14.1 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 and the mechanism by which they incorporate particular cargo molecules. Many components required for the formation and fusion of transport vesicles have been identified 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 live 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 Live Cells

The classic studies of George Palade and his colleagues in the 1960s first established the order in which proteins move from one organelle to the next in the secretory pathway (see Classic Experiment 14-1). These early

studies also showed that secretory proteins are not released into the cytosol — the first indication that transported proteins are always associated with some type of membrane-bounded intermediate. In these experiments, radioactively labeled amino acids were injected into the pancreas of hamsters and the newly synthesized proteins that incorporated the labeled amino acids could be tracked as they made their way to the cell surface. At different times after injection, the animals were sacrificed and the pancreatic cells were immediately fixed with glutaraldehyde, sectioned, and subjected to autoradiography to visualize the locations 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 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 were incorporated into secretory proteins, facilitating the observation of transported proteins.

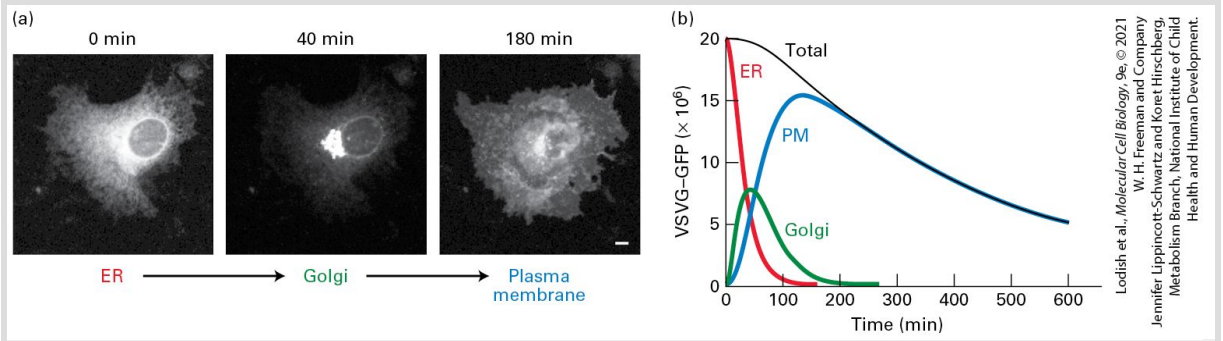
Although autoradiography is rarely used today to localize proteins within cells, these early experiments illustrate the two basic requirements for any assay of intercompartmental transport. First, it is necessary to label a cohort of proteins in an early compartment so that their subsequent transfer to later compartments can be followed over 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 transfected cells efficiently produce the VSV G protein, which is then inserted into the membrane of the ER. The use of a mutant gene encoding a temperature-sensitive VSV G protein allows researchers to turn subsequent transport of this protein on and off. At the restrictive temperature of 40 °C, newly made VSV G protein is misfolded and therefore retained within the ER by the quality-control mechanisms discussed in Chapter 13, whereas at the permissive temperature of 32 °C, the protein is correctly folded and transported through the secretory pathway to the cell surface. Importantly, the misfolding of the temperature-sensitive VSV G protein is reversible; thus when cells synthesizing mutant VSV G protein are grown at 40 °C and then shifted to 32 °C, the misfolded mutant VSV G protein that had accumulated in the ER will refold and be transported normally. 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 came to the same conclusion as Palade's early experiments: 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 the 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 occurring fluorescent protein (see Chapter 4). The hybrid gene is transfected into cultured cells by techniques described in Chapter 6. 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 the 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 complex, which are densely concentrated at the edge of the nucleus, and then to the cell surface (Figure 14-3a). By observing 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 14-3b).



EXPERIMENTAL FIGURE 14-3 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 linked to the gene for green fluorescent protein (GFP). A temperature-sensitive mutant version of the viral gene was used so that newly made hybrid protein

(VSVG-GFP) was retained in the ER at 40 °C but was 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. The scale bar is $5\ \mu m$. (b) Plot of the amount of VSVG-GFP in the endoplasmic reticulum (ER), Golgi, and plasma membrane (PM) at different times after the shift to the permissive 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.

