical approaches described in this section. All studies of intracellular protein trafficking employ some method for assaying the transport of a given protein from one compartment to another. We begin by describing how intracellular protein transport can be followed in living cells and then consider genetic and *in vitro* systems that have proved useful in elucidating the secretory pathway.

Transport of a Protein Through the Secretory Pathway Can Be Assayed in Living Cells

The classic studies of G. Palade and his colleagues in the 1960s first established the order in which proteins move from organelle to organelle in the secretory pathway. These early studies also showed that secretory proteins were never released into the cytosol, the first indication that transported proteins are associated with some type of membrane-bounded intermediate. In these experiments, which combined **pulse-chase** labeling (see Figure 3-36) and **autoradiography**, radioactively labeled amino acids were injected into the pancreas of a hamster. At different times after injection, the animal was sacrificed and the pancreatic cells were chemically fixed, sectioned, and subjected to autoradiography to visualize the location of the radiolabeled proteins. Because the radioactive amino acids were administered in a short pulse, only those proteins synthesized immediately after injection were labeled, forming a distinct group, or cohort, of labeled proteins whose transport could be followed. In addition, because pancreatic acinar cells are dedicated secretory cells, almost all of the labeled amino acids in these cells are incorporated into secretory proteins, facilitating the observation of transported proteins.

Although autoradiography is rarely used today to localize proteins within cells, these early experiments illustrate the two basic requirements for any assay of intercompartmental transport. First, it is necessary to label a cohort of proteins in an early compartment so that their subsequent transfer to later compartments can be followed with time. Second, it is necessary to have a way to identify the compartment in which a labeled protein resides. Here we describe two modern experimental procedures for observing the intracellular trafficking of a secretory protein in almost any type of cell.

In both procedures, a gene encoding an abundant membrane glycoprotein (G protein) from vesicular stomatitis virus (VSV) is introduced into cultured mammalian cells either by **transfection** or simply by infecting the cells with the virus. The treated cells, even those that are not specialized for secretion, rapidly synthesize the VSV G protein on the ER like normal cellular secretory proteins. Use of a mutant encoding a temperature-sensitive VSV G protein allows researchers to turn subsequent protein transport on and off. At the restrictive temperature of 40 °C, newly made VSV G protein is misfolded and therefore retained within the ER by quality control mechanisms discussed in Chapter 16, whereas at the permissive temperature of 32 °C, the accumulated

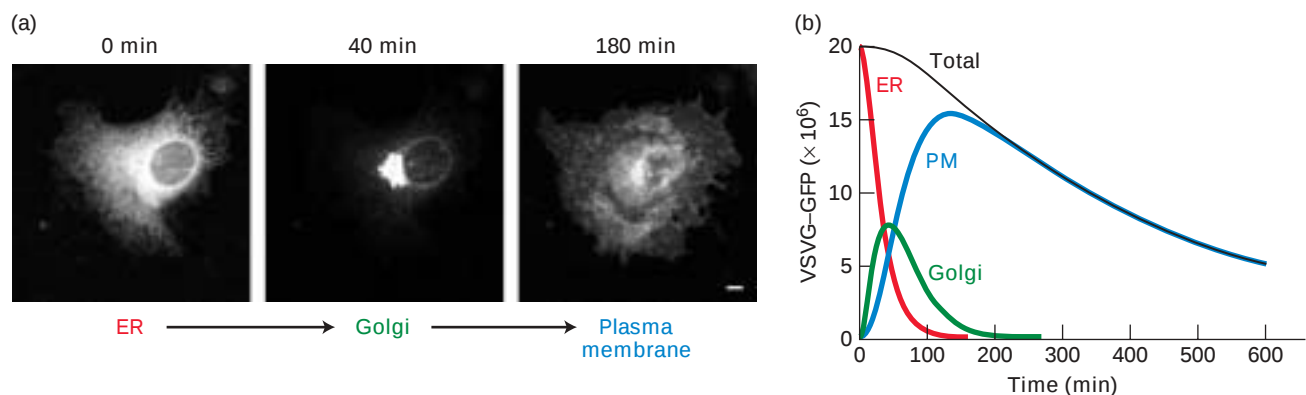
protein is correctly folded and is transported through the secretory pathway to the cell surface. This clever use of a temperature-sensitive mutation in effect defines a protein cohort whose subsequent transport can be followed.

In two variations of this basic procedure, transport of VSV G protein is monitored by different techniques. Studies using both of these modern trafficking assays and Palade's early experiments all came to the same conclusion: in mammalian cells vesicle-mediated transport of a protein molecule from its site of synthesis on the rough ER to its arrival at the plasma membrane takes from 30 to 60 minutes.

Microscopy of GFP-Labeled VSV G Protein One approach for observing transport of VSV G protein employs a hybrid gene in which the viral gene is fused to the gene encoding *green fluorescent protein (GFP)*, a naturally fluorescent protein (Chapter 5). The hybrid gene is transfected into cultured cells by techniques described in Chapter 9. When cells expressing the temperature-sensitive form of the hybrid protein (VSVG-GFP) are grown at the restrictive temperature, VSVG-GFP accumulates in the ER, which appears as a lacy network of membranes when cells are observed in a fluorescent microscope. When the cells are subsequently shifted to a permissive temperature, the VSVG-GFP can be seen to move first to the membranes of the Golgi apparatus, which are densely concentrated at the edge of the nucleus, and then to the cell surface (Figure 17-2a). By analyzing the distribution of VSVG-GFP at different times after shifting cells to the permissive temperature, researchers have determined how long VSVG-GFP resides in each organelle of the secretory pathway (Figure 17-2b).

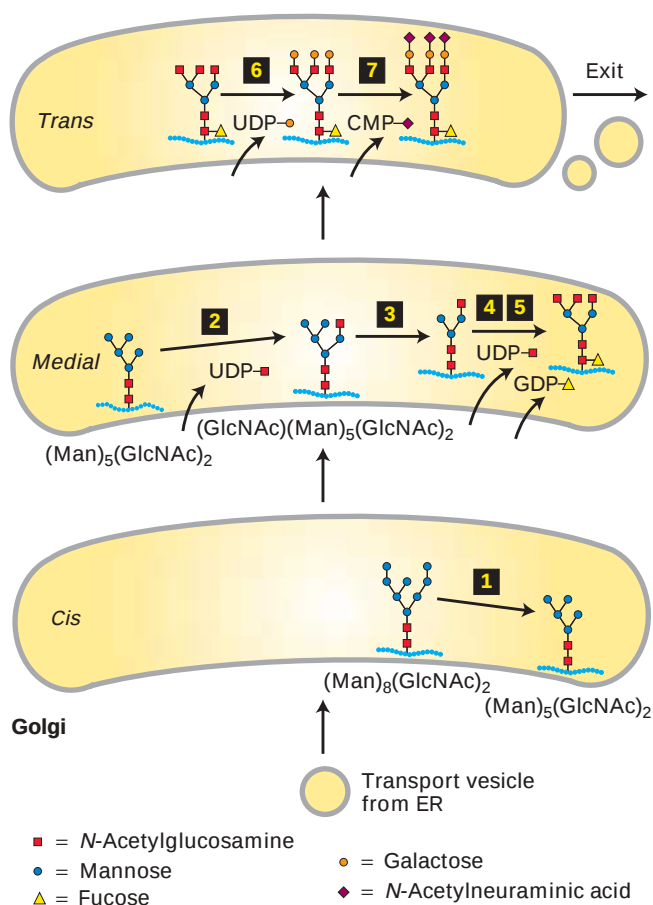
Detection of Compartment-Specific Oligosaccharide Modifications A second way to follow the transport of secretory proteins takes advantage of modifications to their carbohydrate side chains that occur at different stages of the secretory pathway. To understand this approach, recall that many secretory proteins leaving the ER contain one or more copies of the *N-linked oligosaccharide* $\text{Man}_8(\text{GlcNAc})_2$, which are synthesized and attached to secretory proteins in the ER (see Figure 16-18). As a protein moves through the Golgi complex, different enzymes localized to the *cis*-, *medial*-, and *trans*-Golgi cisternae catalyze an ordered series of reactions to these core $\text{Man}_8(\text{GlcNAc})_2$ chains. For instance, glycosidases that reside specifically in the *cis*-Golgi compartment sequentially trim mannose residues off of the core oligosaccharide to yield a "trimmed" form $\text{Man}_5(\text{GlcNAc})_2$ (Figure 17-3, reaction 1). Scientists can use a specialized carbohydrate-cleaving enzyme known as endoglycosidase D to distinguish glycosylated proteins that remain in the ER from those that have entered the *cis*-Golgi: trimmed *cis*-Golgi-specific oligosaccharides are cleaved from proteins by endoglycosidase D, whereas the core (untrimmed) oligosaccharide chains on secretory proteins within the ER are resistant to cleavage by this enzyme. Because a deglycosylated protein produced by endoglycosidase D digestion moves faster on an SDS gel than the corresponding glycosylated protein, they can be readily distinguished.

This type of assay can be used to track movement of VSV G protein in virus-infected cells pulse-labeled with radioactive amino acids. Immediately after labeling, all the extracted labeled VSV G protein is still in the ER and is resistant to digestion by endoglycosidase D, but with time an increasing fraction of the glycoprotein becomes sensitive to digestion



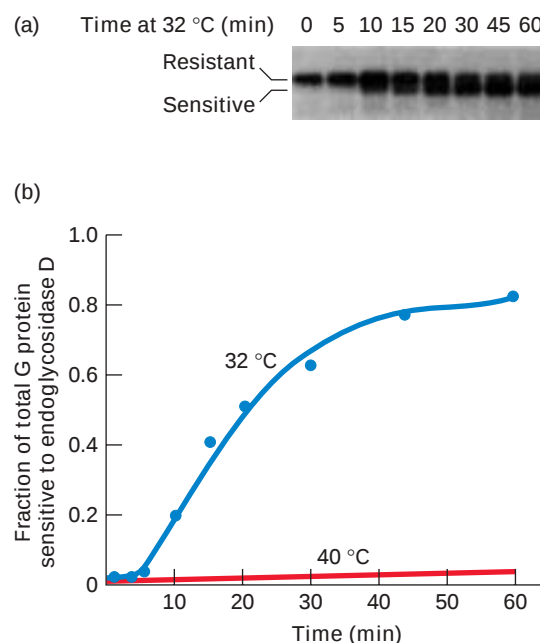
▲ EXPERIMENTAL FIGURE 17-2 Protein transport through the secretory pathway can be visualized by fluorescence microscopy of cells producing a GFP-tagged membrane protein. Cultured cells were transfected with a hybrid gene encoding the viral membrane glycoprotein VSV G protein linked to the gene for green fluorescent protein (GFP). A mutant version of the viral gene was used so that newly made hybrid protein (VSVG-GFP) is retained in the ER at 40 °C but is released for transport at 32 °C. (a) Fluorescence micrographs of cells just before and at two times after

they were shifted to the lower temperature. Movement of VSVG-GFP from the ER to the Golgi and finally to the cell surface occurred within 180 minutes. (b) Plot of the levels of VSVG-GFP in the endoplasmic reticulum (ER), Golgi, and plasma membrane (PM) at different times after shift to lower temperature. The kinetics of transport from one organelle to another can be reconstructed from computer analysis of these data. The decrease in total fluorescence that occurs at later times probably results from slow inactivation of GFP fluorescence. [From Jennifer Lippincott-Schwartz and Koret Hirschberg, Metabolism Branch, National Institute of Child Health and Human Development.]



▲ **FIGURE 17-3 Processing of N-linked oligosaccharide chains on glycoproteins within *cis*-, *medial*-, and *trans*-Golgi cisternae in vertebrate cells.** The enzymes catalyzing each step are localized to the indicated compartments. After removal of three mannose residues in the *cis*-Golgi (step 1), the protein moves by cisternal progression to the *medial*-Golgi. Here, three GlcNAc residues are added (steps 2 and 4), two more mannose residues are removed (step 3), and a single fucose is added (step 5). Processing is completed in the *trans*-Golgi by addition of three galactose residues (step 6) and finally by linkage of an N-acetylneuraminic acid residue to each of the galactose residues (step 7). Specific transferase enzymes add sugars to the oligosaccharide, one at a time, from sugar nucleotide precursors imported from the cytosol. This pathway represents the Golgi processing events for a typical mammalian glycoprotein. Variations in the structure of N-linked oligosaccharides can result from differences in processing steps in the Golgi. [See R. Kornfeld and S. Kornfeld, 1985, *Ann. Rev. Biochem.* **45**:631]

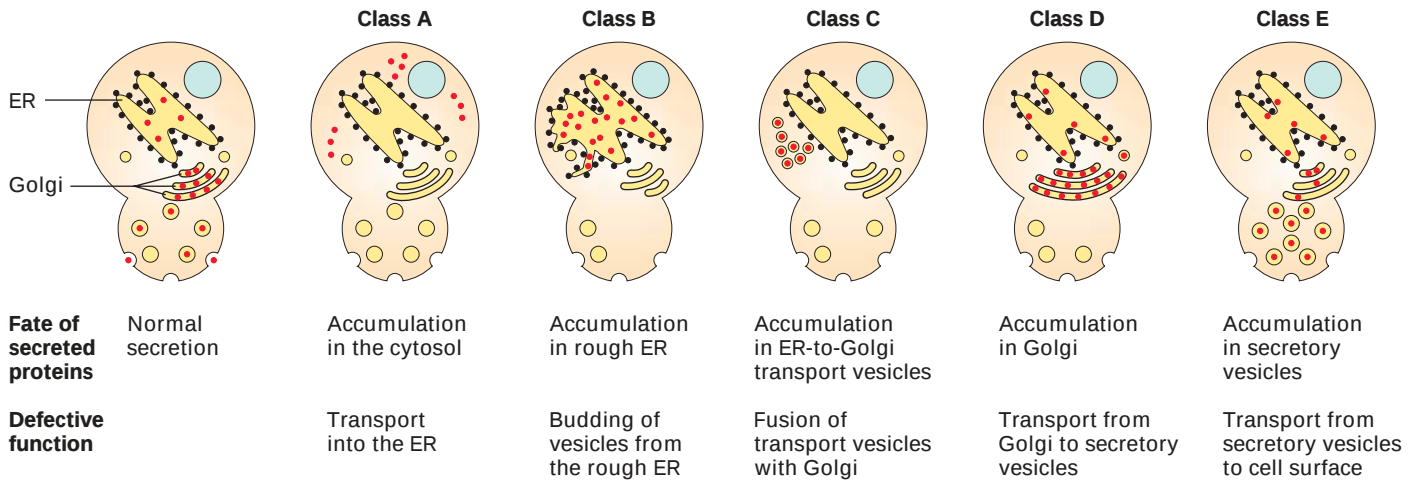
(Figure 17-4). This conversion of VSV G protein from an endoglycosidase D-resistant form to an endoglycosidase D-sensitive form corresponds to vesicular transport of the protein from the ER to the *cis*-Golgi. Note that transport of VSV G protein from the ER to the Golgi takes about 30 minutes as measured by either the assay based on oligosaccharide processing or fluorescence microscopy of VSVG-GFP.



▲ **EXPERIMENTAL FIGURE 17-4 Transport of a membrane glycoprotein from the ER to the Golgi can be assayed based on sensitivity to cleavage by endoglycosidase D.** Cells expressing a temperature-sensitive VSV G protein (VSVG) were labeled with a pulse of radioactive amino acids at the nonpermissive temperature so that labeled protein was retained in the ER. At periodic times after a return to the permissive temperature of 32 °C, VSVG was extracted from cells and digested with endoglycosidase D, which cleaves the oligosaccharide chains from proteins processed in the *cis*-Golgi but not from proteins in the ER. (a) SDS gel electrophoresis of the digestion mixtures resolves the resistant, uncleaved (slower migrating) and sensitive, cleaved (faster migrating) forms of labeled VSVG. As this electrophoretogram shows, initially all of the VSVG was resistant to digestion, but with time an increasing fraction is sensitive to digestion, reflecting protein transported from the ER to the Golgi and processed there. In control cells kept at 40 °C, only slow-moving, digestion-resistant VSVG was detected after 60 minutes (not shown). (b) Plot of the proportion of VSVG that is sensitive to digestion, derived from electrophoretic data, reveals the time course of ER → Golgi transport. [From C. J. Beckers et al., 1987, *Cell* **50**:523.]

Yeast Mutants Define Major Stages and Many Components in Vesicular Transport

The general organization of the secretory pathway and many of the molecular components required for vesicle trafficking are similar in all eukaryotic cells. Because of this conservation, genetic studies with yeast have been useful in confirming the sequence of steps in the secretory pathway and in identifying many of the proteins that participate in vesicular traffic. Although yeasts secrete few proteins into the growth medium, they continuously secrete a number of enzymes that remain



▲ **EXPERIMENTAL FIGURE 17-5 Phenotypes of yeast *sec* mutants identified stages in the secretory pathway.** These temperature-sensitive mutants can be grouped into five classes based on the site where newly made secreted proteins (red dots) accumulate when cells are shifted from the permissive

temperature to the higher nonpermissive one. Analysis of double mutants permitted the sequential order of the steps to be determined. [See P. Novick et al., 1981, *Cell* **25**:461, and C. A. Kaiser and R. Schekman, 1990, *Cell* **61**:723.]

localized in the narrow space between the plasma membrane and the cell wall. The best-studied of these, invertase, hydrolyzes the disaccharide sucrose to glucose and fructose.

A large number of yeast mutants initially were identified based on their ability to secrete proteins at one temperature and inability to do so at a higher, nonpermissive temperature. When these temperature-sensitive *secretion* (*sec*) mutants are transferred from the lower to the higher temperature, they accumulate secreted proteins at the point in the pathway blocked by the mutation. Analysis of such mutants identified five classes (A–E) characterized by protein accumulation in the cytosol, rough ER, small vesicles taking proteins from the ER to the Golgi complex, Golgi cisternae, or constitutive secretory vesicles (Figure 17-5). Subsequent characterization of *sec* mutants in the various classes has helped elucidate the fundamental components and molecular mechanisms of vesicle trafficking that we discuss in later sections.

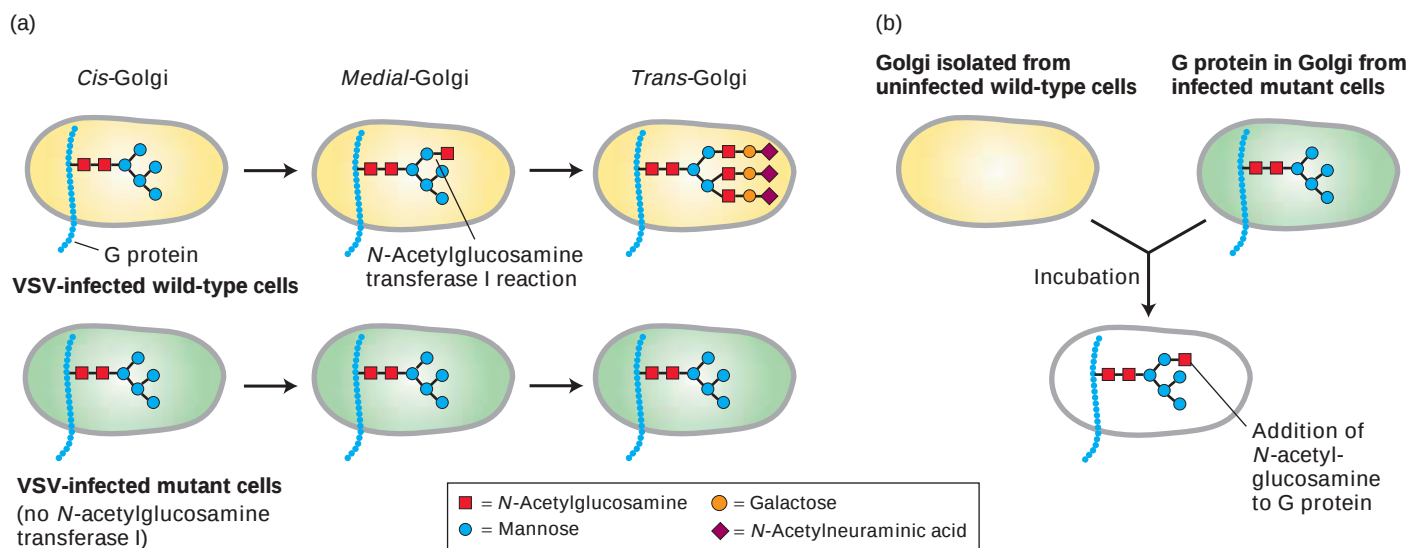
To determine the order of the steps in the pathway, researchers analyzed double *sec* mutants. For instance, when yeast cells contain mutations in both class B and class D functions, proteins accumulate in the rough ER, not in the Golgi cisternae. Since proteins accumulate at the earliest blocked step, this finding shows that class B mutations must act at an earlier point in the secretory pathway than class D mutations do. These studies confirmed that as a secreted protein is synthesized and processed it moves sequentially from the cytosol → rough ER → ER-to-Golgi transport vesicles → Golgi cisternae → secretory vesicles and finally is exocytosed.

Cell-free Transport Assays Allow Dissection of Individual Steps in Vesicular Transport

In vitro assays for intercompartmental transport are powerful complementary approaches to studies with yeast *sec* mu-

tants for identifying and analyzing the cellular components responsible for vesicular trafficking. In one application of this approach, cultured mutant cells lacking one of the enzymes that modify *N*-linked oligosaccharide chains in the Golgi are infected with vesicular stomatitis virus (VSV). For example, if infected cells lack *N*-acetylglucosamine transferase I, they produce abundant amounts of VSV G protein but cannot add *N*-acetylglucosamine residues to the oligosaccharide chains in the *medial*-Golgi as wild-type cells do (Figure 17-6a). When Golgi membranes isolated from such mutant cells are mixed with Golgi membranes from wild-type, uninfected cells, the addition of *N*-acetylglucosamine to VSV G protein is restored (Figure 17-6b). This modification is the consequence of the retrograde vesicular transport of *N*-acetylglucosamine transferase I from the wild-type *medial*-Golgi to the *cis*-Golgi compartment from virally infected mutant cells. Successful intercompartmental transport in this cell-free system depends on requirements that are typical of a normal physiological process including a cytosolic extract, a source of chemical energy in the form of ATP and GTP, and incubation at physiological temperatures.

In addition, under appropriate conditions a uniform population of the retrograde transport vesicles that move *N*-acetylglucosamine transferase I from the *medial*- to *cis*-Golgi can be purified away from the donor wild-type Golgi membranes by centrifugation. By examining the proteins that are enriched in these vesicles, scientists have been able to identify many of the integral membrane proteins and peripheral vesicle coat proteins that are the structural components of this type of vesicle. Moreover, fractionation of the cytosolic extract required for transport in cell-free reaction mixtures has permitted isolation of the various proteins required for formation of transport vesicles and of proteins required for the targeting and fusion of vesicles with appropriate acceptor



▲ **EXPERIMENTAL FIGURE 17-6 Protein transport from one Golgi cisterna to another can be assayed in a cell-free system.** (a) A mutant line of cultured fibroblasts is essential in this type of assay. In this example, the cells lack the enzyme N-acetylglucosamine transferase I (step 2 in Figure 17-3). In wild-type cells, this enzyme is localized to the *medial*-Golgi and modifies N-linked oligosaccharides by the addition of one N-acetylglucosamine. In VSV-infected wild-type cells, the oligosaccharide on the viral G protein is modified to a typical complex oligosaccharide, as shown in the *trans*-Golgi panel. In infected mutant cells, however, the G protein reaches the cell

surface with a simpler high-mannose oligosaccharide containing only two N-acetylglucosamine and five mannose residues. (b) When Golgi cisternae isolated from infected mutant cells are incubated with Golgi cisternae from normal, uninfected cells, the VSV G protein produced *in vitro* contains the additional N-acetylglucosamine. This modification is carried out by transferase enzyme that is moved by retrograde transport vesicles from the wild-type *medial*-Golgi cisternae to the mutant *cis*-Golgi cisternae in the reaction mixture. [See W. E. Balch et al., 1984, *Cell* **39**:405 and 525; W. A. Braell et al., 1984, *Cell* **39**:511; and J. E. Rothman and T. Söllner, 1997, *Science* **276**:1212.]

membranes. In vitro assays similar in general design to the one shown in Figure 17-6 have been used to study various transport steps in the secretory pathway.

KEY CONCEPTS OF SECTION 17.1

Techniques for Studying the Secretory Pathway

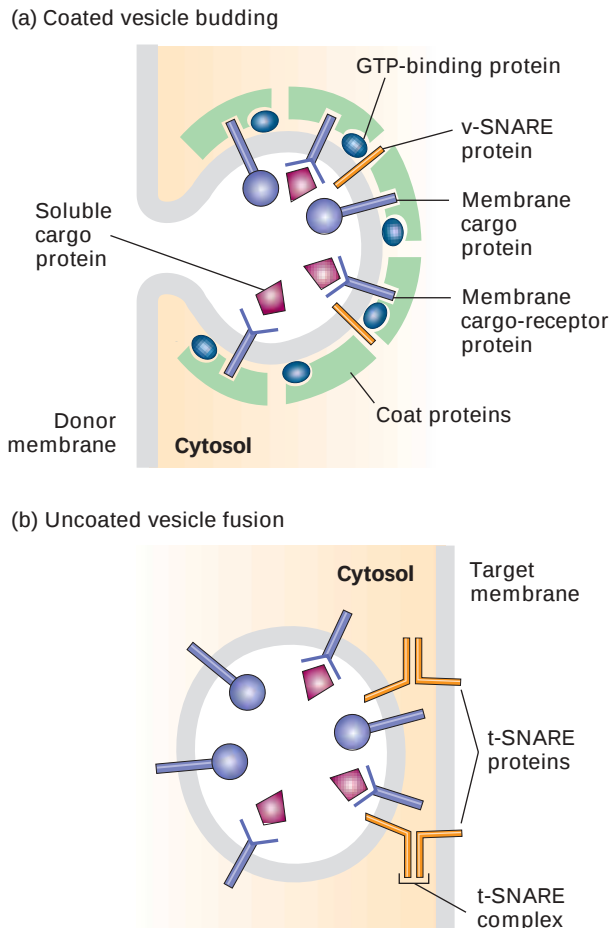
- All assays for following the trafficking of proteins through the secretory pathway in living cells require a way to label a cohort of secretory proteins and a way to identify the compartments where labeled proteins subsequently are located.
- Pulse-labeling with radioactive amino acids can specifically label a cohort of newly made proteins in the ER. Alternatively, a temperature-sensitive mutant protein that is retained in the ER at the nonpermissive temperature will be released as a cohort for transport when cells are shifted to the permissive temperature.
- Transport of a fluorescently labeled protein along the secretory pathway can be observed by microscopy (see Figure 17-2). Transport of a radiolabeled protein commonly is tracked by following compartment-specific covalent modifications to the protein.
- Many of the components required for intracellular protein trafficking have been identified in yeast by analysis

of temperature-sensitive *sec* mutants defective for the secretion of proteins at the nonpermissive temperature (see Figure 17-5).

- Cell-free assays for intercompartmental protein transport have allowed the biochemical dissection of individual steps of the secretory pathway. Such *in vitro* reactions can be used to produce pure transport vesicles and to test the biochemical function of individual transport proteins.

17.2 Molecular Mechanisms of Vesicular Traffic

Small membrane-bounded vesicles that transport proteins from one organelle to another are common elements in the secretory and endocytic pathways (see Figure 17-1). These vesicles bud from the membrane of a particular “parent” (*donor*) organelle and fuse with the membrane of a particular “target” (*destination*) organelle. Although each step in the secretory and endocytic pathways employs a different type of vesicle, studies employing genetic and biochemical techniques described in the previous section have revealed that each of the different vesicular transport steps is simply a variation on a common theme. In this section we explore that common theme, the basic mechanisms underlying vesicle budding and fusion.



▲ **FIGURE 17-7 Overview of vesicle budding and fusion with a target membrane.** (a) Budding is initiated by recruitment of a small GTP-binding protein to a patch of donor membrane. Complexes of coat proteins in the cytosol then bind to the cytosolic domain of membrane cargo proteins, some of which also act as receptors that bind soluble proteins in the lumen, thereby recruiting luminal cargo proteins into the budding vesicle. (b) After being released and shedding its coat, a vesicle fuses with its target membrane in a process that involves interaction of cognate SNARE proteins.

The budding of vesicles from their parent membrane is driven by the polymerization of soluble protein complexes onto the membrane to form a proteinaceous vesicle coat (Figure 17-7a). Interactions between the cytosolic portions of integral membrane proteins and the vesicle coat gather the appropriate cargo proteins into the forming vesicle. Thus the coat not only adds curvature to the membrane to form a vesicle but also acts as the filter to determine which proteins are admitted into the vesicle.

The integral membrane proteins in a budding vesicle include **v-SNAREs**, which are crucial to eventual fusion of the vesicle with the correct target membrane. Shortly after formation of a vesicle is completed, the coat is shed exposing its v-SNARE proteins. The specific joining of v-SNAREs in

the vesicle membrane with cognate **t-SNAREs** in the target membrane brings the membranes into close apposition, allowing the two bilayers to fuse (Figure 17-7b).

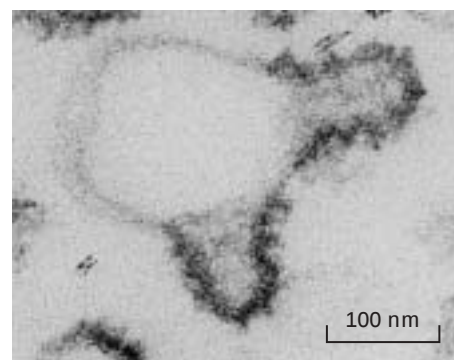
Assembly of a Protein Coat Drives Vesicle Formation and Selection of Cargo Molecules

Three types of coated vesicles have been characterized, each with a different type of protein coat and each formed by reversible polymerization of a distinct set of protein subunits (Table 17-1). Each type of vesicle, named for its primary coat proteins, transports cargo proteins from particular parent organelles to particular destination organelles:

- **COPII** vesicles transport proteins from the rough ER to the Golgi.
- **COPI** vesicles mainly transport proteins in the retrograde direction between Golgi cisternae and from the *cis*-Golgi back to the rough ER.
- **Clathrin** vesicles transport proteins from the plasma membrane (cell surface) and the *trans*-Golgi network to late endosomes.

Researchers have not yet identified the coat proteins surrounding the vesicles that move proteins from the *trans*-Golgi to the plasma membrane during either constitutive or regulated secretion.

The general scheme of vesicle budding shown in Figure 17-7a applies to all three known types of coated vesicles. Experiments with isolated or artificial membranes and purified coat proteins have shown that polymerization of the coat proteins onto the cytosolic face of the parent membrane is necessary to produce the high curvature of the



▲ **EXPERIMENTAL FIGURE 17-8 Vesicle buds can be visualized during in vitro budding reactions.** When purified COPII coat components are incubated with isolated ER vesicles or artificial phospholipid vesicles (liposomes), polymerization of the coat proteins on the vesicle surface induces emergence of highly curved buds. In this electron micrograph of an in vitro budding reaction, note the distinct membrane coat, visible as a dark protein layer, present on the vesicle buds. [From K. Matsuoka et al., 1988, *Cell* **93**(2):263.]

TABLE 17-1 Coated Vesicles Involved in Protein Trafficking

Vesicle Type	Coat Proteins	Associated GTPase	Transport Step Mediated
COPII	Sec23/Sec24 and Sec13/Sec31 complexes, Sec16	Sar1	ER to <i>cis</i> -Golgi
COPI	Coatomers containing seven different COP subunits	ARF	<i>cis</i> -Golgi to ER Later to earlier Golgi cisternae
Clathrin and adapter proteins*	Clathrin + AP1 complexes	ARF	<i>trans</i> -Golgi to endosome
	Clathrin + GGA	ARF	<i>trans</i> -Golgi to endosome
	Clathrin + AP2 complexes	ARF	Plasma membrane to endosome
	AP3 complexes	ARF	Golgi to lysosome, melanosome, or platelet vesicles

*Each type of AP complex consists of four different subunits. It is not known whether the coat of AP3 vesicles contains clathrin.

membrane that is typical of a transport vesicle about 50 nm in diameter. Electron micrographs of *in vitro* budding reactions often reveal structures that exhibit discrete regions of the parent membrane bearing a dense coat accompanied by the curvature characteristic of a completed vesicle (Figure 17-8). Such structures, usually called *vesicle buds*, appear to be intermediates that are visible after the coat has begun to polymerize but before the completed vesicle pinches off from the parent membrane. The polymerized coat proteins are thought to form some type of curved lattice that drives the formation of a vesicle bud by adhering to the cytosolic face of the membrane.

A Conserved Set of GTPase Switch Proteins Controls Assembly of Different Vesicle Coats

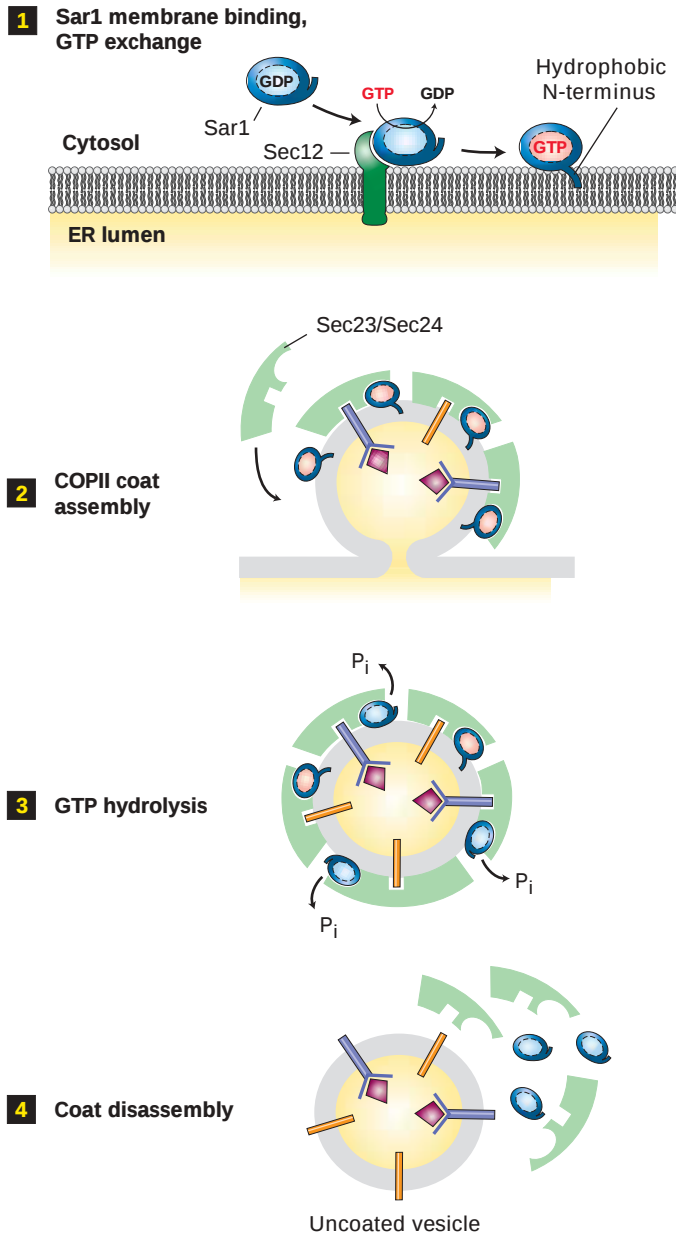
Based on *in vitro* vesicle-budding reactions with isolated membranes and purified coat proteins, scientists have determined the minimum set of coat components required to form each of the three major types of vesicles. Although most of the coat proteins differ considerably from one type of vesicle to another, the coats of all three vesicles contain a small GTP-binding protein that acts as a regulatory subunit to control coat assembly (see Figure 17-7a). For both COPI and clathrin vesicles, this GTP-binding protein is known as *ARF*. A different but related GTP-binding protein known as *Sar1* is present in the coat of COPII vesicles. Both ARF and Sar1 are monomeric proteins with an overall structure similar to that of Ras, a key intracellular signal-transducing protein (see Figure 14-20). ARF and Sar1 proteins, like Ras, belong to the **GTPase superfamily** of switch proteins that cycle between inactive GDP-bound and active GTP-bound forms (see Figure 3-29).

The cycle of GTP binding and hydrolysis by ARF and Sar1 are thought to control the initiation of coat assembly

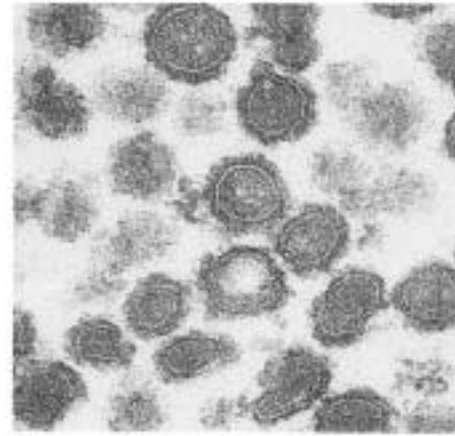
as schematically depicted for the assembly of COPII vesicles in Figure 17-9. First, an ER membrane protein known as Sec12 catalyzes release of GDP from cytosolic Sar1 · GDP and binding of GTP. The Sec12 guanine nucleotide-exchange factor apparently receives and integrates multiple, as yet unknown signals, probably including the presence of cargo proteins in the ER membrane that are ready to be transported. Binding of GTP causes a conformational change in Sar1 that exposes its hydrophobic N-terminus, which then becomes embedded in the phospholipid bilayer and tethers Sar1 · GTP to the ER membrane. The membrane-attached Sar1 · GTP drives polymerization of cytosolic complexes of COPII subunits on the membrane, eventually leading to formation of vesicle buds. Once COPII vesicles are released from the donor membrane, the Sar1 GTPase activity hydrolyzes Sar1 · GTP in the vesicle membrane to Sar1 · GDP with the assistance of one of the coat subunits. This hydrolysis triggers disassembly of the COPII coat. Thus Sar1 couples a cycle of GTP binding and hydrolysis to the formation and then dissociation of the COPII coat.

ARF protein undergoes a similar cycle of nucleotide exchange and hydrolysis coupled to the assembly of vesicle coats composed either of COPI or of clathrin and other coat proteins (AP complexes) discussed later. A myristate anchor covalently attached to the N-terminus of ARF protein weakly tethers ARF · GDP to the Golgi membrane. When GTP is exchanged for the bound GDP by a nucleotide-exchange factor attached to the Golgi membrane, the resulting conformational change in ARF allows hydrophobic residues in its N-terminal segment to insert into the membrane bilayer. The resulting tight association of ARF · GTP with the membrane serves as the foundation for further coat assembly.

Drawing on the structural similarities of Sar1 and ARF to other small GTPase switch proteins, researchers have



▲ **FIGURE 17-9 Model for the role of Sar1 in the assembly and disassembly of COPII coats.** Step **1**: Interaction of soluble GDP-bound Sar1 with the exchange factor Sec12, an ER integral membrane protein, catalyzes exchange of GTP for GDP on Sar1. In the GTP-bound form of Sar1, its hydrophobic N-terminus extends outward from the protein's surface and anchors Sar1 to the ER membrane. Step **2**: Sar1 attached to the membrane serves as a binding site for the Sec23/Sec24 coat protein complex. Cargo proteins are recruited to the forming vesicle bud by binding of specific short sequences (sorting signals) in their cytosolic regions to sites on the Sec23/Sec24 complex. The coat is completed by assembly of a second type of coat complex composed of Sec13/and Sec31 (not shown). Step **3**: After the vesicle coat is complete, the Sec23 coat subunit promotes GTP hydrolysis by Sar1. Step **4**: Release of Sar1 · GDP from the vesicle membrane causes disassembly of the coat. [See S. Springer et al., 1999, *Cell* **97**:145.]



▲ **EXPERIMENTAL FIGURE 17-10 Coated vesicles accumulate during in vitro budding reactions in the presence of a nonhydrolyzable analog of GTP.** When isolated Golgi membranes are incubated with a cytosolic extract containing COPII coat proteins and ATP, vesicles form and bud off from the membranes. Inclusion of a nonhydrolyzable analog of GTP in the budding reaction prevents disassembly of the coat after vesicle release. This micrograph shows COPII vesicles generated in such a reaction and separated from membranes by centrifugation. Coated vesicles prepared in this way can be analyzed to determine their components and properties. [Courtesy of L. Orci.]

constructed genes encoding mutant versions of the two proteins that have predictable effects on vesicular traffic when transfected into cultured cells. For example, in cells expressing mutant versions of Sar1 or ARF that cannot hydrolyze GTP, vesicle coats form and vesicle buds pinch off. However, because the mutant proteins cannot trigger disassembly of the coat, all available coat subunits eventually become permanently assembled into coated vesicles that are unable to fuse with target membranes. Addition of a nonhydrolyzable GTP analog to in vitro vesicle-budding reactions causes a similar blocking of coat disassembly. The vesicles that form in such reactions have coats that never dissociate, allowing their composition and structure to be more readily analyzed. The purified COPII vesicles shown in Figure 17-10 were produced in such a budding reaction.

Targeting Sequences on Cargo Proteins Make Specific Molecular Contacts with Coat Proteins

In order for transport vesicles to move specific proteins from one compartment to the next, vesicle buds must be able to discriminate among potential membrane and soluble cargo proteins, accepting only those cargo proteins that should advance to the next compartment and excluding those that should remain as residents in the donor compartment. In addition to sculpting the curvature of a donor membrane, the vesicle coat also functions in selecting specific proteins as cargo. The primary mechanism by which the vesicle coat selects cargo molecules is by directly binding to specific

TABLE 17-2 Known Sorting Signals That Direct Proteins to Specific Transport Vesicles

Signal Sequence*	Proteins with Signal	Signal Receptor	Vesicles That Incorporate Signal-bearing Protein
Lys-Asp-Glu-Leu (KDEL)	ER-resident luminal proteins	KDEL receptor in <i>cis</i> -Golgi membrane	COPI
Lys-Lys-X-X (KKXX)	ER-resident membrane proteins (cytosolic domain)	COPI α and β subunits	COPI
Di-acidic (e.g., Asp-X-Glu)	Cargo membrane proteins in ER (cytosolic domain)	COPII Sec24 subunit	COPII
Mannose 6-phosphate (M6P)	Soluble lysosomal enzymes after processing in <i>cis</i> -Golgi	M6P receptor in <i>trans</i> -Golgi membrane	Clathrin/AP1
	Secreted lysosomal enzymes	M6P receptor in plasma membrane	Clathrin/AP2
Asn-Pro-X-Tyr (NPXY)	LDL receptor in the plasma membrane (cytosolic domain)	AP2 complex	Clathrin/AP2
Tyr-X-X- Φ (YXX Φ)	Membrane proteins in <i>trans</i> -Golgi (cytosolic domain)	AP1 (μ 1 subunit)	Clathrin/AP1
	Plasma membrane proteins (cytosolic domain)	AP2 (μ 2 subunit)	Clathrin/AP2
Leu-Leu (LL)	Plasma membrane proteins (cytosolic domain)	AP2 complexes	Clathrin/AP2

*X = any amino acid; Φ = hydrophobic amino acid. Single-letter amino acid abbreviations are in parentheses.

sequences, or **sorting signals**, in the cytosolic portion of membrane cargo proteins (see Figure 17-7a). The polymerized coat thus acts as an affinity matrix to cluster selected membrane cargo proteins into forming vesicle buds. Soluble proteins within the lumen of parent organelles can in turn be selected by binding to the luminal domains of certain membrane cargo proteins, which act as receptors for luminal cargo proteins. The properties of several known sorting signals in membrane and soluble proteins are summarized in Table 17-2. We describe the role of these signals in more detail in later sections.

Rab GTPases Control Docking of Vesicles on Target Membranes

A second set of small GTP-binding proteins, known as *Rab proteins*, participate in the targeting of vesicles to the appropriate target membrane. Like Sar1 and ARF, Rab proteins belong to the GTPase superfamily of switch proteins. Conversion of cytosolic Rab · GDP to Rab · GTP, catalyzed by a specific guanine nucleotide-exchange factor, induces a conformational change in Rab that enables it to interact with a surface protein on a particular transport vesicle and insert its isoprenoid anchor into the vesicle membrane. Once

Rab · GTP is tethered to the vesicle surface, it is thought to interact with one of a number of different large proteins, known as Rab effectors, attached to the target membrane. Binding of Rab · GTP to a Rab effector docks the vesicle on an appropriate target membrane (Figure 17-11, step ①). After vesicle fusion occurs, the GTP bound to the Rab protein is hydrolyzed to GDP, triggering the release of Rab · GDP, which then can undergo another cycle of GDP-GTP exchange, binding, and hydrolysis.

Several lines of evidence support the involvement of specific Rab proteins in vesicle-fusion events. For instance, the yeast *SEC4* gene encodes a Rab protein, and yeast cells expressing mutant Sec4 proteins accumulate secretory vesicles that are unable to fuse with the plasma membrane (class E mutants in Figure 17-5). In mammalian cells, Rab5 protein is localized to endocytic vesicles, also known as early endosomes. These uncoated vesicles form from clathrin-coated vesicles just after they bud from the plasma membrane during endocytosis (see Figure 17-1, step ⑨). The fusion of early endosomes with each other in cell-free systems requires the presence of Rab5, and addition of Rab5 and GTP to cell-free extracts accelerates the rate at which these vesicles fuse with each other. A long coiled protein known as EEA1 (early endosome antigen 1), which resides on the membrane of the

early endosome, functions as the effector for Rab5. In this case, Rab5 · GTP on one endocytic vesicle is thought to specifically bind to EEA1 on the membrane of another endocytic vesicle, setting the stage for fusion of the two vesicles.

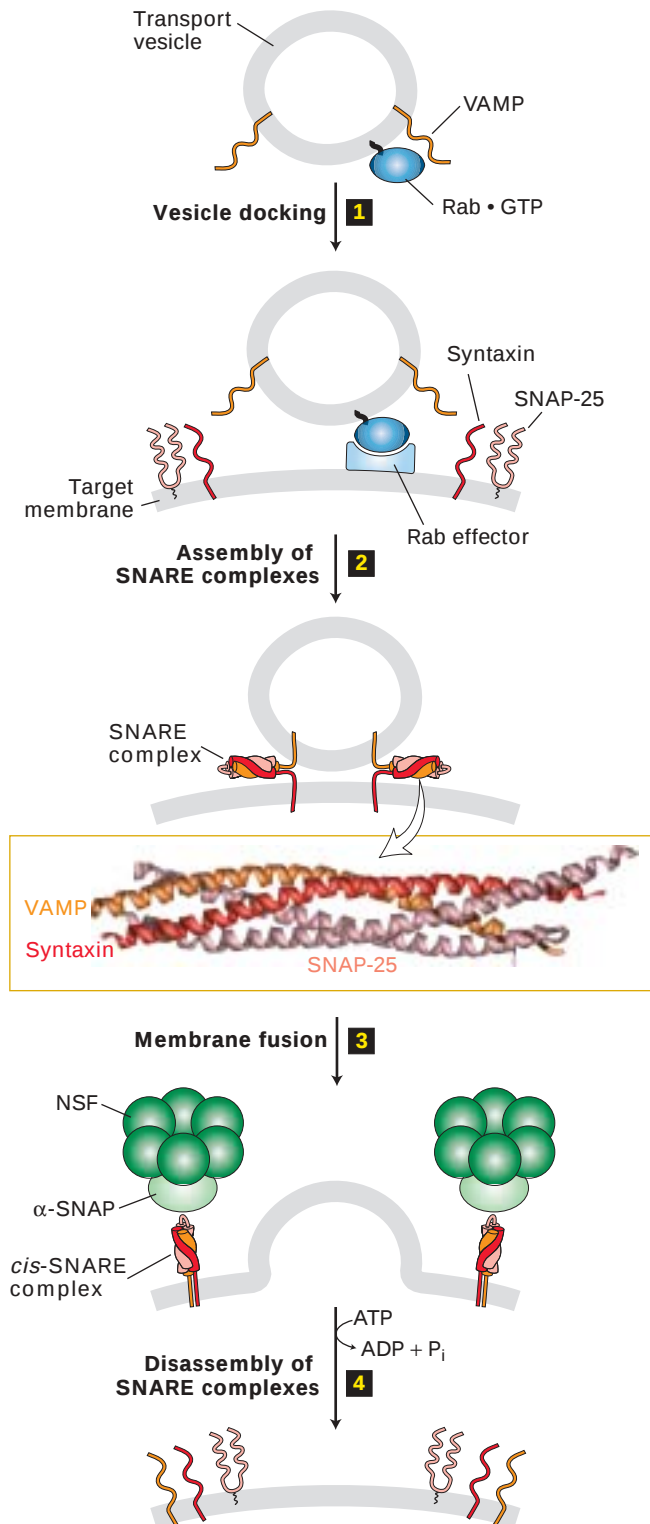
Similarly, Rab1 is essential for ER-to-Golgi transport reactions to occur in cell-free extracts. Rab1 · GTP binds to a

long coiled-coil protein known as p115, which specifically tethers COPII vesicles carrying Rab1 · GTP to the target Golgi membrane. A different type of Rab effector appears to function for each vesicle type and at each step of the secretory pathway. Many questions remain about how Rab proteins are targeted to the correct membrane and how specific complexes form between the different Rab proteins and their corresponding effector proteins.

Paired Sets of SNARE Proteins Mediate Fusion of Vesicles with Target Membranes

As noted previously, shortly after a vesicle buds off from the donor membrane, the vesicle coat disassembles to uncover a vesicle-specific membrane protein, a v-SNARE (see Figure 17-7b). Likewise, each type of target membrane in a cell contains t-SNARE membrane proteins. After Rab-mediated docking of a vesicle on its target (destination) membrane, the interaction of cognate SNAREs brings the two membranes close enough together that they can fuse.

One of the best-understood examples of SNARE-mediated fusion occurs during exocytosis of secreted proteins (Figure 17-11, steps 2 and 3). In this case, the v-SNARE, known as VAMP (vesicle-associated membrane protein), is incorporated into secretory vesicles as they bud from the *trans*-Golgi network. The t-SNAREs are syntaxin, an integral membrane protein in the plasma membrane, and SNAP-25, which is attached to the plasma membrane by a hydrophobic lipid anchor in the middle of the protein. The cytosolic region in each of these three SNARE proteins contains a repeating heptad sequence that allows four α helices—one from VAMP, one



◀ **FIGURE 17-11 Model for docking and fusion of transport vesicles with their target membranes.** The proteins shown in this example participate in fusion of secretory vesicles with the plasma membrane, but similar proteins mediate all vesicle-fusion events. Step **1**: A Rab protein tethered via a lipid anchor to a secretory vesicle binds to an effector protein complex on the plasma membrane, thereby docking the transport vesicle on the appropriate target membrane. Step **2**: A v-SNARE protein (in this case, VAMP) interacts with the cytosolic domains of the cognate t-SNAREs (in this case, syntaxin and SNAP-25). The very stable coiled-coil SNARE complexes that are formed hold the vesicle close to the target membrane. *Inset*: Numerous noncovalent interactions between four long α helices, two from SNAP-25 and one each from syntaxin and VAMP, stabilize the coiled-coil structure. Step **3**: Fusion of the two membranes immediately follows formation of SNARE complexes, but precisely how this occurs is not known. Step **4**: Following membrane fusion, NSF in conjunction with α -SNAP protein binds to the SNARE complexes. The NSF-catalyzed hydrolysis of ATP then drives dissociation of the SNARE complexes, freeing the SNARE proteins for another round of vesicle fusion. [See J. E. Rothman and T. Söhlner, 1997, *Science* **276**:1212, and W. Weis and R. Scheller, 1998, *Nature* **395**:328. Inset from Y. A. Chen and R. H. Scheller, 2001, *Nat. Rev. Mol. Cell Biol.* **2**(2):98.]

from syntaxin, and two from SNAP-25—to coil around one another to form a four-helix bundle. The unusual stability of this bundled SNARE complex is conferred by the arrangement of hydrophobic and charged amino residues in the heptad repeats. The hydrophobic amino acids are buried in the central core of the bundle, and amino acids of opposite charge are aligned to form favorable electrostatic interactions between helices. As the four-helix bundles form, the vesicle and target membranes are drawn into close apposition by the embedded transmembrane domains of VAMP and syntaxin.