Description

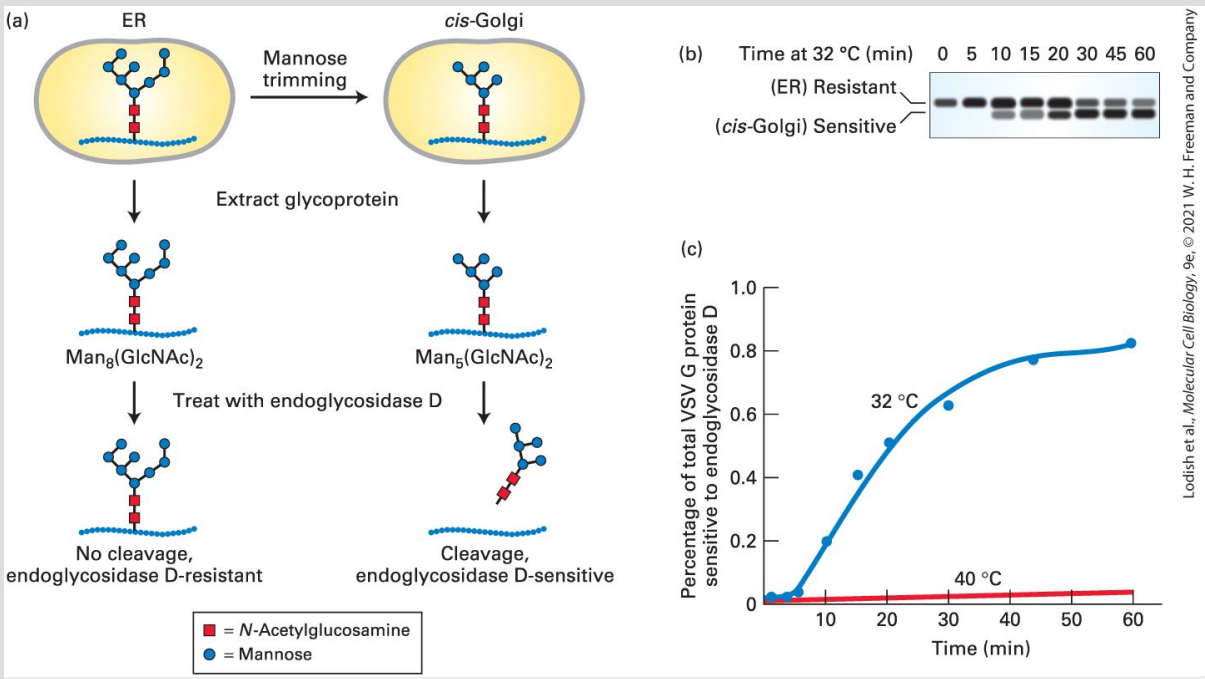
The three micrographs are labeled 0 minutes, 40 minutes, and 180 minutes respectively. In the first micrograph, the fluorescence is restricted to the E R. In the second micrograph, fluorescence is restricted to the Golgi, and in the third micrograph, the fluorescently labeled protein has been delivered to the cell membrane, leading to the fluorescence of the cell surface.

In the graph labeled (b), the vertical axis represents the amount of V Z V G protein labeled with G F P (times 10 to the power 6) ranging from 0 to 20 in increments of 5. The horizontal axis represents time in minutes ranging from 0 to 600 in increments of 100. The red curve corresponding to E R fluorescence starts at (0, 20) and rapidly declines to (0, 100). A green curve, corresponding to fluorescence in the Golgi starts at (0, 0) increases sharply, reaches a maximum at (50, 7.5), and ends at (0,200). The fluorescence of the plasma membrane indicated by a blue curve starts at (0, 0), increases sharply, reaches a maximum at (150, 15), and ends at (6, 600).

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 are carrying one or more copies of the *N*-linked oligosaccharide $\mathrm{Man_8(GlcNAc)_2}$, which are synthesized and attached to secretory proteins in the ER (see Figure 13-18). As a protein moves through the Golgi complex, different enzymes localized to the *cis-, medial-*, and *trans*-Golgi cisternae catalyze an ordered series of modifications to these core $\mathrm{Man_8(GlcNAc)_2}$ chains, as discussed in a later section of this chapter. For instance, glycosidases that reside specifically in the *cis*-Golgi compartment sequentially trim mannose residues off the core oligosaccharide to yield a trimmed form, $\mathrm{Man_5(GlcNAc)_2}$. By monitoring the trimming of the *N*-linked oligosaccharide, scientists can distinguish glycosylated VSV G protein that has entered the *cis*-Golgi from VSV G protein that remains in the ER. A specialized carbohydrate-cleaving enzyme known as *endoglycosidase D* enables the trimming of the *N*-linked oligosaccharide to be readily detected by gel electrophoresis; endoglycosidase D will cleave trimmed *cis*-Golgi-specific oligosaccharides from proteins, but will not cleave the core (untrimmed) oligosaccharide chains on secretory proteins within the ER (Figure 14-4a). Because a deglycosylated VSV G protein produced by endoglycosidase D digestion moves faster on an SDS gel than the corresponding glycosylated protein, these proteins can be readily distinguished (Figure 14-4b).