In vitro experiments have shown that when **liposomes** containing purified VAMP are incubated with other liposomes containing syntaxin and SNAP-25, the two classes of membranes fuse, albeit slowly. This finding is strong evidence that the close apposition of membranes resulting from formation of SNARE complexes is sufficient to bring about membrane fusion. Fusion of a vesicle and target membrane occurs much more rapidly and efficiently in the cell than it does in liposome experiments in which fusion is catalyzed only by SNARE proteins. The likely explanation for this difference is that in the cell the interactions between specific Rab proteins and their effectors promote the formation of specific SNARE bundles by tethering a vesicle to its target membrane.

Yeast cells, like all eukaryotic cells, express more than 20 different related v-SNARE and t-SNARE proteins. Analyses of yeast *sec* mutants defective in each of the SNARE genes have identified the specific membrane-fusion event in which each SNARE protein participates. For all fusion events that have been examined, the SNAREs form four-helix bundled complexes, similar to the VAMP/syntaxin/SNAP-25 complexes that mediate fusion of secretory vesicles with the plasma membrane. However, in other fusion events (e.g., fusion of COPII vesicles with the *cis*-Golgi network), each participating SNARE protein contributes only one α helix to the bundle (unlike SNAP-25, which contributes two helices); in these cases the SNARE complexes comprise one v-SNARE and three t-SNARE molecules.

Using the in vitro liposome fusion assay, researchers have tested the ability of various combinations of individual v-SNARE and t-SNARE proteins to mediate fusion of donor and target membranes. Of the very large number of different combinations tested, only a small number mediated membrane fusion. To a remarkable degree the functional combinations of v-SNAREs and t-SNAREs revealed in these in vitro experiments correspond to the actual SNARE protein interactions that mediate known membrane-fusion events in the yeast cell. Thus the specificity of the interaction between SNARE proteins can account for the specificity of fusion between a particular vesicle and its target membranes.

Dissociation of SNARE Complexes After Membrane Fusion Is Driven by ATP Hydrolysis

After a vesicle and its target membrane have fused, the SNARE complexes must dissociate to make the individual SNARE proteins available for additional fusion events. Be-

cause of the stability of SNARE complexes, which are held together by numerous noncovalent intermolecular interactions, their dissociation depends on additional proteins and the input of energy.

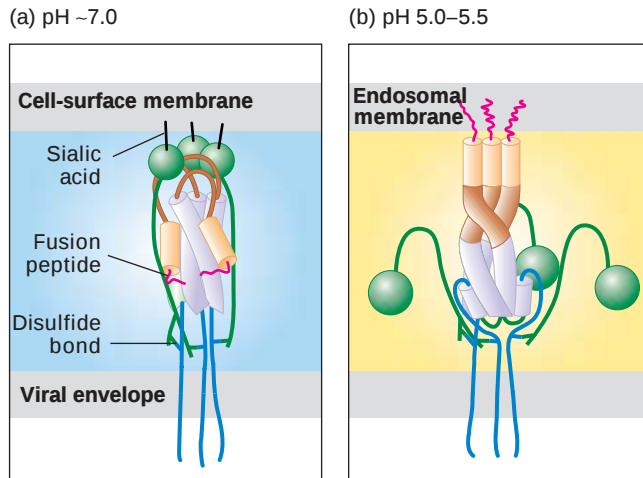
The first clue that dissociation of SNARE complexes required the assistance of other proteins came from in vitro transport reactions depleted of certain cytosolic proteins. The observed accumulation of vesicles in these reactions indicated that vesicles could form but were unable to fuse with a target membrane. Eventually two proteins, designated *NSF* and α -*SNAP*, were found to be required for ongoing vesicle fusion in the in vitro transport reaction. The function of NSF in vivo can be blocked selectively by *N*-ethylmaleimide (NEM), a chemical that reacts with an essential –SH group on NSF (hence the name, *NEM-sensitive factor*).

Among the class C yeast *sec* mutants are strains that lack functional Sec18 or Sec17, the yeast counterparts of mammalian NSF and α -SNAP, respectively. When these class C mutants are placed at the nonpermissive temperature, they accumulate ER-to-Golgi transport vesicles; when the cells are shifted to the lower, permissive temperature, the accumulated vesicles are able to fuse with the *cis*-Golgi.

Subsequent to the initial biochemical and genetic studies identifying NSF and α -SNAP, more sophisticated in vitro transport assays were developed. Using these newer assays, researchers have shown that NSF and α -SNAP proteins are not necessary for actual membrane fusion, but rather are required for regeneration of free SNARE proteins. NSF, a hexamer of identical subunits, associates with a SNARE complex with the aid α -SNAP (soluble NSF attachment protein). The bound NSF then hydrolyzes ATP, releasing sufficient energy to dissociate the SNARE complex (Figure 17-11, step 4). Evidently, the defects in vesicle fusion observed in the earlier in vitro fusion assays and in the yeast mutants after a loss of Sec17 or Sec18 were a consequence of free SNARE proteins rapidly becoming sequestered in undissociated SNARE complexes and thus unavailable to mediate membrane fusion.

Conformational Changes in Viral Envelope Proteins Trigger Membrane Fusion

Some animal viruses, including influenza virus, rabies virus, and human immunodeficiency virus (HIV), have an outer phospholipid bilayer membrane, or **envelope**, surrounding the core of the virus particle composed of viral proteins and genetic material. The viral envelope is derived by budding from the host-cell plasma membrane, which contains virus-encoded glycoproteins. Enveloped viruses enter a host cell by endocytosis following binding of one or more viral envelope glycoproteins with a host's cell-surface molecules. Subsequent fusion of the viral envelope with the endosomal membrane releases the viral genome into the cytosol of the host cell, initiating replication of the virus (see Figure 4-41, step 3). The molecular events of this fusion process have been elucidated in considerable detail in the case of influenza virus.



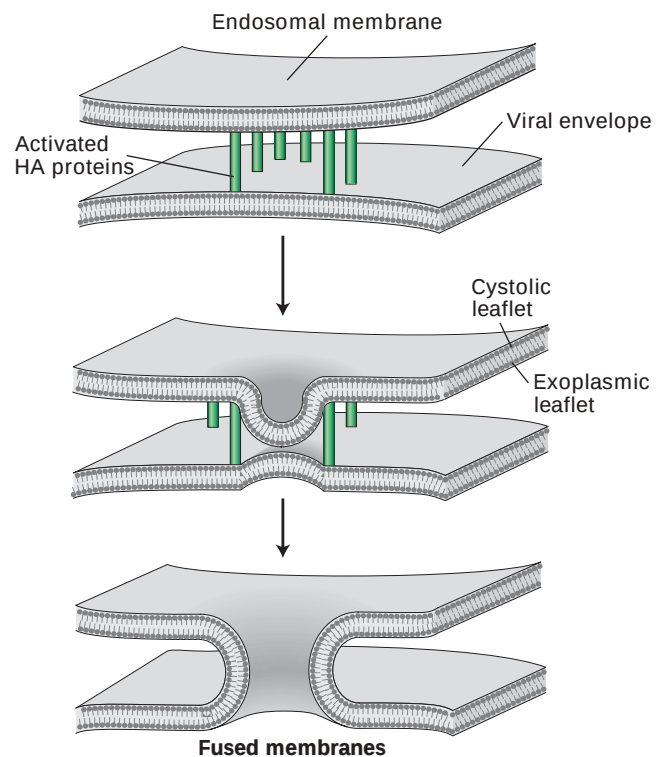
▲ **FIGURE 17-12 Schematic models of the structure of influenza hemagglutinin (HA) at pH 7 and 5.** Three HA₁ and three HA₂ subunits compose a hemagglutinin molecule, which protrudes from the viral envelope like a spike. (a) At pH ≈ 7, part of each HA₁ subunit forms a globular domain (green) at the tip of the native spike. These domains bind to sialic acid residues on the host-cell plasma membrane, initiating viral entry. Each HA₁ subunit is linked to one HA₂ subunit by a disulfide bond at the base of the molecule near the viral envelope. Each HA₂ subunit contains a fusion peptide (red) at its N-terminus (only two are visible), followed by a short α helix (orange cylinder), a nonhelical loop (brown), and a longer α helix (light purple). The longer α helices from the three HA₂ subunits form a three-stranded coiled-coil structure (see Figure 3-7). In this conformation, the fusion peptides are buried within the molecule. (b) At the acidic pH within a late endosome, the binding of the fusion peptide to other segments of HA₂ is disrupted, inducing major structural rearrangements in the protein. First, the three HA₁ globular domains separate from each other but remain tethered to the HA₂ subunits by the disulfide bonds at the base of the molecule. Second, the loop segment of each HA₂ rearranges into an α helix (brown) and combines with the short and long α -helical segments to form a continuous 88-residue α helix. The three long α helices thus form a 13.5-nm-long three-stranded coiled coil that protrudes outward from the viral envelope. In this conformation, the fusion peptides are at the tip of the coiled coil and can insert into the endosomal membrane. [Adapted from C. M. Carr et al., 1997, *Proc. Nat'l. Acad. Sci.* **94**:14306; courtesy of Peter Kim.]

The predominant glycoprotein of the influenza virus is *hemagglutinin (HA)*, which forms the larger spikes on the surface of the virus. There is considerable evidence that following endocytosis of an influenza virion, the low pH within the enclosing late endosome triggers fusion of its membrane with the viral envelope. For instance, viral infection is inhibited by the addition of lipid-soluble bases, such as ammonia or trimethylamine, which raise the normally acidic pH of late endosomes. Also, a conformational change in the HA protein that is critical for infectivity occurs over a very narrow range in pH (5.0–5.5).

Each HA spike on an influenza virion consists of three HA₁ and three HA₂ subunits. At the N-terminus of HA₂ is a strongly hydrophobic 11-residue sequence, called the fusion

peptide. Structural studies have shown that at pH 7.0, the N-terminus of each HA₂ subunit is tucked into a crevice in the spike (Figure 17-12a). This is the normal HA conformation when a viral particle encounters the surface of a host cell. At the acidic pH characteristic of late endosomes, HA undergoes several conformational changes that cause a major rearrangement of the subunits. As a result, the three HA₂ subunits twist together into a three-stranded coiled-coil rod that protrudes more than 13 nm outward from the viral envelope with the fusion peptides at the tip of the rod (Figure 17-12b). In this conformation, the highly hydrophobic fusion peptides are exposed and can insert into the lipid bilayer of the endosomal membrane, triggering fusion of the viral envelope and the membrane. Thus at pH 7 HA can be said to be trapped in a metastable, “spring-loaded” state, which is converted to the lower-energy fusogenic state by shifting the pH to 5–5.5.

Multiple low pH-activated HA spikes are essential for membrane fusion to occur. Figure 17-13 suggests one way by which the protein scaffold formed by many HA spikes, possibly with the assistance of other cellular proteins, could link together the



▲ **FIGURE 17-13 Model for membrane fusion directed by hemagglutinin (HA).** A number of low pH-activated HA spikes, possibly in concert with host-cell membrane proteins, form a scaffold that connects a small region of the viral envelope and the endosomal membrane. By unknown mechanisms, the exoplasmic leaflets of the two membranes fuse and then the cytosolic leaflets fuse, forming a pore that widens until the two membranes are completely joined. A similar interaction between membrane bilayers may be brought about during SNARE-mediated vesicle fusion. [Adapted from J. R. Monck and J. M. Fernandez, 1992, *J. Cell Biol.* **119**:1395.]

viral envelope and endosomal membrane and induce their fusion. This figure also illustrates how cellular membranes brought into close apposition by SNARE complexes might fuse. Note that each HA molecule participates in only one fusion event, whereas the cellular fusion proteins, such as SNAREs, are recycled and catalyze multiple cycles of membrane fusion.

KEY CONCEPTS OF SECTION 17.2

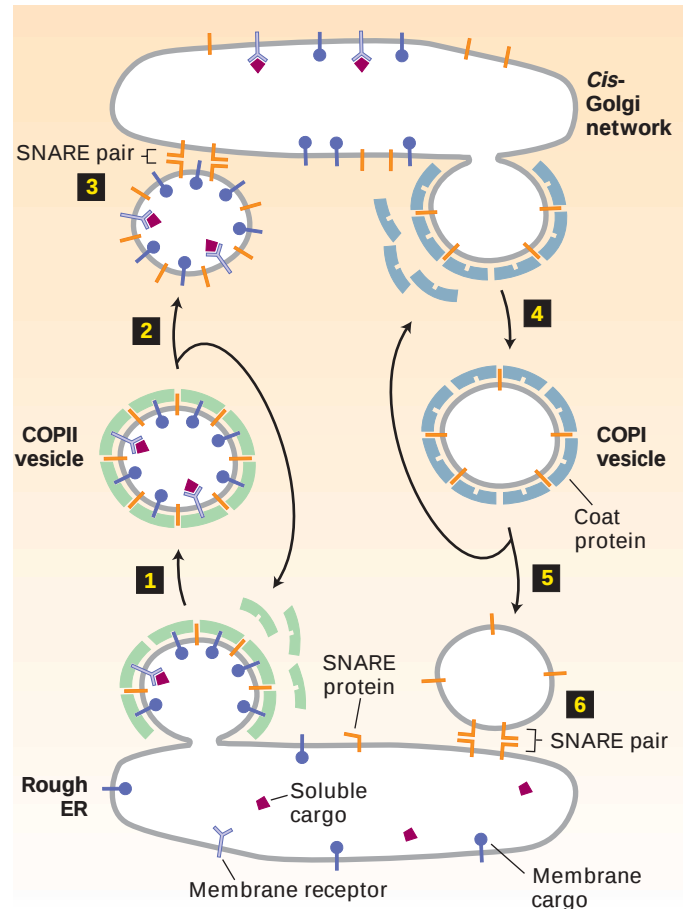
Molecular Mechanisms of Vesicular Traffic

- The three well-characterized transport vesicles—COPI, COPII, and clathrin vesicles—are distinguished by the proteins that form their coats and the transport routes they mediate (see Table 17-1).
- All types of coated vesicles are formed by polymerization of cytosolic coat proteins onto a donor (parent) membrane to form vesicle buds that eventually pinch off from the membrane to release a complete vesicle. Shortly after vesicle release, the coat is shed exposing proteins required for fusion with the target membrane (see Figure 17-7).
- Small GTP-binding proteins (ARF or Sar1) belonging to the GTPase superfamily control polymerization of coat proteins, the initial step in vesicle budding (see Figure 17-9). After vesicles are released from the donor membrane, hydrolysis of GTP bound to ARF or Sar1 triggers disassembly of the vesicle coats.
- Specific sorting signals in membrane and luminal proteins of donor organelles interact with coat proteins during vesicle budding, thereby recruiting cargo proteins to vesicles (see Table 17-2).
- A second set of GTP-binding proteins, the Rab proteins, regulate docking of vesicles with the correct target membrane. Each Rab appears to bind to a specific Rab effector, a typically long coiled-coil protein, associated with the target membrane.
- Each v-SNARE in a vesicular membrane specifically binds to a complex of cognate t-SNARE proteins in the target membrane, inducing fusion of the two membranes. After fusion is completed, the SNARE complex is disassembled in an ATP-dependent reaction mediated by other cytosolic proteins (see Figure 17-11).
- After an enveloped animal virus is endocytosed, the viral envelope fuses with the surrounding endosomal membrane. In the case of influenza virus, the acidic pH within late endosomes causes a conformational change in the HA protein in the viral envelope that permits insertion of HA into the endosomal membrane.

17.3 Early Stages of the Secretory Pathway

In this section we take a closer look at vesicular traffic through the ER and Golgi stages of the secretory pathway

and some of the evidence supporting the general mechanisms discussed in the previous section. Recall that *anterograde transport* from the ER to Golgi, the first step in the secretory pathway, is mediated by COPII vesicles, whereas the reverse *retrograde transport* from the *cis*-Golgi to the ER is mediated by COPI vesicles (Figure 17-14). This retrograde



▲ FIGURE 17-14 Vesicle-mediated protein trafficking between the ER and *cis*-Golgi. Steps **1–3**: Forward (anterograde) transport is mediated by COPII vesicles, which are formed by polymerization of soluble COPII coat protein complexes (blue) on the ER membrane. v-SNAREs (red) and other cargo proteins (green) in the ER membrane are incorporated into the vesicle by interacting with coat proteins. Soluble cargo proteins (purple) are recruited by binding to appropriate receptors in the membrane of budding vesicles. Dissociation of the coat recycles free coat complexes and exposes v-SNARE proteins on the vesicle surface. After the uncoated vesicle becomes tethered to the *cis*-Golgi membrane in a Rab-mediated process, pairing between the exposed v-SNAREs and cognate t-SNAREs in the Golgi membrane allow vesicle fusion, releasing the contents into the *cis*-Golgi compartment (see Figure 17-11). Steps **4–6**: Reverse (retrograde) transport, mediated by vesicles coated with COPI proteins (green), recycles the membrane bilayer and certain proteins, such as v-SNAREs and missorted ER-resident proteins (not shown), from the *cis*-Golgi to the ER. All SNARE proteins are shown in red although each v-SNARE and t-SNARE are distinct proteins.

vesicle transport serves to retrieve v-SNARE proteins and the membrane itself back to the ER to provide the necessary material for additional rounds of vesicle budding from the ER. COPI-mediated retrograde transport also retrieves missorted ER-resident proteins from the *cis*-Golgi to correct sorting mistakes. Proteins that have been correctly delivered to the Golgi advance through successive compartments of the Golgi by cisternal progression.

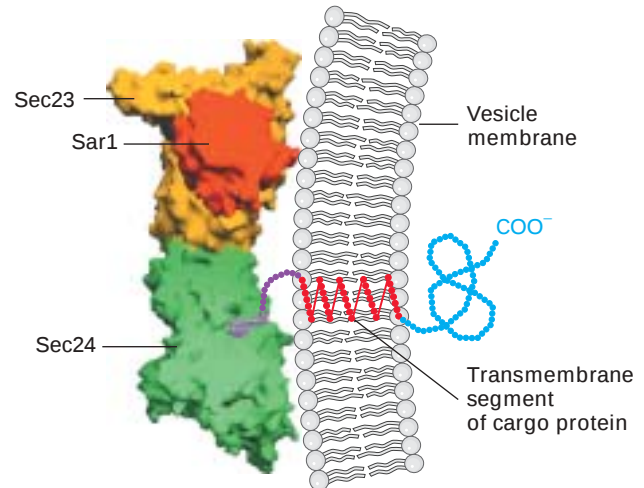
COPII Vesicles Mediate Transport from the ER to the Golgi

COPII vesicles were first recognized when cell-free extracts of yeast rough ER membranes were incubated with cytosol, ATP, and a nonhydrolyzable analog of GTP. The vesicles that formed from the ER membranes had a distinct coat, similar to that on COPI vesicles but composed of different proteins, designated COPII proteins. Yeast cells with mutations in the genes for COPII proteins are class B *sec* mutants and accumulate proteins in the rough ER (see Figure 17-5). Analysis of such mutants has revealed several proteins required for formation of COPII vesicles.

As described previously, formation of COPII vesicles is triggered when Sec12, a guanine nucleotide–exchange factor, catalyzes the exchange of bound GDP for GTP on Sar1. This exchange induces binding of Sar1 to the ER membrane followed by binding of a complex of Sec23 and Sec24 proteins (see Figure 17-9). The resulting ternary complex formed between Sar1·GTP, Sec23, and Sec24 is shown in Figure 17-15. After this complex forms on the ER membrane, a second complex comprising Sec13 and Sec31 proteins then binds to complete the coat structure. A large fibrous protein, called Sec16, which is bound to the cytosolic surface of the ER, interacts with the Sec13/31 and Sec23/24 complexes, and acts to organize the other coat proteins, increasing the efficiency of coat polymerization.

Certain integral ER membrane proteins are specifically recruited into COPII vesicles for transport to the Golgi. The cytosolic segments of many of these proteins contain a *di-acidic sorting signal* (Asp-X-Glu, or DXE in the one-letter code). This sorting signal binds to the Sec24 subunit of the COPII coat and is essential for the selective export of certain membrane proteins from the ER (see Figure 17-15). Biochemical and genetic studies currently are under way to identify additional signals that help direct membrane cargo proteins into COPII vesicles. Other ongoing studies seek to determine how soluble cargo proteins are selectively loaded into COPII vesicles. Although purified COPII vesicles from yeast cells have been found to contain a membrane protein that binds the soluble α mating factor, the receptors for other soluble cargo proteins such as invertase are not yet known.

The experiments described previously in which the transit of VSVG-GFP in cultured mammalian cells is followed by fluorescence microscopy (see Figure 17-2) provided insight into the intermediates in ER-to-Golgi transport. In some cells, small fluorescent vesicles containing VSVG-GFP could be seen to form from the ER, move less than 1 μm , and



▲ **FIGURE 17-15 Three-dimensional structure of ternary complex comprising the COPII coat proteins Sec23 and Sec24 and Sar1·GTP.** Early in the formation of the COPII coat, Sec23 (orange)/Sec24 (green) complexes are recruited to the ER membrane by Sar1 (red) in its GTP-bound state. In order to form a stable ternary complex in solution for structural studies, the nonhydrolyzable GTP analog GppNHp was used. A cargo protein in the ER membrane can be recruited to COPII vesicles by interaction of a tripeptide di-acidic signal (purple) in the cargo's cytosolic domain with Sec24. The likely position of the COPII vesicle membrane and the transmembrane segment of the cargo protein are indicated. The N-terminal segment of Sar1 that tethers it to the membrane is not shown. [See X. Bi et al., 2002, *Nature* **419**:271; interaction with peptide courtesy of J. Goldberg.]

then fuse directly with the *cis*-Golgi. In other cells, in which the ER was located several micrometers from the Golgi complex, several ER-derived vesicles were seen to fuse with each other shortly after their formation, forming what is termed the “ER-to-Golgi intermediate compartment.” These larger structures then were transported along microtubules to the *cis*-Golgi, much in the way vesicles in nerve cells are transported from the cell body, where they are formed, down the long axon to the axon terminus (Chapter 20). Microtubules function much as “railroad tracks” enabling these large aggregates of transport vesicles to move long distances to their *cis*-Golgi destination. At the time the ER-to-Golgi intermediate compartment is formed, some COPI vesicles bud off from it, recycling some proteins back to the ER.

COPI Vesicles Mediate Retrograde Transport within the Golgi and from the Golgi to the ER

COPI vesicles were first discovered when isolated Golgi fractions were incubated in a solution containing ATP, cytosol, and a nonhydrolyzable analog of GTP (see Figure 17-10). Subsequent analysis of these vesicles showed that the coat is formed from large cytosolic complexes, called *coatomers*, composed of seven polypeptide subunits. Yeast cells containing temperature-sensitive mutations in COPI proteins accumulate proteins in the

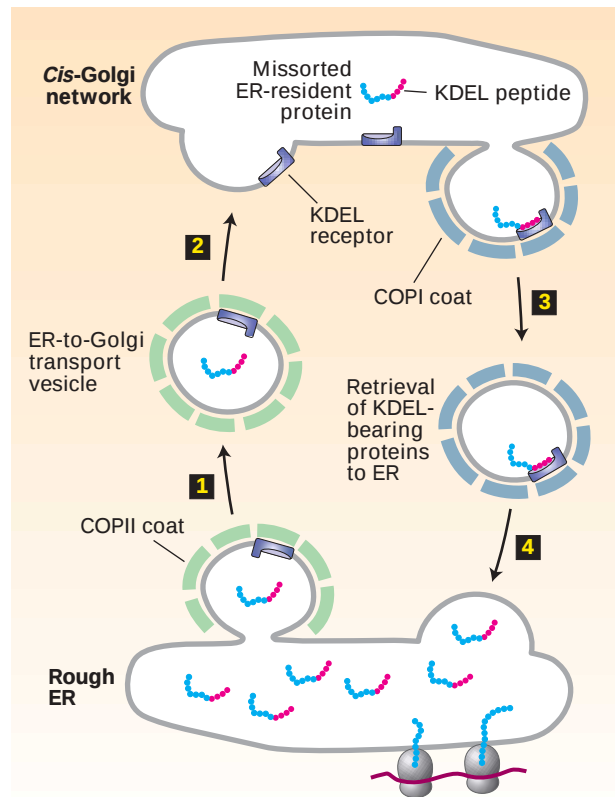
rough ER at the nonpermissive temperature and thus are categorized as class B *sec* mutants (see Figure 17-5). Although discovery of these mutants initially suggested that COPII vesicles mediate ER-to-Golgi transport, subsequent experiments showed that their main function is retrograde transport, both between Golgi cisternae and from the *cis*-Golgi to the rough ER (see Figure 17-14, *right*). Because COPI mutants cannot recycle key membrane proteins back to the rough ER, the ER gradually becomes depleted of ER proteins such as v-SNAREs necessary for COPII vesicle function. Eventually vesicle formation from the rough ER grinds to a halt; secretory proteins continue to be synthesized but accumulate in the ER, the defining characteristic of class B *sec* mutants.

As discussed in Chapter 16, the ER contains several soluble proteins dedicated to the folding and modification of newly synthesized secretory proteins. These include the chaperone BiP and the enzyme protein disulfide isomerase, which are necessary for the ER to carry out its functions. Although such ER-resident luminal proteins are not specifically selected by COPII vesicles, their sheer abundance causes them to be continuously loaded passively into vesicles destined for the *cis*-Golgi. The transport of these soluble proteins back to the ER, mediated by COPI vesicles, prevents their eventual depletion.

Most soluble ER-resident proteins carry a Lys-Asp-Glu-Leu (KDEL in the one-letter code) sequence at their C-terminus (see Table 17-2). Several experiments demonstrated that this *KDEL sorting signal* is both necessary and sufficient for retention in the ER. For instance, when a mutant protein disulfide isomerase lacking these four residues is synthesized in cultured fibroblasts, the protein is secreted. Moreover, if a protein that normally is secreted is altered so that it contains the KDEL signal at its C-terminus, the protein is retained in the ER. The KDEL sorting signal is recognized and bound by the *KDEL receptor*, a transmembrane protein found primarily on small transport vesicles shuttling between the ER and the *cis*-Golgi and on the *cis*-Golgi reticulum. In addition, soluble ER-resident proteins that carry the KDEL signal have oligosaccharide chains with modifications that are catalyzed by enzymes found only in the *cis*-Golgi or *cis*-Golgi reticulum; thus at some time these proteins must have left the ER and been transported at least as far as the *cis*-Golgi network. These findings indicate that the KDEL receptor acts mainly to retrieve soluble proteins containing the KDEL sorting signal that have escaped to the *cis*-Golgi network and return them to the ER (Figure 17-16).

The KDEL receptor and other membrane proteins that are transported back to the ER from the Golgi contain a Lys-Lys-X-X sequence at the very end of their C-terminal segment, which faces the cytosol (see Table 17-2). This *KKXX sorting signal* which binds to a complex of the COPI α and β subunits, is both necessary and sufficient to incorporate membrane proteins into COPI vesicles for retrograde transport to the ER. Temperature-sensitive yeast mutants lacking COPI α or COPI β not only are unable to bind the KKXX signal but also are unable to retrieve proteins bearing this signal back to the ER, indicating that COPI vesicles mediate retrograde Golgi-to-ER transport.

Clearly, the partitioning of proteins between the ER and Golgi complex is a highly selective and regulated process ultimately controlled by the specificity of cargo loading into both COPII (anterograde) and COPI (retrograde) vesicles. The selective entry of proteins into membrane-bounded transport vesicles, the recycling of membrane phospholipids and proteins, and the recycling of soluble luminal proteins between the two compartments are fundamental features of vesicular protein trafficking that also occur in later stages of the secretory pathway.



▲ FIGURE 17-16 Role of the KDEL receptor in retrieval of ER-resident luminal proteins from the Golgi.

ER luminal proteins, especially those present at high levels, can be passively incorporated into COPII vesicles and transported to the Golgi (steps 1 and 2). Many such proteins bear a C-terminal KDEL (Lys-Asp-Glu-Leu) sequence (red) that allows them to be retrieved. The KDEL receptor, located mainly in the *cis*-Golgi network and in both COPII and COPI vesicles, binds proteins bearing the KDEL sorting signal and returns them to the ER (steps 3 and 4). This retrieval system prevents depletion of ER luminal proteins such as those needed for proper folding of newly made secretory proteins. The binding affinity of the KDEL receptor is very sensitive to pH. The small difference in the pH of the ER and Golgi favors binding of KDEL-bearing proteins to the receptor in Golgi-derived vesicles and their release in the ER. [Adapted from J. Semenza et al., 1990, *Cell* 61:1349.]

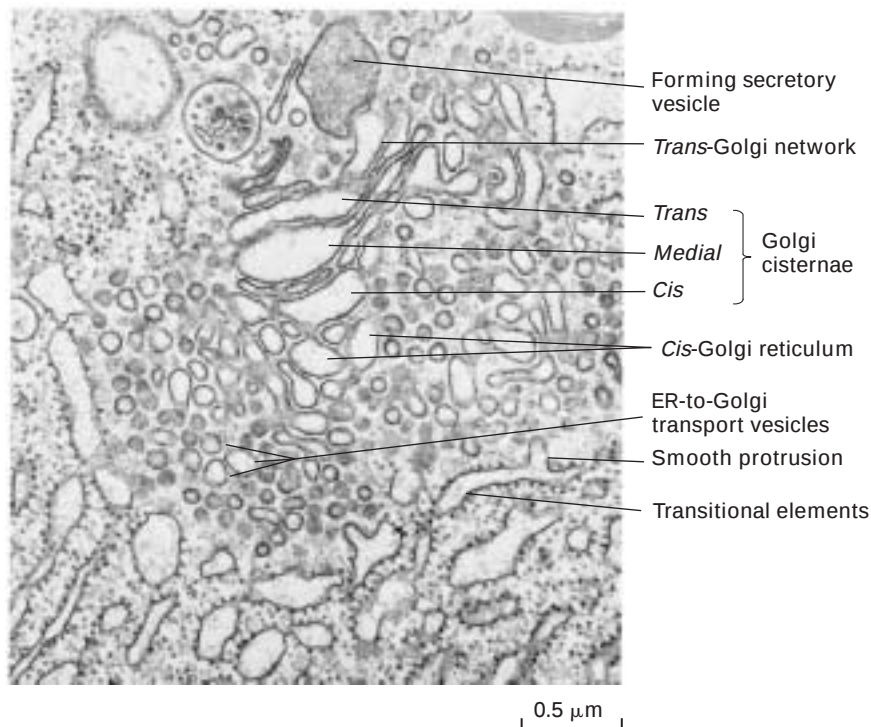
Anterograde Transport Through the Golgi Occurs by Cisternal Progression

At one time it was thought that small transport vesicles carry secretory proteins from the *cis*- to the *medial*-Golgi and from the *medial*- to the *trans*-Golgi. Indeed, electron microscopy reveals many small vesicles associated with the Golgi complex that move proteins from one Golgi compartment to another (Figure 17-17). However, these vesicles most likely mediate retrograde transport, retrieving ER or Golgi enzymes from a later compartment and transporting them to an earlier compartment in the secretory pathway. In this way enzymes that modify secretory proteins come to be localized in the correct compartment.

The first evidence that the forward transport of cargo proteins from the *cis*- to the *trans*-Golgi occurs by a non-vesicular mechanism, called *cisternal progression*, came from careful microscopic analysis of the synthesis of algal scales. These cell-wall glycoproteins are assembled in the *cis*-Golgi into large complexes visible in the electron microscope. Like other secretory proteins, newly made scales move from the *cis*- to the *trans*-Golgi, but they can be 20 times larger than the usual transport vesicles that bud from Golgi cisternae. Similarly, in the synthesis of collagen by fibroblasts, large aggregates of the procollagen precursor often form in the

lumen of the *cis*-Golgi (see Figure 6-20). The procollagen aggregates are too large to be incorporated into small transport vesicles, and investigators could never find such aggregates in transport vesicles. These observations suggested that the forward movement of these and perhaps all secretory proteins from one Golgi compartment to another does *not* occur via small vesicles.

In one test of the cisternal progression model, collagen folding was blocked by an inhibitor of proline hydroxylation, and soon all pre-made, folded procollagen aggregates were secreted from the cell. When the inhibitor was removed, newly made procollagen peptides folded and then formed aggregates in the *cis*-Golgi that subsequently could be seen to move as a “wave” from the *cis*- through the *medial*-Golgi cisternae to the *trans*-Golgi, followed by secretion and incorporation into the extracellular matrix. In these experiments procollagen aggregates were never visible in small transport vesicles. Numerous controversial questions concerning membrane flow within the Golgi stack remain unresolved. Nonetheless, the observed movement of very large macromolecular assemblies through the Golgi stack and the evidence described previously that COPI vesicles mediate retrograde transport have led most researchers in the field to favor the cisternal progression model.



▲ **EXPERIMENTAL FIGURE 17-17** **Electron micrograph of the Golgi complex in an exocrine pancreatic cell reveals both anterograde and retrograde transport vesicles.** A large secretory vesicle can be seen forming from the *trans*-Golgi network. Elements of the rough ER are on the left in this micrograph. Adjacent to the rough ER are transitional elements

from which smooth protrusions appear to be budding. These buds form the small vesicles that transport secretory proteins from the rough ER to the Golgi complex. Interspersed among the Golgi cisternae are other small vesicles now known to function in retrograde, not anterograde, transport. [Courtesy G. Palade.]

KEY CONCEPTS OF SECTION 17.3

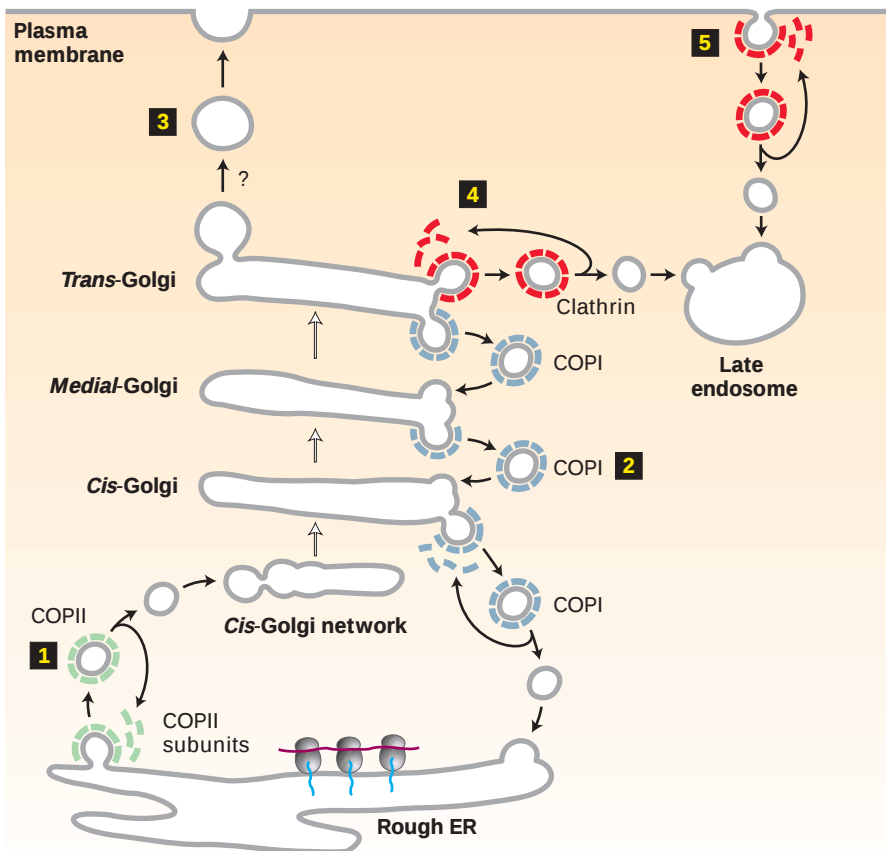
Vesicle Traffic in the Early Stages of the Secretory Pathway

- COPII vesicles transport proteins from the rough ER to the *cis*-Golgi; COPI vesicles transport proteins in the reverse direction (see Figure 17-14).
- COPII coats comprise three components: the small GTP-binding protein Sar1, a Sec23/Sec24 complex, and a Sec13/Sec31 complex.
- Components of the COPII coat bind to membrane cargo proteins containing a di-acidic or other sorting signal in their cytosolic regions (see Figure 17-15). Soluble cargo proteins probably are targeted to COPII vesicles by binding to a membrane protein receptor.
- Membrane proteins needed to form COPII vesicles can be retrieved from the *cis*-Golgi by COPI vesicles. One of the sorting signals that directs membrane proteins into COPI vesicles is a KKXX sequence, which binds to subunits of the COPI coat.
- Many soluble ER-resident proteins contain a KDEL sorting signal. Binding of this retrieval sequence to a specific receptor protein in the *cis*-Golgi membrane recruits missorted ER proteins into retrograde COPI vesicles (see Figure 17-16).

- COPI vesicles also carry Golgi-resident proteins from later to earlier compartments in the Golgi stack.
- Soluble and membrane proteins advance through the Golgi complex by cisternal progression, a nonvesicular process of anterograde transport.

17.4 Later Stages of the Secretory Pathway

As cargo proteins move from the *cis* face to the *trans* face of the Golgi complex by cisternal progression, modifications to their oligosaccharide chains are carried out by Golgi-resident enzymes. The retrograde trafficking of COPI vesicles from later to earlier Golgi compartments maintains sufficient levels of these carbohydrate-modifying enzymes in their functional compartments. Eventually, properly processed cargo proteins reach the *trans*-Golgi network, the most-distal Golgi compartment. Here they are sorted into vesicles for delivery to their final destination. In this section we discuss the different kinds of vesicles that bud from the *trans*-Golgi network, the mechanisms that segregate cargo proteins among them, and key processing events that occur late in the secretory pathway. The transport steps mediated by the major types of coated vesicles are summarized in Figure 17-18.



◀ **FIGURE 17-18 Involvement of the three major types of coat proteins in vesicular traffic in the secretory and endocytic pathways.** After formation of vesicles by budding from a donor membrane, the coats depolymerize into their subunits, which are re-used to form additional transport vesicles. COPII vesicles (1) mediate anterograde transport from the rough ER to the *cis*-Golgi/*cis*-Golgi network. COPI vesicles (2) mediate retrograde transport within the Golgi and from the *cis*-Golgi/*cis*-Golgi network to the rough ER. The coat proteins surrounding secretory vesicles (3) are not yet characterized; these vesicles carry secreted proteins and plasma-membrane proteins from the *trans*-Golgi network to the cell surface. Vesicles coated with clathrin (red) bud from the *trans*-Golgi network (4) and from the plasma membrane (5); after uncoating, these vesicles fuse with late endosomes. The coat on most clathrin vesicles contains additional proteins not indicated here. Note that secretory proteins move from the *cis*- to *trans*-Golgi by cisternal progression, which is not mediated by vesicles. [See H. Pelham, 1997, *Nature* 389:17, and J. F. Presley et al., 1997, *Nature* 389:81]

Vesicles Coated with Clathrin and/or Adapter Proteins Mediate Several Transport Steps

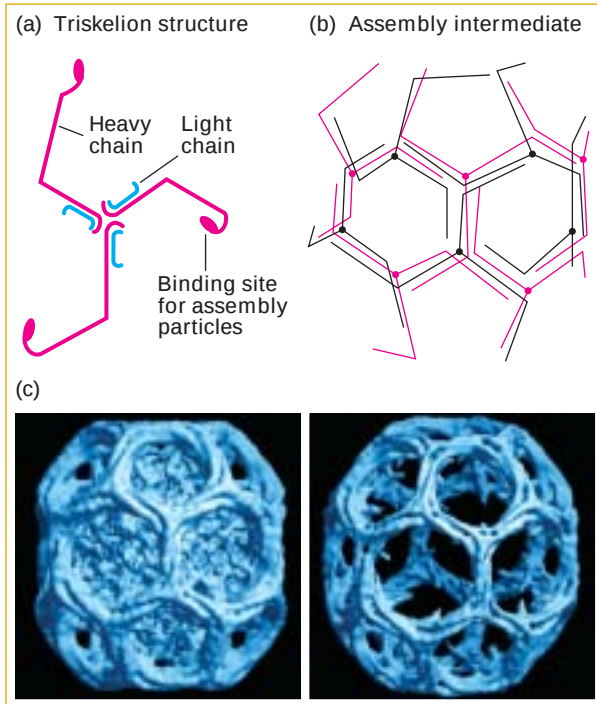
The best-characterized vesicles that bud from the *trans*-Golgi network (TGN) have a two-layered coat: an outer layer composed of the fibrous protein clathrin and an inner layer composed of *adapter protein (AP) complexes*. Purified clathrin molecules, which have a three-limbed shape, are called *triskelions* from the Greek for three-legged (Figure 17-19a). Each limb contains one clathrin heavy chain (180,000 MW) and one clathrin light chain

(≈35,000–40,000 MW). Triskelions polymerize to form a polygonal lattice with an intrinsic curvature (Figure 17-19b). When clathrin polymerizes on a donor membrane, it does so in association with AP complexes, which assemble between the clathrin lattice and the membrane. Each AP complex (340,000 MW) contains one copy each of four different adapter subunit proteins. A specific association between the globular domain at the end of each clathrin heavy chain in a triskelion and one subunit of the AP complex both promotes the co-assembly of clathrin triskelions with AP complexes and adds to the stability of the completed vesicle coat (Figure 17-19c).

By binding to the cytosolic face of membrane proteins, adapter proteins determine which cargo proteins are specifically included in (or excluded from) a budding transport vesicle. Each type of AP complex (e.g., AP1, AP2, AP3) and the recently identified GGAs are composed of different, though related, proteins. Vesicles containing each complex have been found to mediate specific transport steps (see Table 17-1). All vesicles whose coats contain one of these complexes utilize ARF to initiate coat assembly onto the donor membrane. As discussed previously, ARF also initiates assembly of COPI coats. The additional features of the membrane or protein factors that determine which type of coat will assemble after ARF attachment are not well understood at this time.

Vesicles that bud from the *trans*-Golgi network en route to the lysosome by way of the late endosome have clathrin coats associated with either AP1 or GGA. Both AP1 and GGA bind to the cytosolic domain of cargo proteins in the donor membrane, but the functional differences between vesicles that contain AP1 or GGA are unclear. Recent studies have shown that membrane proteins containing a Tyr-X-X-Φ sequence, where X is any amino acid and Φ is a bulky hydrophobic amino acid, are recruited into clathrin/AP1 vesicles budding from the *trans*-Golgi network. This YXXΦ *sorting signal* interacts with one of the AP1 subunits in the vesicle coat. As we discuss in the next section, vesicles with clathrin/AP2 coats, which bud from the plasma membrane during endocytosis, also can recognize the YXXΦ sorting signal.

Some vesicles that bud from the *trans*-Golgi network have coats composed of the AP3 complex. These vesicles mediate trafficking to the lysosome, but they appear to bypass the late endosome and fuse directly with the lysosomal membrane. In certain types of cells, such AP3 vesicles mediate protein transport to specialized storage compartments related to the lysosome. For example, AP3 is required for delivery of proteins to melanosomes, which contain the black pigment melanin in skin cells, and to platelet storage vesicles in megakaryocytes, a large cell that fragments into dozens of platelets. Mice with mutations in either of two different subunits of AP3 not only have abnormal skin pigmentation but also exhibit bleeding disorders. The latter occur because tears in blood vessels cannot be repaired without platelets that contain normal storage vesicles.

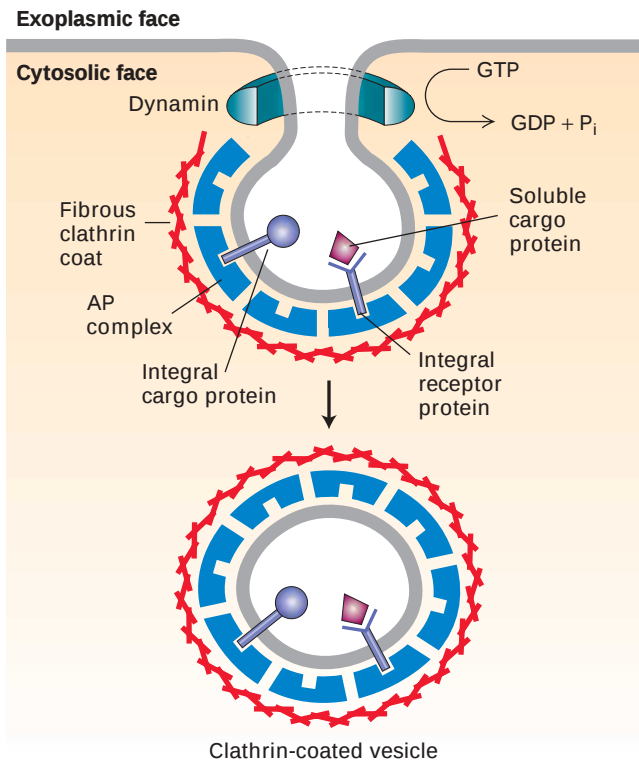


▲ **FIGURE 17-19 Structure of clathrin coats.** (a) A clathrin molecule, called a triskelion, is composed of three heavy and three light chains. It has an intrinsic curvature due to the bend in the heavy chains. (b) The fibrous clathrin coat around vesicles is constructed of 36 clathrin triskelions. Depicted here is an intermediate in assembly of a clathrin coat, containing 10 of the final 36 triskelions, which illustrates the intrinsic curvature and the packing of clathrin triskelions. (c) Clathrin coats were formed *in vitro* by mixing purified clathrin heavy and light chains with AP2 complexes in the absence of membranes. Cryoelectron micrographs of more than 1000 assembled particles were analyzed by digital image processing to generate an average structural representation. The left image shows the reconstructed structure of a complete particle with AP2 complexes packed into the interior of the clathrin cage. In the right image, the AP2 complexes have been subtracted to show only the assembled clathrin heavy and light chains. [See B. Pishvaei and G. Payne, 1998, *Cell* 95:443. Part (c) from Corinne J. Smith, Department of Biological Sciences, University of Warwick.]

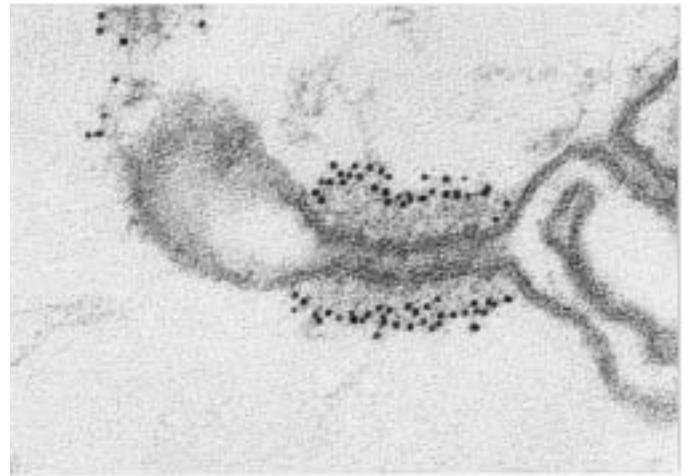
Dynamin Is Required for Pinching Off of Clathrin Vesicles

A fundamental step in the formation of a transport vesicle that we have not yet considered is how a vesicle bud is pinched off from the donor membrane. In the case of clathrin/AP-coated vesicles, a cytosolic protein called *dynamin* is essential for release of complete vesicles. At the later stages of bud formation, dynamin polymerizes around the neck portion and then hydrolyzes GTP. The energy derived from GTP hydrolysis is thought to drive “contraction” of dynamin around the vesicle neck until the vesicle pinches off (Figure 17-20). Interestingly, COPI and COPII vesicles appear to pinch off from donor membranes without the aid of a GTPase such as dynamin. At present this fundamental difference in the process of pinching off among the different types of vesicles is not understood.

Incubation of cell extracts with a nonhydrolyzable derivative of GTP provides dramatic evidence for the importance of dynamin in pinching off of clathrin/AP vesicles during endocytosis. Such treatment leads to accumulation of clathrin-coated vesicle buds with excessively long necks that are



▲ **FIGURE 17-20 Model for dynamin-mediated pinching off of clathrin/AP-coated vesicles.** After a vesicle bud forms, dynamin polymerizes over the neck. By a mechanism that is not well understood, dynamin-catalyzed hydrolysis of GTP leads to release of the vesicle from the donor membrane. Note that membrane proteins in the donor membrane are incorporated into vesicles by interacting with AP complexes in the coat. [Adapted from K. Takel et al., 1995, *Nature* **374**:186.]



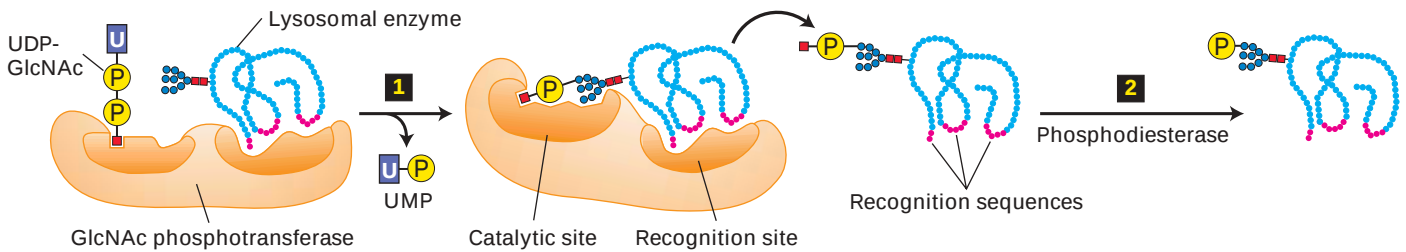
▲ **EXPERIMENTAL FIGURE 17-21 GTP hydrolysis by dynamin is required for pinching off of clathrin-coated vesicles in cell-free extracts.** A preparation of nerve terminals, which undergo extensive endocytosis, was lysed by treatment with distilled water and incubated with GTP- γ -S, a nonhydrolyzable derivative of GTP. After sectioning, the preparation was treated with gold-tagged anti-dynamin antibody and viewed in the electron microscope. This image, which shows a long-necked clathrin/AP-coated bud with polymerized dynamin lining the neck, reveals that buds can form in the absence of GTP hydrolysis, but vesicles cannot pinch off. The extensive polymerization of dynamin that occurs in the presence of GTP- γ -S probably does not occur during the normal budding process. [From K. Takel et al., 1995, *Nature* **374**:186; courtesy of Pietro De Camilli.]

surrounded by polymeric dynamin but do not pinch off (Figure 17-21). Likewise, cells expressing mutant forms of dynamin that cannot bind GTP do not form clathrin-coated vesicles, and instead accumulate similar long-necked vesicle buds encased with polymerized dynamin.

As with COPI and COPII vesicles, clathrin/AP vesicles normally lose their coat soon after their formation. Cytosolic Hsc70, a constitutive chaperone protein found in all eukaryotic cells, is thought to use energy derived from the hydrolysis of ATP to drive depolymerization of the clathrin coat into triskelions. Uncoating not only releases triskelions for reuse in the formation of additional vesicles, but also exposes v-SNAREs for use in fusion with target membranes. Conformational changes that occur when ARF switches from the GTP-bound to GDP-bound state are thought to regulate the timing of clathrin coat depolymerization. How the action of Hsc70 might be coupled to ARF switching is not well understood.