EXPERIMENTAL FIGURE 14-4 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 were labeled with a pulse of radioactive amino acids at the nonpermissive temperature so that the labeled protein was retained in the ER. At periodic times after a return to the permissive temperature of 32 °C, VSV G protein was extracted from cells and digested with endoglycosidase D. (a) As proteins move to the *cis*-Golgi from the ER, the core oligosaccharide $\mathrm{Man_8(GlcNAc)_2}$ is trimmed to $\mathrm{Man_5(GlcNAc)_2}$ by enzymes that reside in the *cis*-Golgi compartment. Endoglycosidase D cleaves the oligosaccharide chains from proteins processed in the *cis*-Golgi but not from proteins in the ER. (b) SDS-polyacrylamide gel electrophoresis of the digestion mixtures resolves the resistant, uncleaved (slower migrating) and sensitive, cleaved (faster migrating) forms of labeled VSV G protein. Initially, as this gel shows, all of the VSV G protein was resistant to digestion, but over time, an increasing fraction was sensitive to digestion, reflecting transport of the protein from the ER to the Golgi and its processing there. In control cells kept at 40 °C, only slow-moving, digestion-resistant VSV G protein was detected after 60 minutes (not shown). (c) A plot of the percentage of VSV G protein that is sensitive to digestion, derived from electrophoretic data, reveals the time course of ER-to-Golgi transport.

[Part (b) Data from C. J. Beckers et al., 1987, "Semi-Intact Cells Permeable to Macromolecules: Use in Reconstitution of Protein Transport from the Endoplasmic

Reticulum to the Golgi Complex," *Cell* **50**(4):523–34.]

Description

The illustration labeled (a) depicts the experimental assaying of transport of proteins from the E R to the Golgi:

The sequence is as follows:

1. A V S V G protein labeled with M a n 8 (G l c N A c) subscript 2 oligosaccharide (made of eight mannose sugars and two N-acetylglucosamines) is present inside the E R.

2. Transport to the cis-Golgi results in mannose trimming of the oligosaccharide.

3. The glycoproteins are extracted.

4. The glycoproteins are treated with endoglycosidase D, which cleaves the oligosaccharides from proteins originating from cis-Golgi, but not those originating from the E R.

The gel documentation image labeled (b) shows the increase in endoglycosidase D sensitive protein over time: The time of incubation at 32 degrees Celsius in minutes is indicated. There are eight bands, corresponding to 0, 5, 10, 15, 20, 30, 45, and 60 minutes respectively. Electrophoresis channels show dark bands. The dark bands in the first row correspond to proteins resistant (E R) to the endoglycosidase and the dark bands in the second row correspond to those sensitive (cis-Golgi) to it. With time, the sensitive band increases in darkness, indicating concentration of the protein in the cis-Golgi.

The graph labeled (c) plots the percentage of total V S V G protein sensitive to endoglycosidase D against time. The vertical axis of the graph represents percentage of total V S V G protein sensitive to endoglycosidase D, ranging from 0 to 1, in increments of 0.2 percent. The horizontal axis represents time in minutes, ranging from 0 to 60 in increments of 10 minutes. Two curves are plotted, a blue curve, corresponding to incubation at 32 degrees Celsius shows an almost linear increase to 0.6 percent in 30 minutes. After this time, the curve begins to plateau, reaching a value

of 0.8 percent at about 45 minutes. A red curve, corresponding to incubation at 40 degrees shows a slow, linear increase from 0 to about 0.5 percent in 60 minutes.