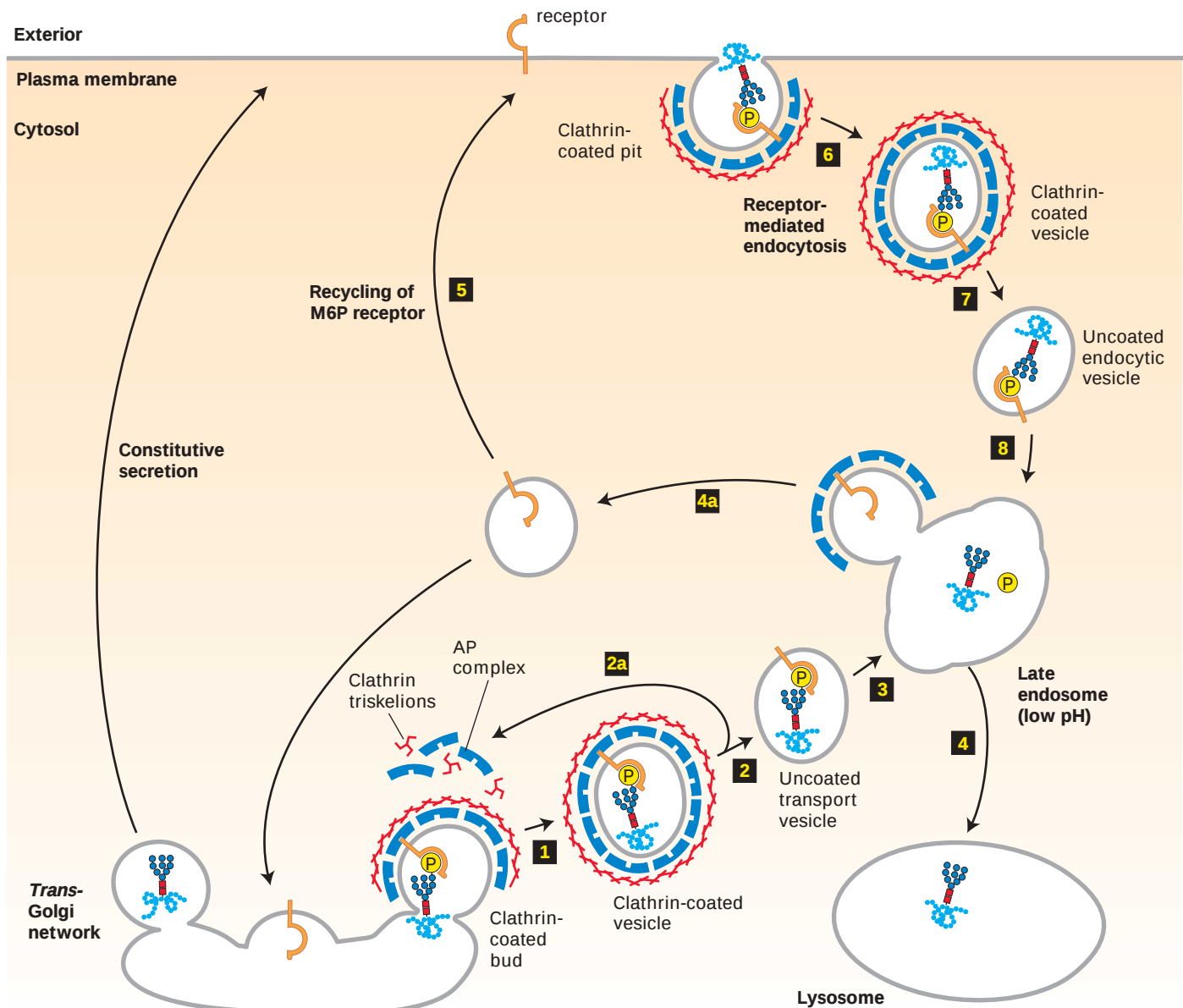
Mannose 6-Phosphate Residues Target Soluble Proteins to Lysosomes

Most of the sorting signals that function in vesicular trafficking are short amino acid sequences in the targeted protein. In contrast, the sorting signal that directs soluble



▲ **FIGURE 17-22 Formation of mannose 6-phosphate (M6P) residues that target soluble enzymes to lysosomes.** The M6P residues that direct proteins to lysosomes are generated in the *cis*-Golgi by two Golgi-resident enzymes. Step **1**: An *N*-acetylglucosamine (GlcNAc) phosphotransferase transfers a phosphorylated GlcNAc group to carbon atom 6 of one or more mannose residues. Because only lysosomal enzymes contain

sequences (red) that are recognized and bound by this enzyme, phosphorylated GlcNAc groups are added specifically to lysosomal enzymes. Step **2**: After release of a modified protein from the phosphotransferase, a phosphodiesterase removes the GlcNAc group, leaving a phosphorylated mannose residue on the lysosomal enzyme. [See A. B. Cantor et al., 1992, *J. Biol. Chem.* **267**:23349, and S. Kornfeld, 1987, *FASEB J.* **1**:462.]



lysosomal enzymes from the *trans*-Golgi network to the late endosome is a carbohydrate residue, *mannose 6-phosphate* (M6P), which is formed in the *cis*-Golgi. The addition and initial processing of one or more preformed *N*-linked oligosaccharide precursors in the rough ER is the same for lysosomal enzymes as for membrane and secreted proteins, yielding core Man₈(GlcNAc)₂ chains (see Figure 16-18). In the *cis*-Golgi, the *N*-linked oligosaccharides present on most lysosomal enzymes undergo a two-step reaction sequence that generates M6P residues (Figure 17-22). The addition of M6P residues to the oligosaccharide chains of soluble lysosomal enzymes prevents these proteins from undergoing the further processing reactions characteristic of secreted and membrane proteins (see Figure 17-3).

As shown in Figure 17-23, the segregation of M6P-bearing lysosomal enzymes from secreted and membrane proteins occurs in the *trans*-Golgi network. Here transmembrane *mannose 6-phosphate receptors* bind the M6P residues on lysosome-destined proteins very tightly and specifically. Clathrin/AP1 vesicles containing the M6P receptor and bound lysosomal enzymes then bud from the *trans*-Golgi network, lose their coats, and subsequently fuse with the late endosome by mechanisms described previously. Because M6P receptors can bind M6P at the slightly acidic pH (≈ 6.5) of the *trans*-Golgi network but not at a pH less than 6, the bound lysosomal enzymes are released within late endosomes, which have an internal pH of 5.0–5.5. Furthermore, a phosphatase within late endosomes usually removes the phosphate from M6P residues on lysosomal enzymes, preventing any rebinding to the M6P receptor that might occur in spite of the low pH in endosomes. Vesicles budding from late endosomes recycle the M6P receptor back to the *trans*-

Golgi network or, on occasion, to the cell surface. Eventually, mature late endosomes fuse with lysosomes, delivering the lysosomal enzymes to their final destination.

The sorting of soluble lysosomal enzymes in the *trans*-Golgi network (see Figure 17-23, steps 1–4) shares many of the features of trafficking between the ER and *cis*-Golgi compartments mediated by COPII and COPI vesicles. First, mannose 6-phosphate acts as a sorting signal by interacting with the luminal domain of a receptor protein in the donor membrane. Second, the membrane-embedded receptors with their bound ligands are incorporated into the appropriate vesicles—in this case, AP1-containing clathrin vesicles—by interacting with the vesicle coat. Third, these transport vesicles fuse only with one specific organelle, here the late endosome, as the result of interactions between specific v-SNAREs and t-SNAREs. And finally, intracellular transport receptors are recycled after dissociating from their bound ligand.

Study of Lysosomal Storage Diseases Revealed Key Components of the Lysosomal Sorting Pathway



A group of genetic disorders, termed *lysosomal storage diseases*, are caused by the absence of one or more lysosomal enzymes. As a result, undigested glycolipids and extracellular components that would normally be degraded by lysosomal enzymes accumulate in lysosomes as large inclusions. *I-cell disease* is a particularly severe type of lysosomal storage disease in which multiple enzymes are missing from the lysosomes. Cells from affected individuals lack the *N*-acetylglucosamine phosphotransferase that is required for formation of M6P residues on lysosomal enzymes in the *cis*-Golgi (see Figure 17-22). Biochemical comparison of lysosomal enzymes from normal individuals with those from patients with I-cell disease led to the initial discovery of mannose 6-phosphate as the lysosomal sorting signal. Lacking the M6P sorting signal, the lysosomal enzymes in I-cell patients are secreted rather than being sorted to and sequestered in lysosomes.

When fibroblasts from patients with I-cell disease are grown in a medium containing lysosomal enzymes bearing M6P residues, the diseased cells acquire a nearly normal intracellular content of lysosomal enzymes. This finding indicates that the plasma membrane of these cells contain M6P receptors, which can internalize extracellular phosphorylated lysosomal enzymes by receptor-mediated endocytosis. This process, used by many cell-surface receptors to bring bound proteins or particles into the cell, is discussed in detail in the next section. It is now known that even in normal cells, some M6P receptors are transported to the plasma membrane and some phosphorylated lysosomal enzymes are secreted (see Figure 17-23). The secreted enzymes can be retrieved by receptor-mediated endocytosis and directed to lysosomes. This pathway thus scavenges any lysosomal enzymes that escape the usual M6P sorting pathway.

◀ FIGURE 17-23 Trafficking of soluble lysosomal enzymes from the *trans*-Golgi network and cell surface to lysosomes.

Newly synthesized lysosomal enzymes, produced in the ER, acquire mannose 6-phosphate (M6P) residues in the *cis*-Golgi (see Figure 17-22). For simplicity, only one phosphorylated oligosaccharide chain is depicted, although lysosomal enzymes typically have many such chains. In the *trans*-Golgi network, proteins that bear the M6P sorting signal interact with M6P receptors in the membrane and thereby are directed into clathrin/AP1 vesicles (step 1). The coat surrounding released vesicles is rapidly depolymerized (step 2), and the uncoated transport vesicles fuse with late endosomes (step 3). After the phosphorylated enzymes dissociate from the M6P receptors and are dephosphorylated, late endosomes subsequently fuse with a lysosome (step 4). Note that coat proteins and M6P receptors are recycled (steps 2a and 4a), and some receptors are delivered to the cell surface (step 5). Phosphorylated lysosomal enzymes occasionally are sorted from the *trans*-Golgi to the cell surface and secreted. These secreted enzymes can be retrieved by receptor-mediated endocytosis (steps 6–8), a process that closely parallels trafficking of lysosomal enzymes from the *trans*-Golgi network to lysosomes. [See G. Griffiths et al., 1988, *Cell* 52:329; S. Kornfeld, 1992, *Ann. Rev. Biochem.* 61:307; and G. Griffiths and J. Gruenberg, 1991, *Trends Cell Biol.* 1:5.]

Hepatocytes from patients with I-cell disease contain a normal complement of lysosomal enzymes and no inclusions, even though these cells are defective in mannose phosphorylation. This finding implies that hepatocytes (the most abundant type of liver cell) employ a M6P-independent pathway for sorting lysosomal enzymes. The nature of this pathway, which also may operate in other cells types, is unknown. ■

Protein Aggregation in the *Trans*-Golgi May Function in Sorting Proteins to Regulated Secretory Vesicles

As noted in the chapter introduction, all eukaryotic cells continuously secrete certain proteins, a process commonly called *constitutive secretion*. Specialized secretory cells also store other proteins in vesicles and secrete them only when triggered by a specific stimulus. One example of such *regulated secretion* occurs in pancreatic β cells, which store newly made insulin in special secretory vesicles and secrete insulin in response to an elevation in blood glucose (see Figure 15-7). These and other secretory cells simultaneously utilize two different types of vesicles to move proteins from the *trans*-Golgi network to the cell surface: regulated transport vesicles, often simply called secretory vesicles, and unregulated transport vesicles, also called constitutive secretory vesicles.

A common mechanism appears to sort regulated proteins as diverse as ACTH (adrenocorticotrophic hormone), insulin, and trypsinogen into regulated secretory vesicles. Evidence for a common mechanism comes from experiments in which recombinant DNA techniques are used to induce the synthesis of insulin and trypsinogen in pituitary tumor cells already synthesizing ACTH. In these cells all three proteins segregate into the same regulated secretory vesicles and are secreted together when a hormone binds to a receptor on the pituitary cells and causes a rise in cytosolic Ca^{2+} . Although these three proteins share no identical amino acid sequences that might serve as a sorting sequence, they obviously have some common feature that signals their incorporation into regulated secretory vesicles.

Morphologic evidence suggests that sorting into the regulated pathway is controlled by selective protein aggregation. For instance, immature vesicles in this pathway—those that have just budded from the *trans*-Golgi network—contain diffuse aggregates of secreted protein that are visible in the electron microscope. These aggregates also are found in vesicles that are in the process of budding, indicating that proteins destined for regulated secretory vesicles selectively aggregate together before their incorporation into the vesicles.

Other studies have shown that regulated secretory vesicles from mammalian secretory cells contain three proteins, *chromogranin A*, *chromogranin B*, and *secretogranin II*, that together form aggregates when incubated at the ionic conditions ($\text{pH} \approx 6.5$ and $1 \text{ mM } \text{Ca}^{2+}$) thought to occur in the *trans*-Golgi network; such aggregates do not form at the neutral pH of the ER. The selective aggregation of regulated secreted proteins together with chromogranin A, chromogranin B, or secretogranin II could be the basis for sorting of these proteins into

regulated secretory vesicles. Secreted proteins that do not associate with these proteins, and thus do not form aggregates, would be sorted into unregulated transport vesicles by default.

Some Proteins Undergo Proteolytic Processing After Leaving the *Trans*-Golgi

For some secretory proteins (e.g., growth hormone) and certain viral membrane proteins (e.g., the VSV glycoprotein), removal of the N-terminal ER signal sequence from the nascent chain is the only known proteolytic cleavage required to convert the polypeptide to the mature, active species (see Figure 16-6). However, some membrane and many soluble secretory proteins initially are synthesized as relatively long-lived, inactive precursors, termed *proproteins*, that require further proteolytic processing to generate the mature, active proteins. Examples of proteins that undergo such processing are soluble lysosomal enzymes, many membrane proteins such as influenza hemagglutinin (HA), and secreted proteins such as serum albumin, insulin, glucagon, and the yeast α mating factor. In general, the proteolytic conversion of a proprotein to the corresponding mature protein occurs after the proprotein has been sorted in the *trans*-Golgi network to appropriate vesicles.

In the case of soluble lysosomal enzymes, the proproteins are called *proenzymes*, which are sorted by the M6P receptor as catalytically inactive enzymes. In the late endosome or lysosome a proenzyme undergoes a proteolytic cleavage that generates a smaller but enzymatically active polypeptide. Delaying the activation of lysosomal proenzymes until they reach the lysosome prevents them from digesting macromolecules in earlier compartments of the secretory pathway.

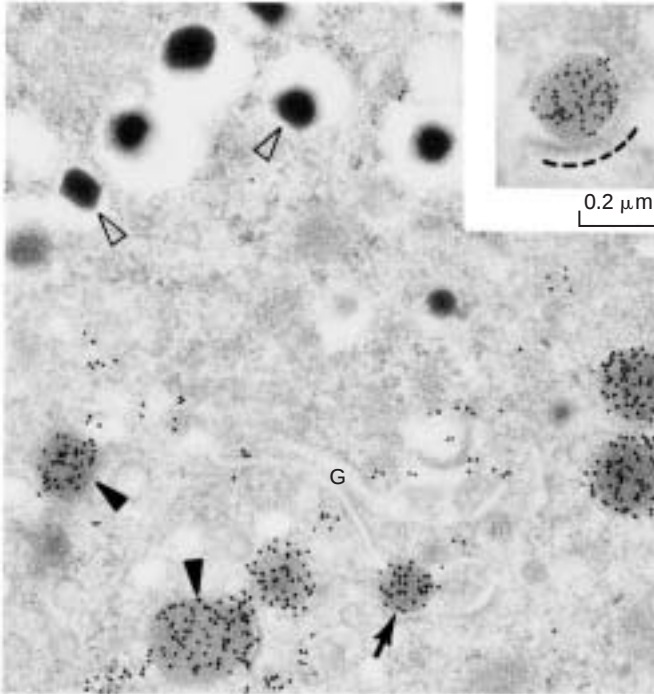
Normally, mature vesicles carrying secreted proteins to the cell surface are formed by fusion of several immature

► EXPERIMENTAL FIGURE 17-24 Proteolytic cleavage of proinsulin occurs in secretory vesicles after they have budded from the *trans*-Golgi network.

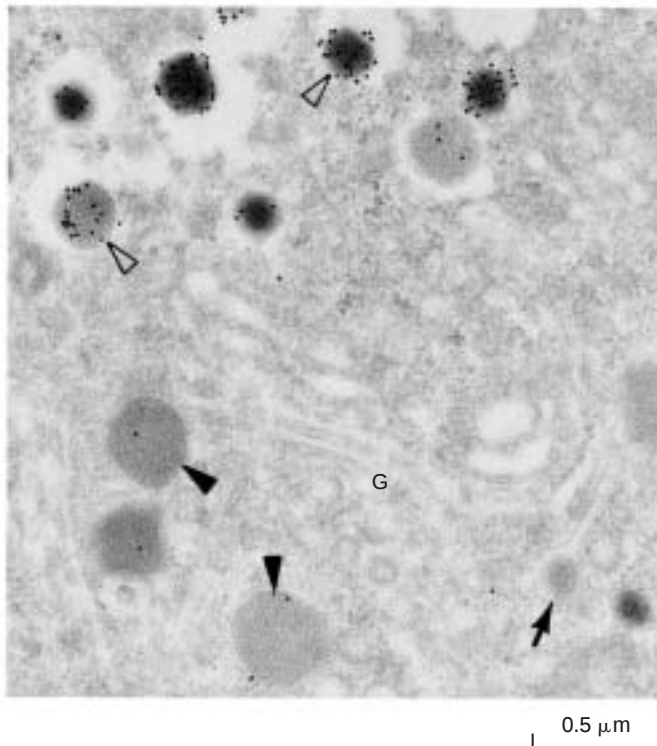
Serial sections of the Golgi region of an insulin-secreting cell were stained with (a) a monoclonal antibody that recognizes proinsulin but not insulin or (b) a different antibody that recognizes insulin but not proinsulin. The antibodies, which were bound to electron-opaque gold particles, appear as dark dots in these electron micrographs (see Figure 5-51). Immature secretory vesicles (closed arrowheads) and vesicles budding from the *trans*-Golgi (arrows) stain with the proinsulin antibody but not with insulin antibody. These vesicles contain diffuse protein aggregates that include proinsulin and other regulated secreted proteins. Mature vesicles (open arrowheads) stain with insulin antibody but not with proinsulin antibody and have a dense core of almost crystalline insulin. Since budding and immature secretory vesicles contain proinsulin (not insulin), the proteolytic conversion of proinsulin to insulin must take place in these vesicles after they bud from the *trans*-Golgi network. The inset in (a) shows a proinsulin-rich secretory vesicle surrounded by a protein coat (dashed line). [From L. Orci et al., 1987, *Cell* 49:865; courtesy of L. Orci.]

ones containing proprotein. Proteolytic cleavage of proproteins, such as proinsulin, occurs in vesicles after they move away from the *trans*-Golgi network (Figure 17-24). The proproteins of most constitutively secreted proteins (e.g., albumin) are cleaved only once at a site C-terminal to a dibasic

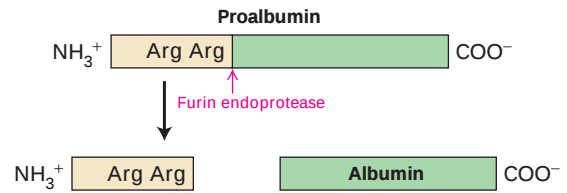
(a) Proinsulin antibody



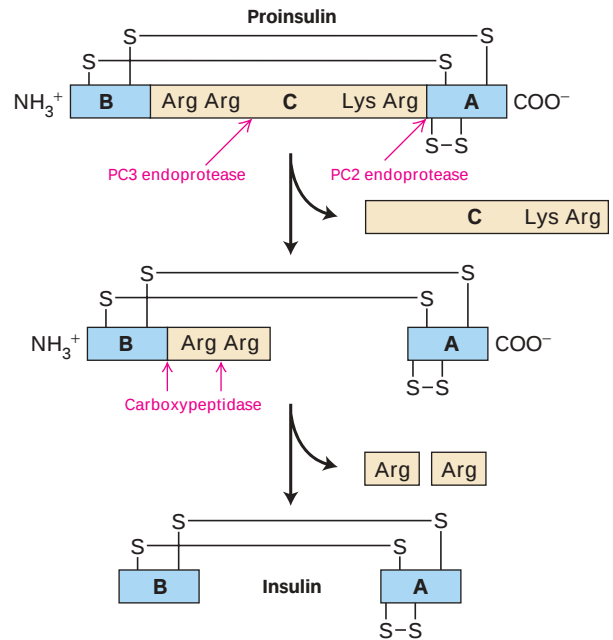
(b) Insulin antibody



(a) Constitutive secreted proteins



(b) Regulated secreted proteins



▲ FIGURE 17-25 Proteolytic processing of proproteins in the constitutive and regulated secretion pathways. The processing of proalbumin and proinsulin is typical of the constitutive and regulated pathways, respectively. The endoproteases that function in such processing cleave C-terminal to sequences of two consecutive basic amino acids. (a) The endoprotease furin acts on the precursors of constitutive secreted proteins. (b) Two endoproteases, PC2 and PC3, act on the precursors of regulated secreted proteins. The final processing of many such proteins is catalyzed by a carboxypeptidase that sequentially removes two basic amino acid residues at the C-terminus of a polypeptide. [See D. Steiner et al., 1992, *J. Biol. Chem.* **267**:23435.]

recognition sequence such as Arg-Arg or Lys-Arg (Figure 17-25a). Proteolytic processing of proteins whose secretion is regulated generally entails additional cleavages. In the case of proinsulin, multiple cleavages of the single polypeptide chain yields the N-terminal B chain and the C-terminal A chain of mature insulin, which are linked by disulfide bonds, and the central C peptide, which is lost and subsequently degraded (Figure 17-25b).

The breakthrough in identifying the proteases responsible for such processing of secreted proteins came from analysis

of yeast with a mutation in the *KEX2* gene. These mutant cells synthesized the precursor of the α mating factor but could not proteolytically process it to the functional form, and thus were unable to mate with cells of the opposite mating type (see Figure 22-13). The wild-type *KEX2* gene encodes an endoprotease that cleaves the α -factor precursor at a site C-terminal to Arg-Arg and Lys-Arg residues. Using the *KEX2* gene as a DNA probe, researchers were able to clone a family of mammalian endoproteases, all of which cleave a protein chain on the C-terminal side of an Arg-Arg or Lys-Arg sequence. One, called *furin*, is found in all mammalian cells; it processes proteins such as albumin that are secreted by the continuous pathway. In contrast, the *PC2* and *PC3* endoproteases are found only in cells that exhibit regulated secretion; these enzymes are localized to regulated secretory vesicles and proteolytically cleave the precursors of many hormones at specific sites.

Several Pathways Sort Membrane Proteins to the Apical or Basolateral Region of Polarized Cells

The plasma membrane of polarized epithelial cells is divided into two domains, **apical** and **basolateral**; tight junctions located between the two domains prevent the movement of plasma-membrane proteins between the domains (see Figure 6-5). Several sorting mechanisms direct newly synthesized membrane proteins to either the apical or basolateral domain of epithelial cells, and any one protein may be sorted by more than one mechanism. Although these sorting mechanisms are understood in general terms, the molecular signals underlying the vesicle-mediated transport of membrane proteins in polarized cells are not yet

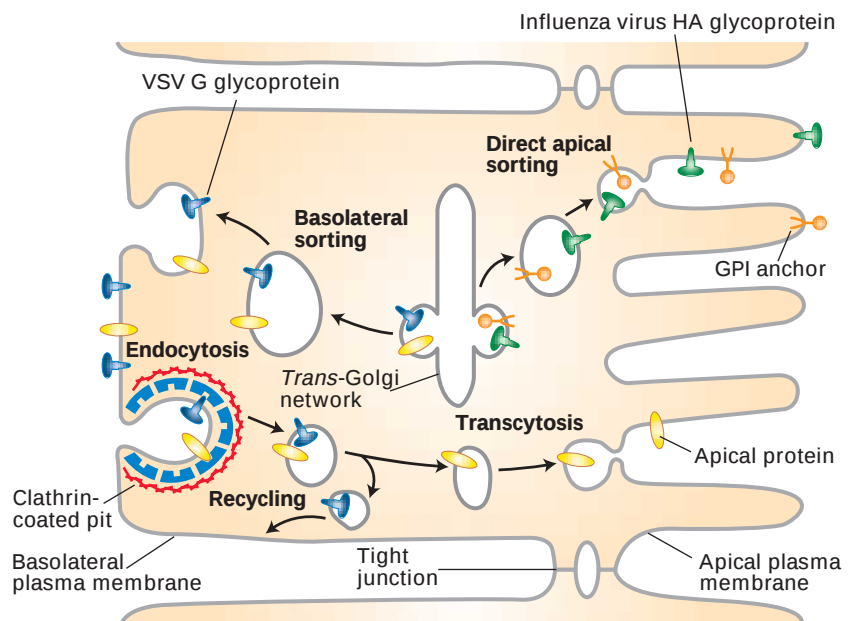
known. As a result of this sorting and the restriction on protein movement within the plasma membrane due to tight junctions, distinct sets of proteins are found in the apical or basolateral domain. This preferential localization of certain transport proteins is critical to a variety of important physiological functions, such as absorption of nutrients from the intestinal lumen and acidification of the stomach lumen (see Figures 7-27 and 7-28).

Microscopic and cell-fractionation studies indicate that proteins destined for either the apical or the basolateral membranes are initially located together within the membranes of the *trans*-Golgi network. In some cases, proteins destined for the apical membrane are sorted into their own transport vesicles that bud from the *trans*-Golgi network and then move to the apical region, whereas proteins destined for the basolateral membrane are sorted into other vesicles that move to the basolateral region. The different vesicle types can be distinguished by their protein constituents, including distinct Rab and v-SNARE proteins, which apparently target them to the appropriate plasma-membrane domain. In this mechanism, segregation of proteins destined for either the apical or basolateral membranes occurs as cargo proteins are incorporated into particular types of vesicles budding from the *trans*-Golgi network.

Such direct basolateral-apical sorting has been investigated in cultured Madin-Darby canine kidney (MDCK) cells, a line of cultured polarized epithelial cells (see Figure 6-6). In MDCK cells infected with the influenza virus, progeny viruses bud only from the apical membrane, whereas in cells infected with vesicular stomatitis virus (VSV), progeny viruses bud only from the basolateral membrane. This difference occurs because the HA glycoprotein of influenza

► FIGURE 17-26 Sorting of proteins destined for the apical and basolateral plasma membranes of polarized cells.

When cultured MDCK cells are infected simultaneously with VSV and influenza virus, the VSV G glycoprotein (purple) is found only on the basolateral membrane, whereas the influenza HA glycoprotein (green) is found only on the apical membrane. Some cellular proteins (orange circle), especially those with a GPI anchor, are likewise sorted directly to the apical membrane and others to the basolateral membrane (not shown) via specific transport vesicles that bud from the *trans*-Golgi network. In certain polarized cells, some apical and basolateral proteins are transported together to the basolateral surface; the apical proteins (orange oval) then move selectively, by endocytosis and transcytosis, to the apical membrane. [After K. Simons and A. Wandinger-Ness, 1990, *Cell* 62:207, and K. Mostov et al., 1992, *J. Cell Biol.* 116:577.]



virus is transported from the Golgi complex exclusively to the apical membrane, and the VSV G protein is transported only to the basolateral membrane (Figure 17-26). Furthermore, when the gene encoding HA protein is introduced into uninfected cells by recombinant DNA techniques, all the expressed HA accumulates in the apical membrane, indicating that the sorting signal resides in the HA glycoprotein itself and not in other viral proteins produced during viral infection.

Among the cellular proteins that undergo similar apical-basolateral sorting in the Golgi are those with a *glycosylphosphatidylinositol (GPI) membrane anchor*. In MDCK cells and most other types of epithelial cells, GPI-anchored proteins are targeted to the apical membrane. In membranes GPI-anchored proteins are clustered into **lipid rafts**, which are rich in sphingolipids (see Figure 5-10). This finding suggests that lipid rafts are localized to the apical membrane along with proteins that preferentially partition into them in many cells. However, the GPI anchor is not an apical sorting signal in all polarized cells; in thyroid cells, for example, GPI-anchored proteins are targeted to the basolateral membrane. Other than GPI anchors no unique sequences have been identified that are both necessary and sufficient to target proteins to either the apical or basolateral domain. Instead, each membrane protein may contain multiple sorting signals, any one of which can target it to the appropriate plasma-membrane domain. The identification of such complex signals and of the vesicle coat proteins that recognize them is currently being pursued for a number of different proteins that are sorted to specific plasma-membrane domains of polarized epithelial cells.

Another mechanism for sorting apical and basolateral proteins, also illustrated in Figure 17-26, operates in hepatocytes. The basolateral membranes of hepatocytes face the blood (as in intestinal epithelial cells), and the apical membranes line the small intercellular channels into which bile is secreted. In hepatocytes, newly made apical and basolateral proteins are first transported in vesicles from the *trans*-Golgi network to the basolateral region and incorporated into the plasma membrane by exocytosis (i.e., fusion of the vesicle membrane with the plasma membrane). From there, both basolateral and apical proteins are endocytosed in the same vesicles, but then their paths diverge. The endocytosed basolateral proteins are sorted into transport vesicles that recycle them to the basolateral membrane. In contrast, the apically destined endocytosed proteins are sorted into transport vesicles that move across the cell and fuse with the apical membrane, a process called **transcytosis**. As discussed in the next section, transcytosis also is used to move extracellular materials from one side of an epithelium to another. Even in epithelial cells, such as MDCK cells, in which apical-basolateral protein sorting occurs in the Golgi, transcytosis may provide a “fail-safe” sorting mechanism. That is, an apical protein sorted incorrectly to the basolateral membrane would be subjected to endocytosis and then correctly delivered to the apical membrane.

KEY CONCEPTS OF SECTION 17.4

Protein Sorting and Processing in Later Stages of the Secretory Pathway

- The *trans*-Golgi network (TGN) is a major branch point in the secretory pathway where soluble secreted proteins, lysosomal proteins, and in some cells membrane proteins destined for the basolateral or apical plasma membrane are segregated into different transport vesicles.
- Many vesicles that bud from the *trans*-Golgi network as well as endocytic vesicles bear a coat composed of AP (adapter protein) complexes and clathrin (see Figure 17-19).
- Pinching off of clathrin-coated vesicles requires dynamin, which forms a collar around the neck of the vesicle bud and hydrolyzes GTP (see Figure 17-20).
- Soluble enzymes destined for lysosomes are modified in the *cis*-Golgi yielding multiple mannose 6-phosphate (M6P) residues on their oligosaccharide chains.
- M6P receptors in the membrane of the *trans*-Golgi network bind proteins bearing M6P residues and direct their transfer to late endosomes, where receptors and their ligands dissociate. The receptors then are recycled to the Golgi or plasma membrane, and the lysosomal enzymes are delivered to lysosomes (see Figure 17-23).
- Regulated secreted proteins are concentrated and stored in secretory vesicles to await a neural or hormonal signal for exocytosis. Protein aggregation within the *trans*-Golgi network may play a role in sorting secreted proteins to the regulated pathway.
- Many proteins transported through the secretory pathway undergo post-Golgi proteolytic cleavages that yield the mature, active proteins. Generally, proteolytic maturation can occur in vesicles carrying proteins from the *trans*-Golgi network to the cell surface, in the late endosome, or in the lysosomal.
- In polarized epithelial cells, membrane proteins destined for the apical or basolateral domains of the plasma membrane are sorted in the *trans*-Golgi network into different transport vesicles (see Figure 17-26). The GPI anchor is the only apical-basolateral sorting signal identified so far.
- In hepatocytes and some other polarized cells, all plasma-membrane proteins are directed first to the basolateral membrane. Apically destined proteins then are endocytosed and moved across the cell to the apical membrane (transcytosis).

17.5 Receptor-Mediated Endocytosis and the Sorting of Internalized Proteins

In previous sections we have explored the main pathways whereby secretory and membrane proteins synthesized on

the rough ER are delivered to the cell surface or other destinations. Cells also can internalize materials from their surroundings and sort these to particular destinations. A few cell types (e.g., macrophages) can take up whole bacteria and other large particles by **phagocytosis**, a nonselective actin-mediated process in which extensions of the plasma membrane envelop the ingested material, forming large vesicles called phagosomes (see Figure 5-20). In contrast, all eukaryotic cells continually engage in endocytosis, a process in which a small region of the plasma membrane invaginates to form a membrane-limited vesicle about 0.05–0.1 μm in diameter. In one form of endocytosis, called *pinocytosis*, small droplets of extracellular fluid and any material dissolved in it are nonspecifically taken up. Our focus in this section, however, is on **receptor-mediated endocytosis** in which a specific receptor on the cell surface binds tightly to an extracellular macromolecular ligand that it recognizes; the plasma-membrane region containing the receptor-ligand complex then buds inward and pinches off, becoming a transport vesicle.

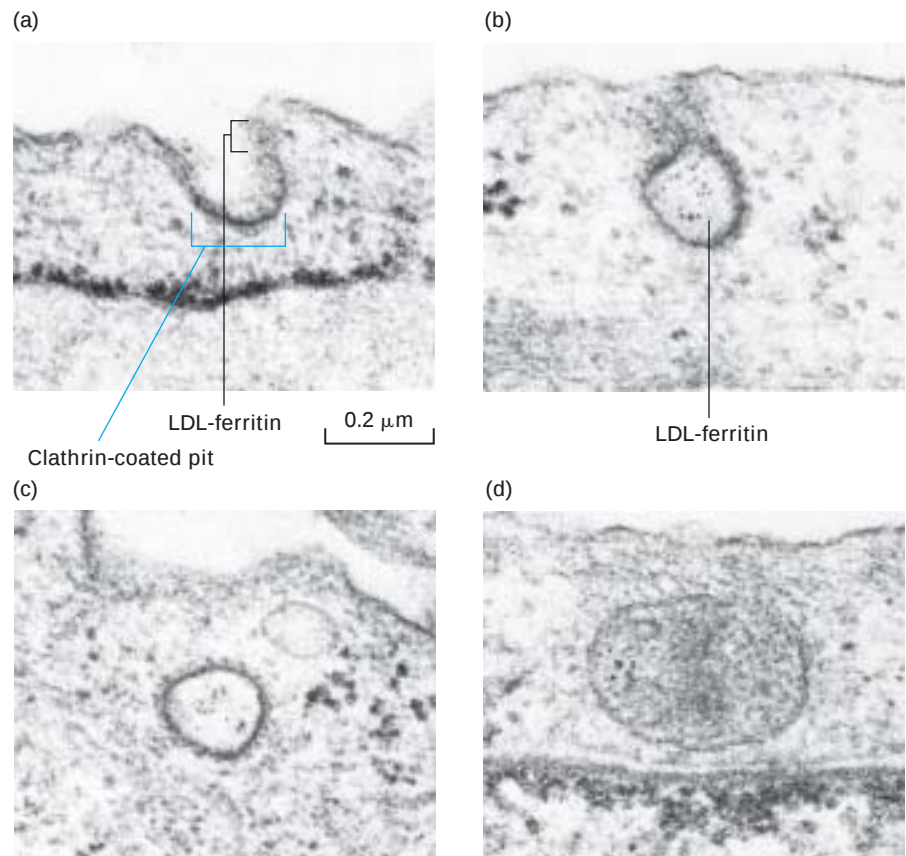
Among the common macromolecules that vertebrate cells internalize by receptor-mediated endocytosis are cholesterol-containing particles called low-density lipoprotein (LDL); the iron-binding protein transferrin; many protein hormones (e.g., insulin); and certain glycoproteins. Receptor-mediated endocytosis of such ligands generally occurs via clathrin/AP2-

coated pits and vesicles in a process similar to the packaging of lysosomal enzymes by mannose 6-phosphate (M6P) in the *trans*-Golgi network (see Figure 17-23). As noted earlier, some M6P receptors are found on the cell surface, and these participate in the receptor-mediated endocytosis of lysosomal enzymes that are secreted. In general, transmembrane receptor proteins that function in the uptake of extracellular ligands are internalized from the cell surface during endocytosis and are then sorted and recycled back to the cell surface, much like the recycling of M6P receptors to the plasma membrane and *trans*-Golgi. The rate at which a ligand is internalized is limited by the amount of its corresponding receptor on the cell surface.

Clathrin/AP2 pits make up about 2 percent of the surface of cells such as hepatocytes and fibroblasts. Many internalized ligands have been observed in these pits and vesicles, which are thought to function as intermediates in the endocytosis of most (though not all) ligands bound to cell-surface receptors (Figure 17-27). Some receptors are clustered over clathrin-coated pits even in the absence of ligand. Other receptors diffuse freely in the plane of the plasma membrane but undergo a conformational change when binding to ligand, so that when the receptor-ligand complex diffuses into a clathrin-coated pit, it is retained there. Two or more types of receptor-bound ligands, such as LDL and transferrin, can be seen in the same coated pit or vesicle.

► **EXPERIMENTAL FIGURE 17-27**
The initial stages of receptor-mediated endocytosis of low-density lipoprotein (LDL) particles are revealed by electron microscopy.

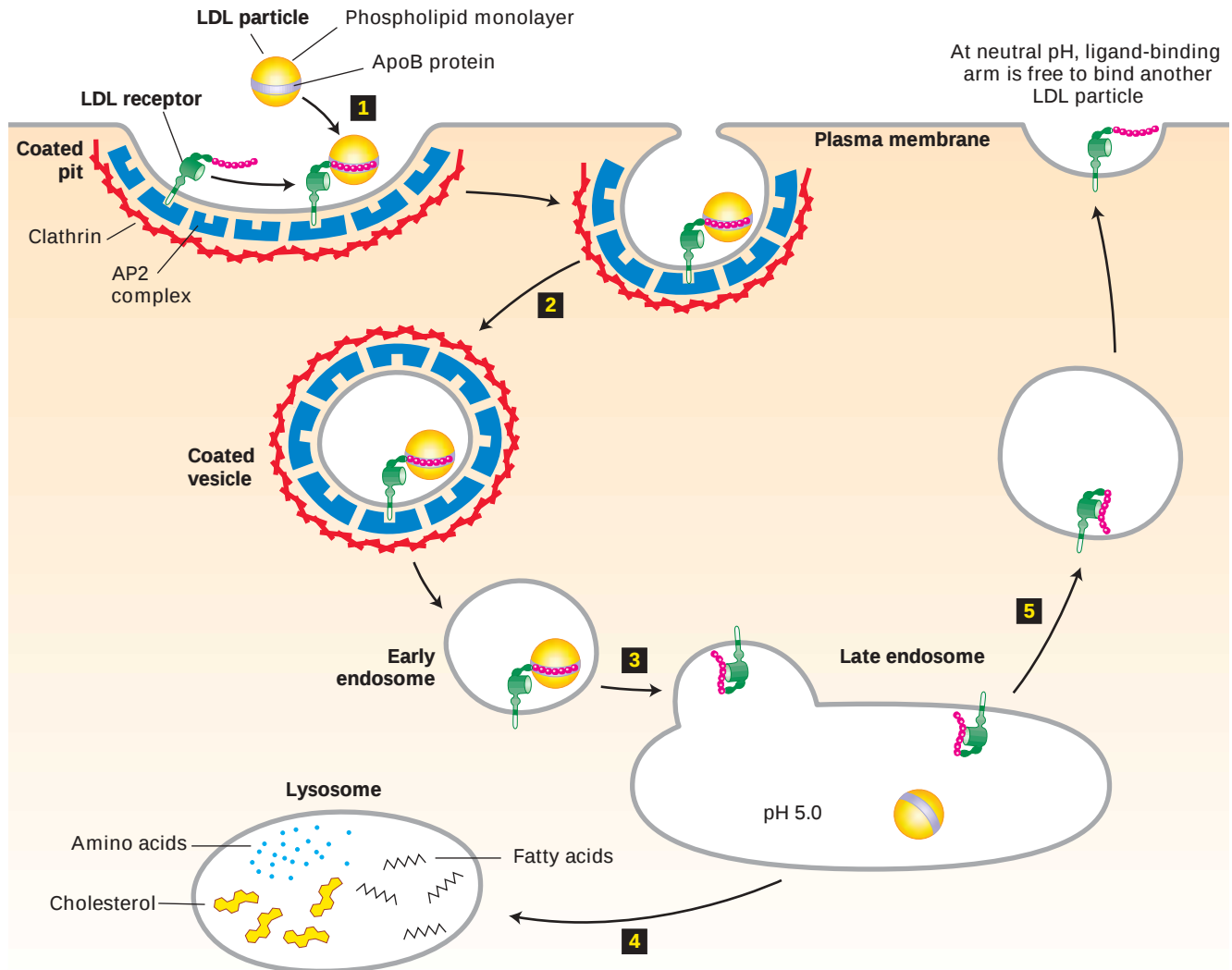
Cultured human fibroblasts were incubated in a medium containing LDL particles covalently linked to the electron-dense, iron-containing protein ferritin; each small iron particle in ferritin is visible as a small dot under the electron microscope. Cells initially were incubated at 4 °C; at this temperature LDL can bind to its receptor but internalization does not occur. After excess LDL not bound to the cells was washed away, the cells were warmed to 37 °C and then prepared for microscopy at periodic intervals. (a) A coated pit, showing the clathrin coat on the inner (cytosolic) surface of the pit, soon after the temperature was raised. (b) A pit containing LDL apparently closing on itself to form a coated vesicle. (c) A coated vesicle containing ferritin-tagged LDL particles. (d) Ferritin-tagged LDL particles in a smooth-surfaced early endosome 6 minutes after internalization began. [Photographs courtesy of R. Anderson. Reprinted by permission from J. Goldstein et al., *Nature* 279:679. Copyright 1979, Macmillan Journals Limited. See also M. S. Brown and J. Goldstein, 1986, *Science* 232:34.]



Receptors for Low-Density Lipoprotein and Other Ligands Contain Sorting Signals That Target Them for Endocytosis

As will be discussed in detail in the next chapter, **low-density lipoprotein (LDL)** is one of several complexes that carry cholesterol through the bloodstream (see Figure 17-28). A LDL particle, a sphere 20–25 nm in diameter, has an outer phospholipid shell containing a single molecule of a large

protein known as *apoB-100*; the core of a particle is packed with cholesterol in the form of cholesteryl esters (see Figure 18-12). Most mammalian cells produce cell-surface receptors that specifically bind to apoB-100 and internalize LDL particles by receptor-mediated endocytosis. After endocytosis, the LDL particles are transported to lysosomes via the endocytic pathway and then are degraded by lysosomal hydrolases. LDL receptors, which dissociate from their ligands in the late endosome, recycle to the cell surface.



▲ **FIGURE 17-28 Endocytic pathway for internalizing low-density lipoprotein (LDL).** Step **1**: Cell-surface LDL receptors bind to an apoB protein embedded in the phospholipid outer layer of LDL particles. Interaction between the NPXY sorting signal in the cytosolic tail of the LDL receptor and the AP2 complex incorporates the receptor-ligand complex into forming endocytic vesicles. Step **2**: Clathrin-coated pits (or buds) containing receptor-LDL complexes are pinched off by the same dynamin-mediated mechanism used to form clathrin/AP1 vesicles on the *trans*-Golgi network (see Figure 17-20). Step **3**: After the vesicle coat is shed, the uncoated endocytic vesicle (early

endosome) fuses with the late endosome. The acidic pH in this compartment causes a conformational change in the LDL receptor that leads to release of the bound LDL particle. Step **4**: The late endosome fuses with the lysosome, and the proteins and lipids of the free LDL particle are broken down to their constituent parts by enzymes in the lysosome. Step **5**: The LDL receptor recycles to the cell surface where at the neutral pH of the exterior medium the receptor undergoes a conformational change so that it can bind another LDL particle. [See M. S. Brown and J. L. Goldstein, 1986, *Science* **232**:34, and G. Rudenko et al., 2002, *Science* **298**:2353.]

Studies of the inherited disorder *familial hypercholesterolemia* led to discovery of the LDL receptor and the initial understanding of the endocytic pathway. An individual with this disorder produces one of several mutant forms of the LDL receptor, causing impaired endocytosis of LDL and high serum levels of cholesterol (Chapter 18). The major features of the LDL endocytic pathway as currently understood are depicted in Figure 17-28. The LDL receptor is an 839-residue glycoprotein with a single transmembrane segment; it has a short C-terminal cytosolic segment and a long N-terminal exoplasmic segment that contains a β -propeller domain and a ligand-binding domain. Seven cysteine-rich imperfect repeats form the ligand-binding domain, which interacts with the apoB-100 molecule in a LDL particle.



Mutant receptors from some individuals with familial hypercholesterolemia bind LDL normally, but the LDL-receptor complex cannot be internalized by the cell and is distributed evenly over the cell surface rather than being confined to clathrin/AP2-coated pits. In individuals with this type of defect, plasma-membrane receptors for other ligands are internalized normally, but the mutant LDL receptor apparently is not recruited into coated pits. Analysis of this mutant receptor and other mutant LDL receptors generated experimentally and expressed in fibroblasts identified a four-residue motif in the cytosolic segment of the receptor that is crucial for its internalization: Asn-Pro-X-Tyr where X can be any amino acid. This *NPXY sorting signal* binds to the AP2 complex, linking the clathrin/AP2 coat to the cytosolic segment of the LDL receptor in forming coated pits. A mutation in any of the conserved residues of the NPXY signal will abolish the ability of the LDL receptor to be incorporated into coated pits.

A small number of individuals who exhibit the usual symptoms associated with familial hypercholesterolemia produce normal LDL receptors. In these individuals, the gene encoding the AP2 subunit protein that binds the NPXY sorting signal is defective. As a result, LDL receptors are not incorporated into clathrin/AP2 vesicles and endocytosis of LDL particles is compromised. Analysis of patients with this genetic disorder highlights the importance of adapter proteins in protein trafficking mediated by clathrin vesicles. ■

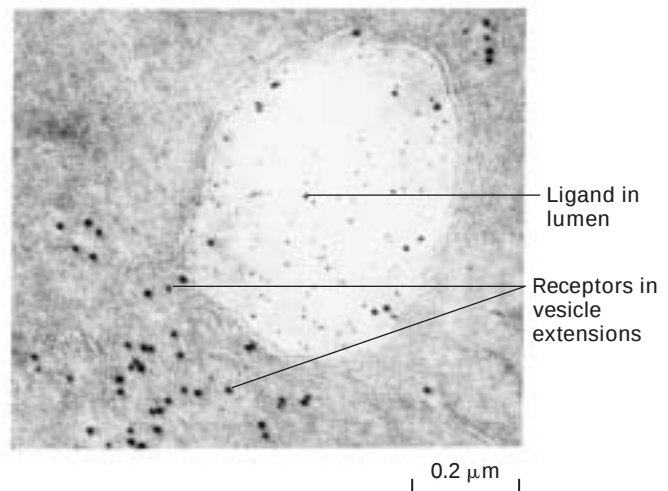
Mutational studies have shown that other cell-surface receptors can be directed into forming clathrin/AP2 pits by a different sorting signal: Tyr-X-X- Φ , where X can be any amino acid and Φ is a bulky hydrophobic amino acid. This YXX Φ sorting signal in the cytosolic segment of a receptor protein binds to a specific cleft in the μ 2 subunit of the AP2 complex. Because the tyrosine and Φ residues mediate this binding, a mutation in either one reduces or abolishes the ability of the receptor to be incorporated into clathrin/AP2-coated pits. Moreover, if influenza HA protein, which is not normally endocytosed, is genetically engineered to contain this four-residue sequence in its cytosolic domain, the mutant HA is internalized. Recall from our earlier discussion that

this same sorting signal recruits membrane proteins into clathrin/AP1 vesicles that bud from the *trans*-Golgi network by binding to the μ 1 subunit of AP1 (see Table 17-2). All these observations indicate that YXX Φ is a widely used signal for sorting membrane proteins to clathrin-coated vesicles.

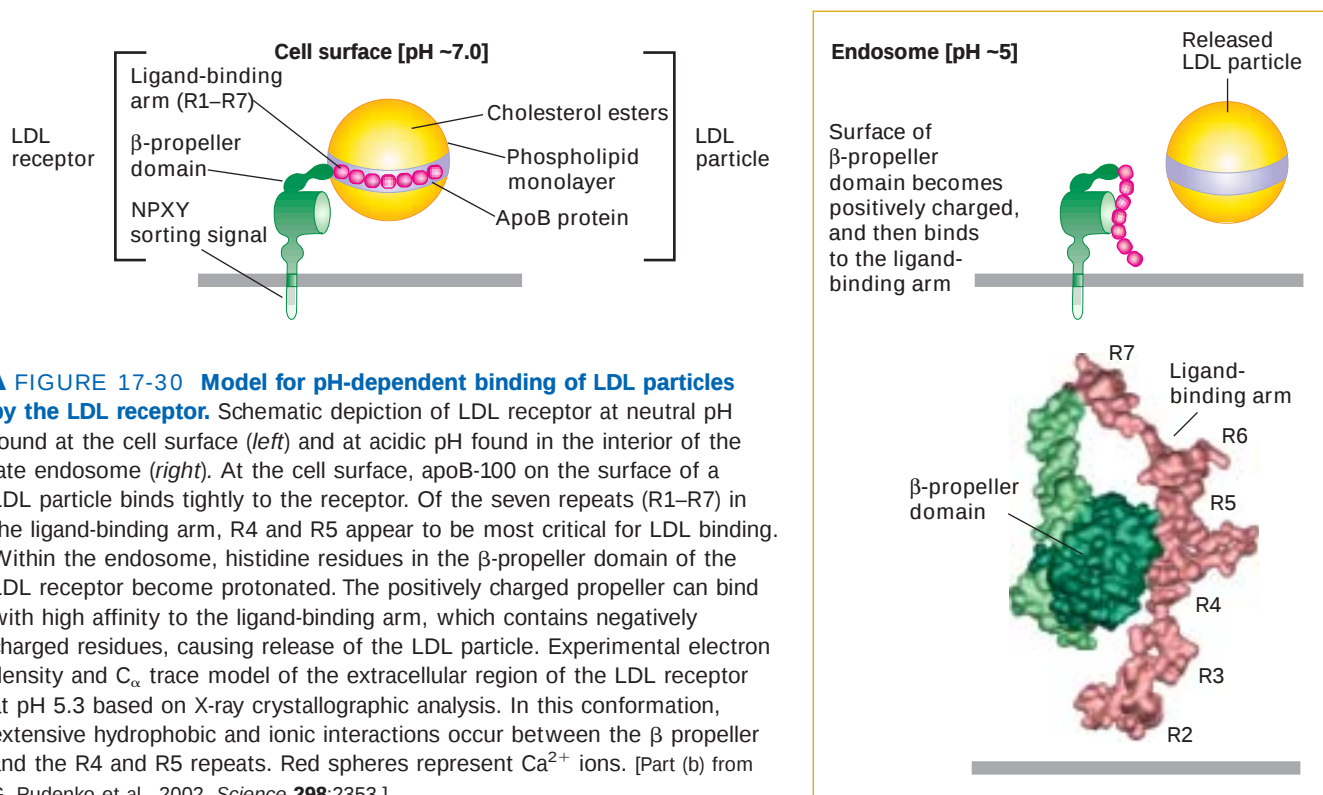
In some cell-surface proteins, however, other sequences (e.g., Leu-Leu) or covalently linked ubiquitin molecules signal endocytosis. Among the proteins associated with clathrin/AP2 vesicles, several contain domains that specifically bind to ubiquitin, and it has been hypothesized that these vesicle-associated proteins mediate the selective incorporation of ubiquitinated membrane proteins into endocytic vesicles. As described later, the ubiquitin tag on endocytosed membrane proteins is also recognized at a later stage in the endocytic pathway and plays a role in delivering these proteins into the interior of the lysosome where they are degraded.

The Acidic pH of Late Endosomes Causes Most Receptor-Ligand Complexes to Dissociate

The overall rate of endocytic internalization of the plasma membrane is quite high; cultured fibroblasts regularly internalize 50 percent of their cell-surface proteins and phospholipids each hour. Most cell-surface receptors that undergo endocytosis will repeatedly deposit their ligands within the



▲ **EXPERIMENTAL FIGURE 17-29 Electron microscopy demonstrates that endocytosed receptor-ligand complexes dissociate in late endosomes.** Liver cells were perfused with an asialoglycoprotein ligand and then were fixed and sectioned for electron microscopy. The sections were stained with receptor-specific antibodies, tagged with gold particles 8 nm in diameter, to localize the receptor and with asialoglycoprotein-specific antibody, linked to gold particles 5 nm in diameter, to localize the ligand (see Figure 5-51). As seen in this electron micrograph of a late endosome, the ligand (smaller dark grains) is localized in the vesicle lumen and the asialoglycoprotein receptor (larger dark grains) is localized in the tubular extensions budding off from the vesicle. [Courtesy of H. J. Geuze. Copyright 1983, M.I.T. See H. J. Geuze et al., 1983, *Cell* **32**:277.]



▲ **FIGURE 17-30 Model for pH-dependent binding of LDL particles by the LDL receptor.** Schematic depiction of LDL receptor at neutral pH found at the cell surface (*left*) and at acidic pH found in the interior of the late endosome (*right*). At the cell surface, apoB-100 on the surface of a LDL particle binds tightly to the receptor. Of the seven repeats (R1–R7) in the ligand-binding arm, R4 and R5 appear to be most critical for LDL binding. Within the endosome, histidine residues in the β -propeller domain of the LDL receptor become protonated. The positively charged propeller can bind with high affinity to the ligand-binding arm, which contains negatively charged residues, causing release of the LDL particle. Experimental electron density and C_α trace model of the extracellular region of the LDL receptor at pH 5.3 based on X-ray crystallographic analysis. In this conformation, extensive hydrophobic and ionic interactions occur between the β propeller and the R4 and R5 repeats. Red spheres represent Ca^{2+} ions. [Part (b) from G. Rudenko et al., 2002, *Science* **298**:2353.]

cell and then recycle to the plasma membrane, once again to mediate internalization of ligand molecules. For instance, the LDL receptor makes one round trip into and out of the cell every 10–20 minutes, for a total of several hundred trips in its 20-hour life span.

Internalized receptor-ligand complexes commonly follow the pathway depicted for the M6P receptor in Figure 17-23 and the LDL receptor in Figure 17-28. Endocytosed cell-surface receptors typically dissociate from their ligands within late endosomes, which appear as spherical vesicles with tubular branching membranes located a few micrometers from the cell surface. The original experiments that defined the late endosome sorting vesicle utilized the asialoglycoprotein receptor. This liver-specific protein mediates the binding and internalization of abnormal glycoproteins whose oligosaccharides terminate in galactose rather than the normal sialic acid, hence the name *asialoglycoprotein*. Electron microscopy of liver cells perfused with asialoglycoprotein reveal that 5–10 minutes after internalization, ligand molecules are found in the lumen of late endosomes, while the tubular membrane extensions are rich in receptor and rarely contain ligand (Figure 17-29). These findings indicate that the late endosome is the organelle in which receptors and ligands are uncoupled.

The dissociation of receptor-ligand complexes in late endosomes occurs not only in the endocytic pathway but also in the delivery of soluble lysosomal enzymes via the secretory pathway (see Figure 17-23). As discussed in Chapter 7, the membranes of late endosomes and lysosomes contain V-class

proton pumps that act in concert with Cl^- channels to acidify the vesicle lumen (see Figure 7-10). Most receptors, including the M6P receptor and cell-surface receptors for LDL particles and asialoglycoprotein, bind their ligands tightly at neutral pH but release their ligands if the pH is lowered to 6.0 or below. The late endosome is the first vesicle encountered by receptor-ligand complexes whose luminal pH is sufficiently acidic to promote dissociation of most endocytosed receptors from their tightly bound ligands.

The mechanism by which the LDL receptor releases bound LDL particles is now understood in detail (Figure 17-30). At the endosomal pH of 5.0–5.5, histidine residues in the β -propeller domain of the receptor become protonated, forming a site that can bind with high affinity to the negatively charged repeats in the ligand-binding domain. This intramolecular interaction sequesters the repeats in a conformation that cannot simultaneously bind to apoB-100, thus causing release of the bound LDL particle.

The Endocytic Pathway Delivers Iron to Cells Without Dissociation of Receptor-Transferrin Complex in Endosomes

An exception to the general theme of pH-dependent receptor-ligand dissociation in the late endosome occurs in the endocytic pathway that delivers transferrin-bound iron to cells. A major glycoprotein in the blood, transferrin transports iron to all tissue cells from the liver (the main site of iron

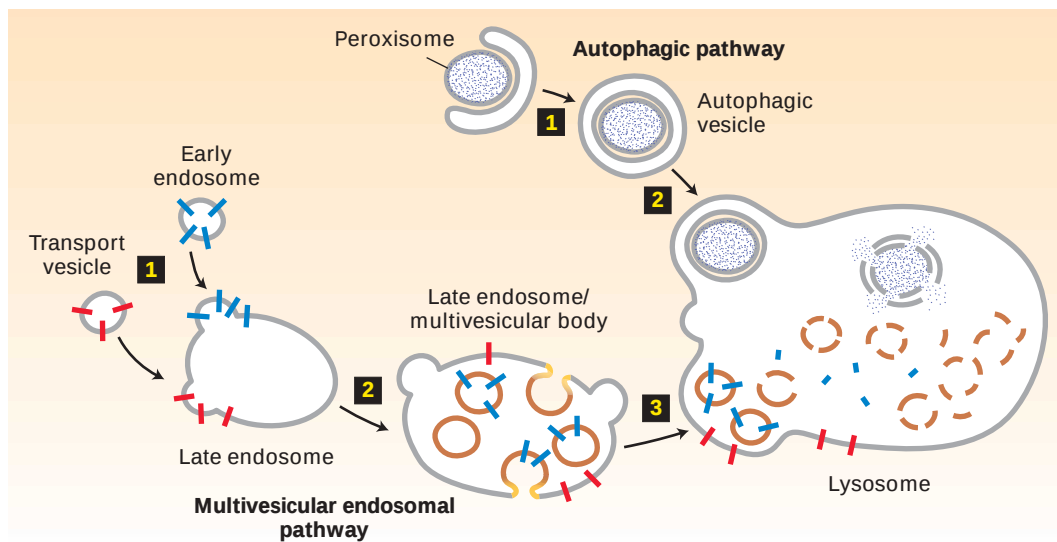
storage in the body) and from the intestine (the site of iron absorption). The iron-free form, *apotransferrin*, binds two Fe^{3+} ions very tightly to form *ferrotransferrin*. All mammalian cells contain cell-surface transferrin receptors that avidly bind ferrotransferrin at neutral pH, after which the receptor-bound ferrotransferrin is subjected to endocytosis. Like the components of a LDL particle, the two bound Fe^{3+} atoms remain in the cell, but the apotransferrin part of the ligand does not dissociate from the receptor and is secreted from the cell within minutes after being endocytosed.

Although apotransferrin remains bound to the transferrin receptor at the low pH of late endosomes, changes in pH are critical to functioning of the transferrin endocytic pathway. At a pH below 6.0, the two bound Fe^{3+} atoms dissociate from ferrotransferrin, are reduced to Fe^{2+} by an unknown mechanism, and then are exported into the cytosol by an endosomal transporter specific for divalent metal ions. The receptor-apotransferrin complex remaining after dissociation of the iron atoms is recycled back to the cell surface. Although apotransferrin binds tightly to its receptor at a pH of 5.0 or 6.0, it does not bind at neutral pH. Hence the bound apotransferrin dissociates from the transferrin receptor when the recycling vesicles fuse with the plasma membrane and the receptor-ligand complex encounters the

neutral pH of the extracellular interstitial fluid or growth medium. The recycled receptor is then free to bind another molecule of ferrotransferrin, and the released apotransferrin is carried in the bloodstream to the liver or intestine to be reloaded with iron.

Specialized Vesicles Deliver Cell Components to the Lysosome for Degradation

The major function of lysosomes is to degrade extracellular materials taken up by the cell and intracellular components under certain conditions. Materials to be degraded must be delivered to the lumen of the lysosome where the various degradative enzymes reside. As just discussed, endocytosed ligands (e.g., LDL particles) that dissociate from their receptors in the late endosome subsequently enter the lysosomal lumen when the membrane of the late endosome fuses with the membrane of the lysosome (see Figure 17-28). Likewise, phagosomes carrying bacteria or other particulate matter can fuse with lysosomes, releasing their contents into the lumen for degradation. However, the delivery of endocytosed membrane proteins and of cytoplasmic materials to lysosomes for degradation poses special problems and involves two unusual types of vesicles.



▲ **FIGURE 17-31 Delivery of plasma-membrane proteins and cytoplasmic components to the lysosomal interior for degradation.** *Left:* Early endosomes carrying endocytosed plasma-membrane proteins (blue) and vesicles carrying lysosomal membrane proteins (red) from the *trans*-Golgi network fuse with the late endosome, transferring their membrane proteins to the endosomal membrane (step 1). Proteins to be degraded are incorporated into vesicles that bud *into* the interior of the late endosome, eventually forming a multivesicular endosome containing many such internal vesicles (step 2). Fusion of a multivesicular endosome directly with a lysosome releases the internal vesicles into the lumen of the lysosome where they can be degraded (step 3). Because proton pumps

and other lysosomal membrane proteins normally are not incorporated into internal endosomal vesicles, they are delivered to the lysosomal membrane and are protected from degradation. *Right:* In the autophagic pathway, a cup-shaped structure forms around portions of the cytosol or an organelle such as a peroxisome, as shown here. Continued addition of membrane eventually leads to the formation of an autophagic vesicle that envelopes its contents by two complete membranes (step 1). Fusion of the outer membrane with the membrane of a lysosome releases a single-layer vesicle and its contents into the lysosome interior (step 2). [See F. Reggiori and D. J. Klionsky, 2002, *Eukaryot. Cell* 1:11, and D. J. Katzmann et al., 2002, *Nature Rev. Mol. Cell Biol.* 3:893.]

Multivesicular Endosomes Resident lysosomal proteins, such as V-class proton pumps and other lysosomal membrane proteins, can carry out their functions and remain in the lysosomal membrane where they are protected from degradation by the soluble hydrolytic enzymes in the lumen. Such proteins are delivered to the lysosomal membrane by transport vesicles that bud from the *trans*-Golgi network by the same basic mechanisms described in earlier sections. In contrast, endocytosed membrane proteins to be degraded are transferred in their entirety to the interior of the lysosome by a specialized delivery mechanism. Lysosomal degradation of cell-surface receptors for extracellular signaling molecules is a common mechanism for controlling the sensitivity of cells to such signals (Chapter 13). Receptors that become damaged also are targeted for lysosomal degradation.

Early evidence that membranes can be delivered to the lumen of compartments came from electron micrographs showing membrane vesicles and fragments of membranes within endosomes and lysosomes (see Figure 5-20c). Parallel experiments in yeast revealed that endocytosed receptor proteins targeted to the vacuole (the yeast organelle equivalent to the lysosome) were primarily associated with membrane fragments and small vesicles within the interior of the vacuole rather than with the vacuole surface membrane.

These observations suggest that endocytosed membrane proteins can be incorporated into specialized vesicles that form at the endosomal membrane (Figure 17-31, *left*). Although these vesicles are similar in size and appearance to transport vesicles, they differ topologically. Transport vesicles bud *outward* from the surface of a donor organelle into the cytosol, whereas vesicles within the endosome bud *inward* from the surface into the lumen (away from the cytosol). Mature endosomes containing numerous vesicles in their interior are usually called *multivesicular endosomes* (or bodies). Eventually the surface membrane of a multivesicular endosome fuses with the membrane of a lysosome, thereby delivering its internal vesicles and the membrane proteins they contain into the lysosome interior for degradation. Thus the sorting of proteins in the endosomal membrane determines which ones will remain on the lysosome surface (e.g., pumps and transporters) and which ones will be incorporated into internal vesicles and ultimately degraded in lysosomes.

Autophagic Vesicles The delivery of bulk amounts of cytosol or entire organelles to lysosomes and their subsequent degradation is known as *autophagy* (“eating oneself”). Autophagy is often a regulated process and is typically induced in cells placed under conditions of starvation or other types of stress, allowing the cell to recycle macromolecules for use as nutrients.

The autophagic pathway begins with the formation of a flattened double-membraned cup-shaped structure (Figure 17-31, *right*). This structure can grow by vesicle fusion and eventually seals to form an autophagic vesicle that envelops a region of the cytosol or an entire organelle (e.g., peroxisome, mitochondrion). Unknown at this time is the origin

of the membranes that form the initial cup-shaped organelle and the vesicles that are added to it, but the endosome itself is a likely candidate. The outer membrane of an autophagic vesicle can fuse with the lysosome delivering a large vesicle, bounded by a single membrane bilayer, to the interior of the lysosome. The lipases and proteases within the lysosome eventually will degrade this vesicle and its contents into their molecular components.

Retroviruses Bud from the Plasma Membrane by a Process Similar to Formation of Multivesicular Endosomes

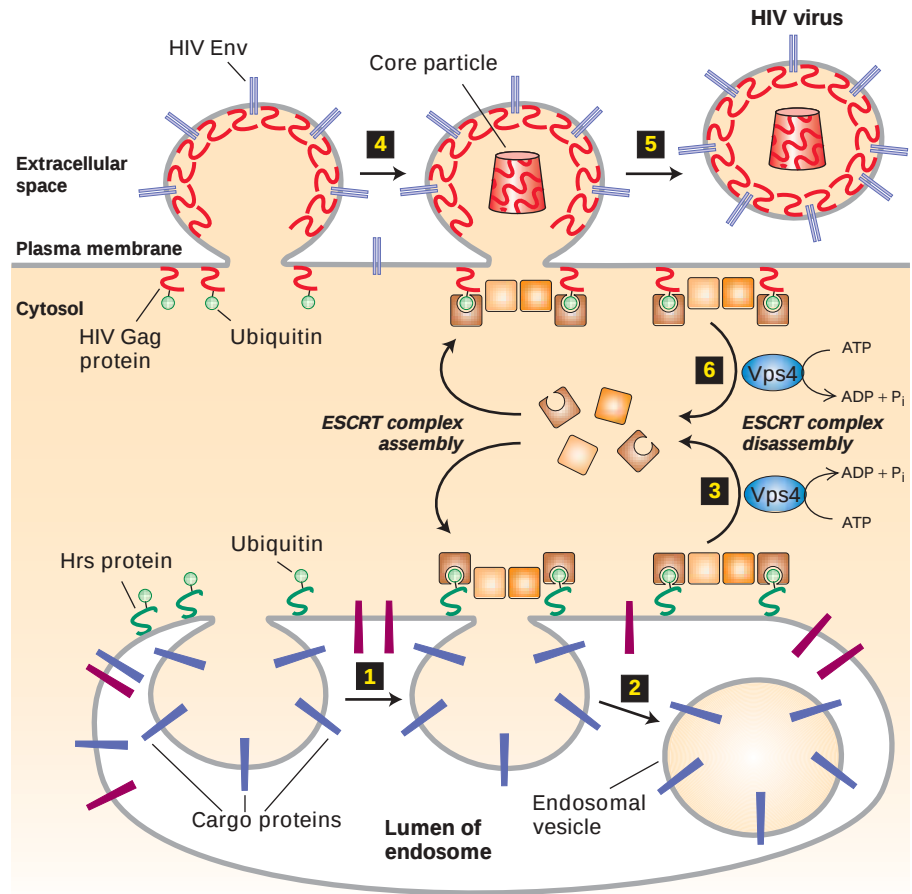
The vesicles that bud into the interior of endosomes have a topology similar to that of enveloped virus particles that bud from the plasma membrane of virus-infected cells. Moreover, recent experiments demonstrate that a common set of proteins are required for both types of membrane-budding events. In fact, the two processes so closely parallel one another in mechanistic detail as to suggest that enveloped viruses have evolved mechanisms to recruit the cellular proteins used in inward endosomal budding for their own purposes.

Many of the proteins required for inward budding of the endosomal membrane were first identified by mutations in yeast that blocked delivery of membrane proteins to the interior of the vacuole. More than 10 such “budding” proteins have been identified in yeast, most with significant similarities to mammalian proteins that evidently perform the same function in mammalian cells. The current model of endosomal budding to form multivesicular endosomes in mammalian cells is based primarily on studies in yeast (Figure 17-32). A ubiquitin-tagged peripheral membrane protein of the endosome, known as Hrs, facilitates loading of specific ubiquitinated membrane cargo proteins into vesicle buds directed into the interior of the endosome. The ubiquitinated Hrs protein then recruits a set of three different protein complexes to the membrane. These *ESCRT* (endosomal sorting complexes required for transport) *complexes* include the ubiquitin-binding protein Tsg101. The membrane-associated ESCRT complexes act to complete vesicle budding, leading to release of a vesicle carrying specific membrane cargo into the interior of the endosome. Finally, an ATPase, known as Vps4, uses the energy from ATP hydrolysis to disassemble the ESCRT complexes, releasing them into the cytosol for another round of budding. In the fusion event that pinches off a completed endosomal vesicle, the ESCRT proteins and Vps4 may function like SNAREs and NSF, respectively, in the typical membrane-fusion process discussed previously (see Figure 17-11).

The human immunodeficiency virus (HIV) is an enveloped retrovirus that buds from the plasma membrane of infected cells in a process driven by viral Gag protein, the major structural component of completed virus particles. Gag protein binds to the plasma membrane of an infected cell and ≈4000 Gag molecules polymerize into a spherical

► **FIGURE 17-32 Model of the common mechanism for formation of multivesicular endosomes and budding of HIV from the plasma membrane.**

Bottom: In endosomal budding, ubiquitinated Hrs on the endosomal membrane directs loading of specific membrane cargo proteins (blue) into vesicle buds and then recruits cytosolic ESCRT complexes to the membrane (step **1**). Note that both Hrs and the recruited cargo proteins are tagged with ubiquitin. After the set of bound ESCRT complexes mediate membrane fusion and pinching off of the completed vesicle (step **2**), they are disassembled by the ATPase Vps4 and returned to the cytosol (step **3**). **Top:** Budding of HIV particles from HIV-infected cells occurs by a similar mechanism using the virally encoded Gag protein and cellular ESCRT complexes and Vps4 (steps **4–6**). Ubiquitinated Gag near a budding particle functions like Hrs. See text for discussion. [Adapted from O. Pornillos et al., 2002, *Trends Cell Biol.* **12**:569.]

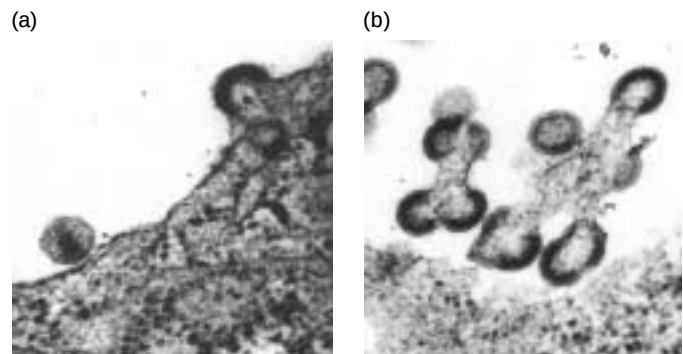


shell, producing a structure that looks like a vesicle bud protruding outward from the plasma membrane. Mutational studies with HIV have revealed that the N-terminal segment of Gag protein is required for association with the plasma membrane, whereas the C-terminal segment is required for pinching off of complete HIV particles. For instance, if the portion of the viral genome encoding the C-terminus of Gag is removed, HIV buds will form in infected cells, but pinching off does not occur and thus no free virus particles are released.

The first indication that HIV budding employs the same molecular machinery as vesicle budding into endosomes

came from the observation that Tsg101, a component of the ESCRT complex, binds to the C-terminus of Gag protein. Subsequent findings have clearly established the mechanistic parallels between the two processes (Figure 17-32). For example, Gag is ubiquitinated as part of the process of virus budding, and in cells with mutations in Tsg101 or Vps4, HIV virus buds accumulate but cannot pinch off from the membrane (Figure 17-33). Moreover, when a segment from the cellular Hrs protein is added to a truncated Gag protein, proper budding and release of virus particles is restored. Taken together, these results indicate that Gag protein mimics the function of Hrs, redirecting ESCRT complexes to the

► **FIGURE 17-33 Electron micrographs of virus budding from wild-type and ESCRT-deficient HIV-infected cells.** (a) In wild-type cells infected with HIV, virus particles bud from the plasma membrane and are rapidly released into the extracellular space. (b) In cells that lack the functional ESCRT protein Tsg101, the viral Gag protein forms dense virus-like structures, but budding of these structures from the plasma membrane cannot be completed and chains of incomplete viral buds still attached to the plasma membrane accumulate. [Wes Sundquist, University of Utah.]



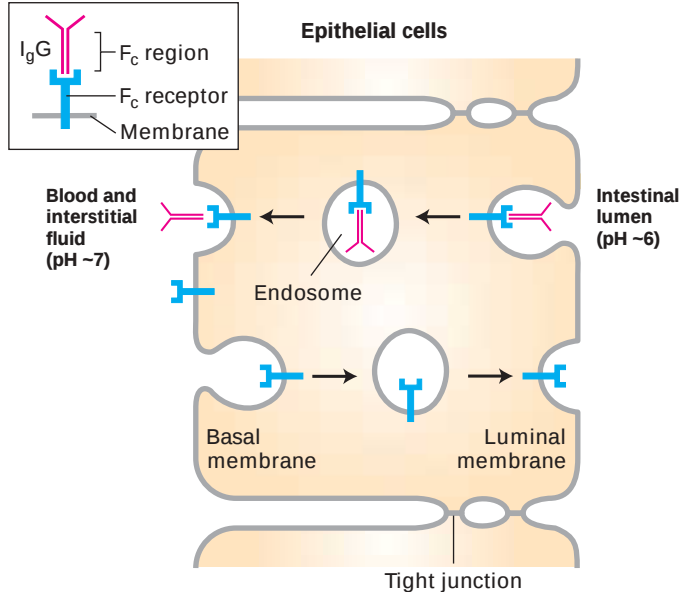
plasma membrane where they can function in the budding of virus particles.

Other enveloped retroviruses such as murine leukemia virus and Rous sarcoma virus also have been shown to require ESCRT complexes for their budding, although each virus appears to have evolved a somewhat different mechanism to recruit ESCRT complexes to the site of virus budding.

Transcytosis Moves Some Endocytosed Ligands Across an Epithelial Cell Layer

As noted previously, transcytosis is used by some cells in the apical-basolateral sorting of certain membrane proteins (see Figure 17-26). This process of transcellular transport, which combines endocytosis and exocytosis, also can be employed to import an extracellular ligand from one side of a cell, transport it across the cytoplasm, and secrete it from the plasma membrane at the opposite side. Transcytosis occurs mainly in sheets of polarized epithelial cells.

Maternal immunoglobulins (antibodies) contained in ingested breast milk are transported across the intestinal epithelial cells of the newborn mouse and human by transcytosis (Figure 17-34). The F_c receptor that mediates this movement binds antibodies at the acidic pH of 6 found in the intestinal lumen but not at the neutral pH of the extracellular fluid on the basal side of the intestinal epithelium. This difference in the pH of the extracellular media on the two sides of intes-



▲ **FIGURE 17-34 Transcytosis of maternal IgG immunoglobulins across the intestinal epithelial cells of newborn mice.** This transcellular movement of a ligand involves both endocytosis and exocytosis. The one-way movement of ligand from the intestinal lumen to the blood depends on the differential affinity of the F_c receptor for antibody at pH 6 (strong binding) and at pH 7 (weak binding). Transcytosis in the opposite direction returns the empty F_c receptor to the luminal membrane. See text for discussion.

tinal epithelial cells allows maternal immunoglobulins to move in one direction—from the lumen to the blood. The same process also moves circulating maternal immunoglobulins across mammalian yolk-sac cells into the fetus.

KEY CONCEPTS OF SECTION 17.5

Receptor-Mediated Endocytosis and the Sorting of Internalized Proteins

- Some extracellular ligands that bind to specific cell-surface receptors are internalized, along with their receptors, in clathrin-coated vesicles whose coats also contain AP2 complexes.
- Sorting signals in the cytosolic domain of cell-surface receptors target them into clathrin/AP2-coated pits for internalization. Known signals include the Asn-Pro-X-Tyr, Tyr-X-X-Φ, and Leu-Leu sequences (see Table 17-2).
- The endocytic pathway delivers some ligands (e.g., LDL particles) to lysosomes where they are degraded. Most receptor-ligand complexes dissociate in the acidic milieu of the late endosome; the receptors are recycled to the plasma membrane, while the ligands are sorted to lysosomes (see Figure 17-28).
- Iron is imported into cells by an endocytic pathway in which Fe^{3+} ions are released from ferrotransferrin in the late endosome. The receptor-apotransferrin complex is recycled to the cell surface where the complex dissociates, releasing both the receptor and apotransferrin for reuse.
- Endocytosed membrane proteins destined for degradation in the lysosome are incorporated into vesicles that bud into the interior of the endosome. Multivesicular endosomes, which contain many of these internal vesicles, can fuse with the lysosome to deliver the vesicles to the interior of the lysosome (see Figure 17-31).
- A portion of the cytoplasm or an entire organelle (e.g., peroxisome) can be enveloped in a flattened membrane and eventually incorporated into a double-membraned autophagic vesicle. Fusion of the outer vesicle membrane with the lysosome delivers the enveloped contents to the interior of the lysosome for degradation.
- Some of the cellular components (e.g., ESCRT complexes) that mediate inward budding of endosomal membranes are used in the budding and pinching off of enveloped viruses such as HIV from the plasma membrane of virus-infected cells (see Figure 17-32).

17.6 Synaptic Vesicle Function and Formation

In this final section we consider the regulated secretion of **neurotransmitters** that is the basis for signaling by many nerve cells. These small, water-soluble molecules (e.g., acetyl-

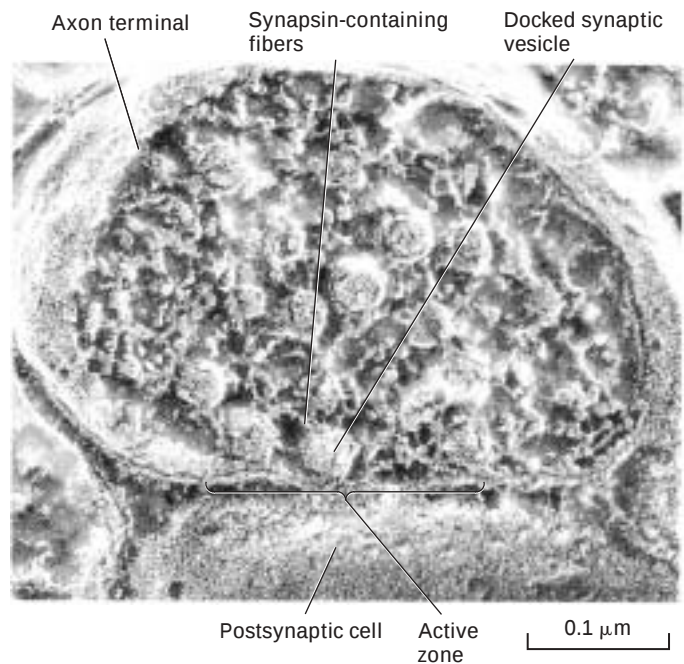
choline, dopamine) are released at chemical **synapses**, specialized sites of contact between a signaling neuron and a receiving cell. Generally signals are transmitted in only one direction: an axon terminal from a *presynaptic cell* releases neurotransmitter molecules that diffuse through a narrow extracellular space (the synaptic cleft) and bind to receptors on a *postsynaptic cell* (see Figure 7-31). The membrane of the postsynaptic cell, which can be another neuron, a muscle cell, or a gland cell, is located within approximately 50 nm of the presynaptic membrane.

Neurotransmitters are stored in specialized regulated secretory vesicles, known as *synaptic vesicles*, which are 40–50 nm in diameter. Exocytosis of these vesicles and release of neurotransmitters is initiated when a stimulatory electrical impulse (**action potential**) travels down the axon of a presynaptic cell to the axon terminal where it triggers opening of voltage-gated Ca^{2+} channels. The subsequent localized rise in the cytosolic Ca^{2+} concentration induces some synaptic vesicles to fuse with the plasma membrane, releasing their contents into the synaptic cleft. We described the major events in signal transmission at chemical synapses and the effects of neurotransmitter binding on postsynaptic cells in Chapter 7. Here we focus on the regulated secretion of neurotransmitters and the formation of synaptic vesicles in the context of the basic principles of vesicular trafficking already outlined in this chapter.

Synaptic Vesicles Loaded with Neurotransmitter Are Localized Near the Plasma Membrane

The exocytosis of neurotransmitters from synaptic vesicles involves targeting and fusion events similar to those that lead to release of secreted proteins in the secretory pathway. However, several unique features permit the very rapid release of neurotransmitters in response to arrival of an action potential at the presynaptic axon terminal. For example, in resting neurons some neurotransmitter-filled synaptic vesicles are “docked” at the plasma membrane; others are in reserve in the *active zone* near the plasma membrane at the synaptic cleft. In addition, the membrane of synaptic vesicles contains a specialized Ca^{2+} -binding protein that senses the rise in cytosolic Ca^{2+} after arrival of an action potential, triggering rapid fusion of docked vesicles with the presynaptic membrane.

A highly organized arrangement of cytoskeletal fibers in the axon terminal helps localize synaptic vesicles in the active zone (Figure 17-35). The vesicles themselves are linked together by *synapsin*, a fibrous phosphoprotein associated with the cytosolic surface of all synaptic-vesicle membranes. Filaments of synapsin also radiate from the plasma membrane and bind to vesicle-associated synapsin. These interactions probably keep synaptic vesicles close to the part of the plasma membrane facing the synapse. Indeed, synapsin knockout mice, although viable, are prone to seizures; during repetitive stimulation of many neurons in such mice, the number of synaptic vesicles that fuse with the plasma mem-



▲ **EXPERIMENTAL FIGURE 17-35 Fibrous proteins help localize synaptic vesicles to the active zone of axon terminals.** In this micrograph of an axon terminal obtained by the rapid-freezing deep-etch technique, synapsin fibers can be seen to interconnect the vesicles and to connect some to the active zone of the plasma membrane. Docked vesicles are ready to be exocytosed. Those toward the center of the terminal are in the process of being filled with neurotransmitter. [From D. M. D. Landis et al., 1988, *Neuron* 1:201]

brane is greatly reduced. Thus synapsins are thought to recruit synaptic vesicles to the active zone.

Rab3A, a GTP-binding protein located in the membrane of synaptic vesicles, also is required for targeting of neurotransmitter-filled vesicles to the active zone of presynaptic cells facing the synaptic cleft. Rab3A knockout mice, like synapsin-deficient mice, exhibit a reduced number of synaptic vesicles able to fuse with the plasma membrane after repetitive stimulation. The neuron-specific Rab3 is similar in sequence and function to other Rab proteins that participate in docking vesicles on particular target membranes in the secretory pathway.

A Calcium-Binding Protein Regulates Fusion of Synaptic Vesicles with the Plasma Membrane

Fusion of synaptic vesicles with the plasma membrane of axon terminals depends on the same proteins that mediate membrane fusion of other regulated secretory vesicles. The principal v-SNARE in synaptic vesicles (VAMP) tightly binds syntaxin and SNAP-25, the principal t-SNAREs in the plasma membrane of axon terminals, to form four-helix SNARE complexes. After fusion, SNAP proteins and NSF

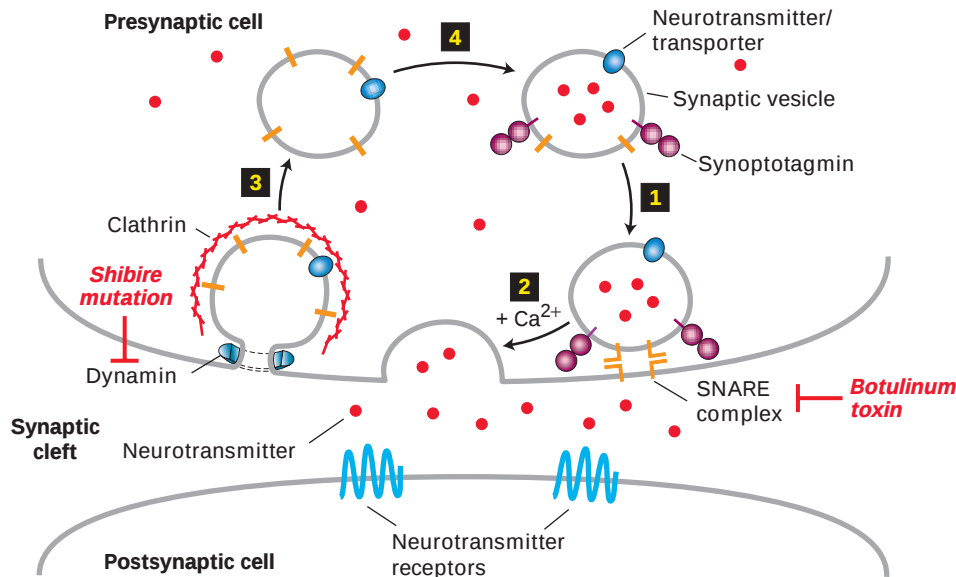
within the axon terminal promote disassociation of VAMP from t-SNAREs, as in the fusion of secretory vesicles depicted previously (see Figure 17-11).



Strong evidence for the role of VAMP in neurotransmitter exocytosis is provided by the mechanism of action of botulinum toxin, a bacterial protein that can cause the paralysis and death characteristic of *botulism*, a type of food poisoning. The toxin is composed of two polypeptides: One binds to motor neurons that release acetylcholine at synapses with muscle cells, facilitating entry of the other polypeptide, a protease, into the cytosol of the axon terminal. The only protein this protease cleaves is VAMP. After the botulinum protease enters an axon terminal, synaptic vesicles that are not already docked rapidly lose their ability to fuse with the plasma membrane because cleavage of VAMP prevents assembly of SNARE complexes. The resulting block in acetylcholine release at neuromuscular synapses causes paralysis. However, vesicles that are already docked exhibit remarkable resistance to the toxin indicating that SNARE complexes may already be in a partially assembled, protease-resistant state when vesicles are docked on the presynaptic membrane. ■

The signal that triggers exocytosis of docked synaptic vesicles is a rise in the Ca^{2+} concentration in the cytosol near vesicles from $<0.1 \mu\text{M}$, characteristic of resting cells, to $1\text{--}100 \mu\text{M}$ following arrival of an action potential in stimulated cells. The speed with which synaptic vesicles fuse with the presynaptic membrane after a rise in cytosolic Ca^{2+} (less than 1 msec) indicates that the fusion machinery is entirely assembled in the resting state and can rapidly undergo a conformational change leading to exocytosis of neurotransmitter. A Ca^{2+} -binding protein called *synaptotagmin*, located in the membrane of synaptic vesicles, is thought to be a key component of the vesicle fusion machinery that triggers exocytosis in response to Ca^{2+} (Figure 17-36).

Several lines of evidence support a role for synaptotagmin as the Ca^{2+} sensor for exocytosis of neurotransmitters. For instance, mutant embryos of *Drosophila* and *C. elegans* that completely lack synaptotagmin fail to hatch and exhibit very reduced, uncoordinated muscle contractions. Larvae with partial loss-of-function mutations of synaptotagmin survive, but their neurons are defective in Ca^{2+} -stimulated vesicle exocytosis. Moreover, in mice, mutations in synaptotagmin that decrease its affinity for Ca^{2+} cause a corresponding



▲ **FIGURE 17-36 Release of neurotransmitters and the recycling of synaptic vesicles.** Step **1**: Synaptic vesicles loaded with neurotransmitter (red circles) move to the active zone and then dock at defined sites on the plasma membrane of a presynaptic cell. Synaptotagmin prevents membrane fusion and release of neurotransmitter. Botulinum toxin prevents exocytosis by proteolytically cleaving VAMP, the v-SNARE on vesicles. Step **2**: In response to a nerve impulse (action potential), voltage-gated Ca^{2+} channels in the plasma membrane open, allowing an influx of Ca^{2+} from the extracellular medium. The resulting Ca^{2+} -induced conformational change in synaptotagmin leads to fusion of docked vesicles with the plasma membrane and release of neurotransmitters into the synaptic cleft. Step **3**: After

clathrin/AP vesicles containing v-SNARE and neurotransmitter transporter proteins bud inward and are pinched off in a dynamin-mediated process, they lose their coat proteins. Dynamin mutations such as *shibire* in *Drosophila* block the re-formation of synaptic vesicles, leading to paralysis. Step **4**: The uncoated vesicles import neurotransmitters from the cytosol, generating fully reconstituted synaptic vesicles and completing the cycle. Most synaptic vesicles are formed by endocytic recycling as depicted here. However, endocytic vesicles containing membrane from the axon terminus can fuse with the endosome; budding from this compartment can then form “new” synaptic vesicles. [See K. Takei et al., 1996, *J. Cell. Biol.* **133**:1237; V. Murthy and C. Stevens, 1998, *Nature* **392**:497; and R. Jahn et al., 2003, *Cell* **112**:519.]

increase in the amount of cytosolic Ca^{2+} needed to trigger rapid exocytosis.

Several hypotheses concerning how synaptotagmin promotes neurotransmitter exocytosis have been proposed, but the precise mechanism of its function is still unresolved. Synaptotagmin is known to bind phospholipids and after undergoing a Ca^{2+} -induced conformational change, it may promote association of the phospholipids in the vesicle and plasma membranes. Synaptotagmin also binds to SNARE proteins and may catalyze a late stage in assembly of SNARE complexes when bound to Ca^{2+} . Finally, synaptotagmin may also act to inhibit inappropriate exocytosis in resting cells. At the low cytosolic Ca^{2+} levels found in resting nerve cells, synaptotagmin apparently binds to a complex of the plasma-membrane proteins neuexin and syntaxin. The presence of synaptotagmin blocks binding of other essential fusion proteins to the neuexin-syntaxin complex, thereby preventing vesicle fusion. When synaptotagmin binds Ca^{2+} , it is displaced from the complex, allowing other proteins to bind and thus initiating membrane docking or fusion. Thus synaptotagmin may operate as a “clamp” to prevent fusion from proceeding in the absence of a Ca^{2+} signal.

Fly Mutants Lacking Dynamin Cannot Recycle Synaptic Vesicles

Synaptic vesicles are formed primarily by endocytic budding from the plasma membrane of axon terminals. Endocytosis usually involves clathrin-coated pits and is quite specific, in that several membrane proteins unique to the synaptic vesicles (e.g., neurotransmitter transporters) are specifically incorporated into the endocytosed vesicles. In this way, synaptic-vesicle membrane proteins can be reused and the recycled vesicles refilled with neurotransmitter (see Figure 17-36).

As in the formation of other clathrin/AP-coated vesicles, pinching off of endocytosed synaptic vesicles requires the GTP-binding protein dynamin (see Figure 17-20). Indeed, analysis of a temperature-sensitive *Drosophila* mutant called *shibire* (*shi*), which encodes the fly dynamin protein, provided early evidence for the role of dynamin in endocytosis. At the permissive temperature of 20°C, the mutant flies are normal, but at the nonpermissive temperature of 30°C, they are paralyzed (*shibire*, paralyzed in Japanese) because pinching off of clathrin-coated pits in neurons and other cells is blocked. When viewed in the electron microscope, the *shi* neurons at 30°C show abundant clathrin-coated pits with long necks but few clathrin-coated vesicles. The appearance of nerve terminals in *shi* mutants at the nonpermissive temperature is similar to that of terminals from normal neurons incubated in the presence of a nonhydrolyzable analog of GTP (see Figure 17-21). Because of their inability to pinch off new synaptic vesicles, the neurons in *shi* mutants eventually become depleted of synaptic vesicles when flies are shifted to the nonpermissive temperature, leading to a cessation of synaptic signaling and paralysis.

KEY CONCEPTS OF SECTION 17.6

Synaptic Vesicle Formation and Function

- Transmission of nerve impulses at chemical synapses depends on the exocytosis of neurotransmitter-filled synaptic vesicles and the regeneration of empty vesicles by endocytosis.
- Efficient recruitment of vesicles to the presynaptic membrane adjacent to the synaptic cleft requires cytosolic proteins, such as synapsin, and Rab3a, a GTP-binding protein that is tethered to the vesicle membrane.
- In resting neurons, synaptotagmin in the synaptic-vesicle membrane prevents fusion of docked vesicles with the membrane. The influx of Ca^{2+} following arrival of an action potential at the axon terminus leads to Ca^{2+} binding by synaptotagmin, causing a change in its conformation that permits vesicle fusion to proceed (see Figure 17-36).
- Synaptic vesicles are rapidly regenerated by endocytic budding of clathrin-coated vesicles from the plasma membrane, a process that requires dynamin. After the clathrin coat is shed, vesicles are refilled with neurotransmitter and move to the active zone for another round of docking and fusion.

PERSPECTIVES FOR THE FUTURE

The biochemical, genetic, and structural information presented in this chapter shows that we now have a basic understanding of how protein traffic flows from one membrane-bounded compartment to another. Our understanding of these processes has come largely from experiments on the function of various types of transport vesicles. These studies have led to the identification of many vesicle components and the discovery of how these components work together to drive vesicle budding, to incorporate the correct set of cargo molecules from the donor organelle, and then to mediate fusion of a completed vesicle with the membrane of a target organelle.

Despite these advances, there remain important stages of the secretory and endocytic pathways about which we know relatively little. For example, we do not yet know what types of proteins form the coats of either the regulated or constitutive secretory vesicles that bud from the *trans*-Golgi network. Indeed, it is not clear whether assembly of a cytosolic coat drives their budding at all. Moreover, the types of signals on cargo proteins that might target them for packaging into secretory vesicles have not yet been defined. Another baffling process is the formation of vesicles that bud away from the cytosol, such as the vesicles that enter multivesicular endosomes. Although some of the proteins that participate in formation of these “internal” endosome vesicles are known, we do not know what de-

termines their shape or what type of process causes them to pinch off from the donor membrane. In the future, it should be possible for these and other poorly understood vesicle-trafficking steps to be dissected through the use of the same powerful combination of biochemical and genetic methods that have delineated the working parts of COPI, COPII, and clathrin/AP vesicles.

Questions still remain about vesicle trafficking between the ER and *cis*-Golgi, between Golgi stacks, and between the *trans*-Golgi and endosome, the best-characterized transport steps. In particular, our understanding of how proteins are actually sorted between these organelles is incomplete largely because of the highly dynamic nature of all the organelles along the secretory pathway. Although we know many of the details of how particular vesicle components function, we cannot account for why their functions are restricted to specific stages in the overall flow of anterograde and retrograde transport steps. For example, we cannot explain why COPII vesicles fuse with one another to form a new *cis*-Golgi stack, whereas COPI vesicles fuse with the membrane of the ER, since both vesicle types appear to contain similar sets of v-SNARE proteins. In the same vein, we do not know what feature of the Golgi membrane actually distinguishes a COPI-coated vesicle bud from a clathrin/AP-coated bud. In both cases binding of ARF protein to the Golgi membrane appears to initiate vesicle budding. The solution to these problems will require a more integrated understanding of the flow of vesicular traffic in the context of the entire secretory pathway. Recent improvements in our ability to image vesicular transport of cargo proteins in live cells gives hope that some of these more subtle aspects of vesicle function may be clarified in the near future.

KEY TERMS

AP (adapter protein) complexes 720	Rab proteins 711
anterograde transport 715	receptor-mediated endocytosis 728
ARF protein 709	regulated secretion 724
autophagy 733	retrograde transport 715
cisternal progression 715	<i>sec</i> mutants 706
clathrin 708	secretory pathway 701
constitutive secretion 724	sorting signals 711
COPI 708	synaptotagmin 737
COPII 708	transcytosis 727
dynamain 721	<i>trans</i> -Golgi network (TGN) 701
ESCRT complexes 733	transport vesicles 701
late endosome 702	t-SNAREs 708
mannose 6-phosphate (M6P) 723	v-SNAREs 708
multivesicular endosomes 733	

REVIEW THE CONCEPTS

1. The studies of Palade and colleagues using pulse-chase labeling with radioactively labeled amino acids and autoradiography to visualize the location of the radiolabeled proteins is a classic case of the experimentalist realizing what works well in his or her biological system. These experiments were done with pancreatic acinar cells. Alternatively, HeLa cells can be used. HeLa cells are a classic human cell line originating from a cervical carcinoma. When applied to HeLa cells, the same experimental protocols are a dismal failure with respect to tracking secretion. What would you expect autoradiography of a HeLa cell to look like after pulse-labeling with radioactive amino acids?

2. *Sec18* is a yeast gene that encodes NSF. It is a class C mutant in the yeast secretory pathway. What is the mechanistic role of NSF in membrane trafficking. As indicated by its class C phenotype, why does an NSF mutation produce accumulation of vesicles at what appears to be only one stage of the secretory pathway?

3. Vesicle budding is associated with coat proteins. What is the role of coat proteins in vesicle budding? How are coat proteins recruited to membranes? What kinds of molecules are likely to be included or excluded from newly formed vesicles? What is the best-known example of a protein likely to be involved in vesicle pinching off?

4. Treatment of cells with the drug brefeldin A (BFA) has the effect of decoating Golgi apparatus membranes, resulting in a cell in which the vast majority of Golgi proteins are found in the ER. What inferences can be made from this observation regarding roles of coat proteins other than promoting vesicle formation? Predict what type of mutation in Arf1 might have the same effect as treating cells with BFA.

5. An antibody to an exposed “hinge” region of β COPI known as EAGE blocks the function of β COPI when microinjected into HeLa cells. Predict what the consequences of this functional block might be for anterograde transport from the ER to the plasma membrane. Propose an experiment to test whether the effect of EAGE microinjection is initially on anterograde or retrograde transport.

6. Specificity in fusion between vesicles involves two discrete and sequential processes. Describe the first of the two processes and its regulation by GTPase switch proteins. What effect on the size of early endosomes might result from overexpression of a mutant form of Rab5 that is stuck in the GTP-bound state?

7. Two different protein-mediated membrane fusion processes are described in this chapter, SNARE- and viral HA-mediated fusion. Compare and contrast the two. In each example give particular attention to what the direct effect of polypeptide sequences in membrane fusion is and to what controls the specificity of membrane fusion in each.

8. Sorting signals that cause retrograde transport of a protein in the secretory pathway are sometimes known as retrieval sequences. List the two known examples of retrieval sequences for soluble and membrane proteins of the ER? How does the presence of a retrieval sequence on a soluble ER protein result in its retrieval from the *cis*-Golgi complex? Describe how the concept of a retrieval sequence is essential to the cisternal-progression model.

9. Clathrin adapter protein (AP) complexes bind directly to the cytosolic face of membrane proteins and also interact with clathrin. What are the four known adapter protein complexes? Why may clathrin be considered to be an accessory protein to a core coat composed of adapter proteins?

10. I-cell disease is a classic example of an inherited human defect in protein targeting that affects an entire class of proteins, soluble enzymes of the lysosome. What is the molecular defect in I-cell disease? Why does it affect the targeting of an entire class of proteins? What other types of mutations might produce the same phenotype?

11. The TGN, *trans*-Golgi network, is the site of multiple sorting processes as proteins and lipids exit the Golgi complex. Compare and contrast the sorting of proteins to lysosomes versus the packaging of proteins into regulated secretory granules such as those containing insulin. Compare and contrast the sorting of proteins to the basolateral versus apical cell surfaces in MDCK cells versus hepatocytes.

12. The efficiency of bacterial phagocytosis by macrophages is increased greatly by first binding antibody molecules to the bacterial surface. On the basis of prior descriptions of antibody structure, to what portion of the immunoglobulin molecule do you predict a macrophage receptor for antibody bound to bacteria might be directed? Design an experiment to test this prediction.

13. Describe how pH plays a key role in regulating the interaction between mannose 6-phosphate and the mannose 6-phosphate receptor. Why does elevating endosomal pH lead to the secretion of newly synthesized lysosomal enzymes into the extracellular medium?

14. What mechanistic features are shared by (a) the formation of multivesicular endosomes by budding into the interior of the endosome and (b) the outward budding of HIV virus at the cell surface? You wish to design a peptide inhibitor/competitor of HIV budding and decide to mimic in a synthetic peptide a portion of the HIV Gag protein. Which portion of the HIV Gag protein would be a logical choice? What normal cellular process might this inhibitor block?

15. The exocytosis of neurotransmitter-filled synaptic vesicles is an example of regulated exocytosis. How is the influx of Ca^{2+} following arrival of an action potential at the axon terminus sensed and linked to the exocytosis of

synaptic vesicles? Why do normal *Drosophila* neurons incubated in the presence of a nonhydrolyzable analog of GTP have the same appearance as nerve terminals in *shi* mutants?

ANALYZE THE DATA

A variety of protein toxins, such as the bacterial toxin *Pseudomonas* and Shiga toxin and the plant toxin ricin, are heteromeric proteins consisting of A and B subunits. The A subunit is catalytic. For Shiga toxin, the proximal cause of food poisoning due to bacterially contaminated hamburger, the A subunit is an *N*-glycosidase and specifically cleaves 28S ribosomal RNA, thereby intoxicating cells by inhibiting protein synthesis. Amazingly, only one molecule of A subunit when introduced into the cytosol is sufficient to kill a cell. Interestingly the A subunit of Shiga toxin is transferred into the cytosol from the lumen of the ER by the Sec61 protein translocon. The B subunit targets Shiga toxin to the ER by binding to a glycolipid GM3 on the cell surface that acts as the Shiga toxin internalization receptor. Shiga toxin is internalized into endosomes, from endosomes is transferred to the Golgi complex, and from the Golgi complex goes to the ER where the A and B subunits dissociate, permitting the A subunit to translocate into the cytosol.

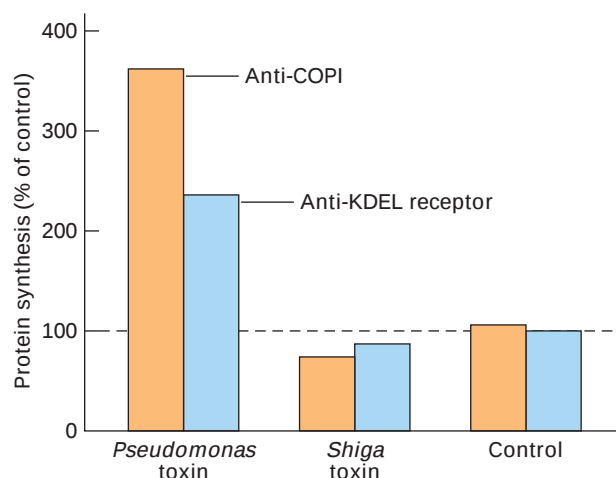
In a series of experiments designed to characterize the comparative mechanisms of *Pseudomonas* and Shiga toxin transfer from the Golgi complex to the ER, investigators first sequenced the respective targeting subunits. The C-terminal 24 amino acids of the B subunits of *Pseudomonas* toxin and Shiga toxin are shown below:

C-terminal 24 amino acids of *Pseudomonas* toxin B subunit
KEQAISALPD YASQPGKPPR KDEL

C-terminal 24 amino acids of Shiga toxin B subunit
TGMTVTIKTN ACHNGGGFSE VIFR

From inspection of these sequences, what is the probable targeting receptor for transfer of *Pseudomonas* toxins from the Golgi apparatus to the ER?

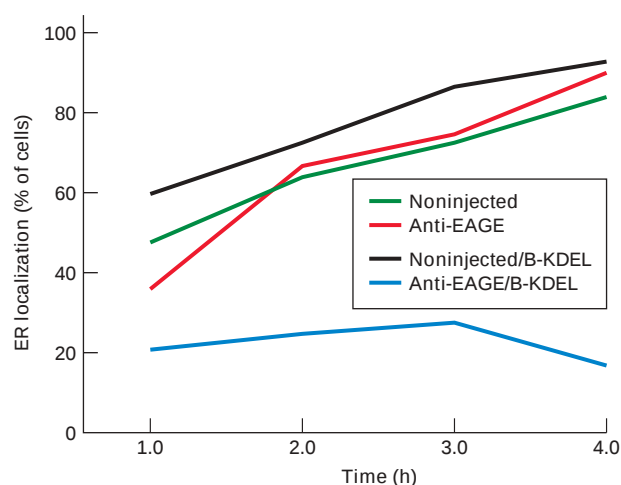
To test this prediction directly, investigators experimentally characterized the role of COPI coat proteins and KDEL receptors in intoxication. Monkey cells were microinjected with antibodies directed against either COPI coat proteins or the cytosolic domain of KDEL receptors. Cells then were incubated with *Pseudomonas* or Shiga toxin for 4 h. Protein synthesis was determined following a 30-minute pulse labeling with [^{35}S]methionine. Results are shown in the accompanying figure, with controls showing the low level of protein synthesis caused by incubation with either *Pseudomonas* or Shiga toxin without antibody injection.



Effect of microinjected antibodies on cell intoxication by *Pseudomonas* and Shiga toxin.

How do these results support your sequence-based predictions and the known role of COPI coat protein in retrograde transport? Can you formulate a hypothesis for how Shiga toxin is transported from the Golgi to the ER?

To explore further whether or not Shiga toxin transfer from the Golgi apparatus to the ER depends on COPI coat proteins, investigators prepared two different fluorescent dye-conjugated Shiga toxin B subunits and then assessed by fluorescence microscopy transport of the B subunits from the Golgi complex to the ER. The first preparation was Cy3-conjugated wild-type B subunit. The second preparation was Cy3-conjugated B subunit in which the C terminus was extended by the four amino acids KDEL (B-KDEL). Cells were microinjected with antibody directed against COPI coat proteins. Following microinjection, cells were incubated with fluorescent B subunit for various periods of time and B subunit distributions scored. The results are shown in the figure below.



Effect of microinjected antibodies directed against COPI proteins on the transport of wild type Shiga toxin B-subunit or Shiga toxin B-KDEL from Golgi complex to the ER.

What evidence do these results provide for or against transport of wild-type Shiga toxin B subunit from the Golgi complex to the ER in COPI-coated vesicles? What is the importance of the results with B-KDEL in interpreting the overall results of these experiments?

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