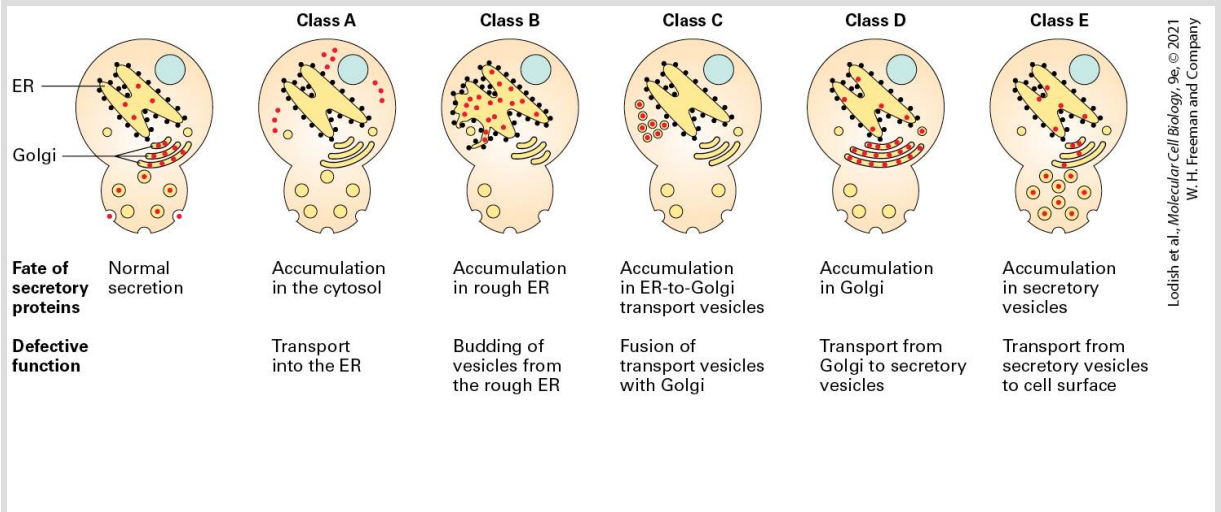
This type of assay can be used to track movement of VSV G protein from the ER to the *cis*-Golgi in virus-infected cells pulse-labeled with radioactive amino acids. Immediately after labeling, all the labeled VSV G protein is still in the ER and, upon extraction, is resistant to digestion by endoglycosidase D. Over time, however, the fraction of the extracted glycoprotein that has received carbohydrate trimming in the *cis*-Golgi and is thus sensitive to digestion by endoglycosidase D increases. Note that transport of VSV G protein from the ER to the Golgi takes about 30 minutes, as measured either by the assay based on oligosaccharide processing or by fluorescence microscopy of VSVG-GFP (Figure 14-4c). A variety of assays based on specific carbohydrate modifications that occur in later Golgi compartments have been developed to measure progression of VSV G protein through each compartment of the Golgi complex.

Yeast Mutants Define Major Stages and Components of 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 identifying many of the proteins that participate in

vesicular traffic. For yeast cells, as for all cells, the secretory pathway is essential for transport and delivery of new protein and membrane to the cell surface. Thus genes encoding important components of the secretory pathway are essential for cell growth and can be studied only as conditional temperature-sensitive mutants, as described in Chapter 8.

A large number of yeast mutants were initially identified by their inability to secrete proteins at a nonpermissive temperature. When these temperature-sensitive ***secretion (sec)* mutants** are transferred from the lower permissive temperature to the higher nonpermissive temperature, they accumulate secretory proteins at the point in the secretory 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 14-5). Subsequent characterization of *sec* mutants in these various classes has helped elucidate the fundamental components and molecular mechanisms of vesicle trafficking that we discuss in later sections.



EXPERIMENTAL FIGURE 14-5 Phenotypes of yeast *sec* mutants identified five stages in the secretory pathway. These temperature-sensitive mutants can be grouped into five classes (A–E) based on the site where newly made secretory 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. Note that organelle structures before the stage blocked by a mutant are exaggerated because the *sec* mutants block movement of membranes as well as cargo. See P. Novick et al., 1981, *Cell* **25**:461; and C. A. Kaiser and R. Schekman, 1990, *Cell* **61**:723.

Description
A normal yeast cell shows proteins in the E R, the Golgi, and in vesicles that are being transported in or out of the cell. The classes of mutants are listed from A to E. Class A mutants accumulated proteins in the cytosol due to defective transport into the E R. Class B mutants accumulated proteins in the rough E R due to defective budding of vesicles from the rough E R. Class C mutants accumulated vesicles used for transport from the E R to the Golgi due to defective fusion of transport vesicles with the Golgi. Class D mutants accumulate proteins in the Golgi due to defective transport from the Golgi to secretory vesicles. Class E mutants accumulate proteins in secretory vesicles due to defected transport of secretory vesicles to the cell surface.

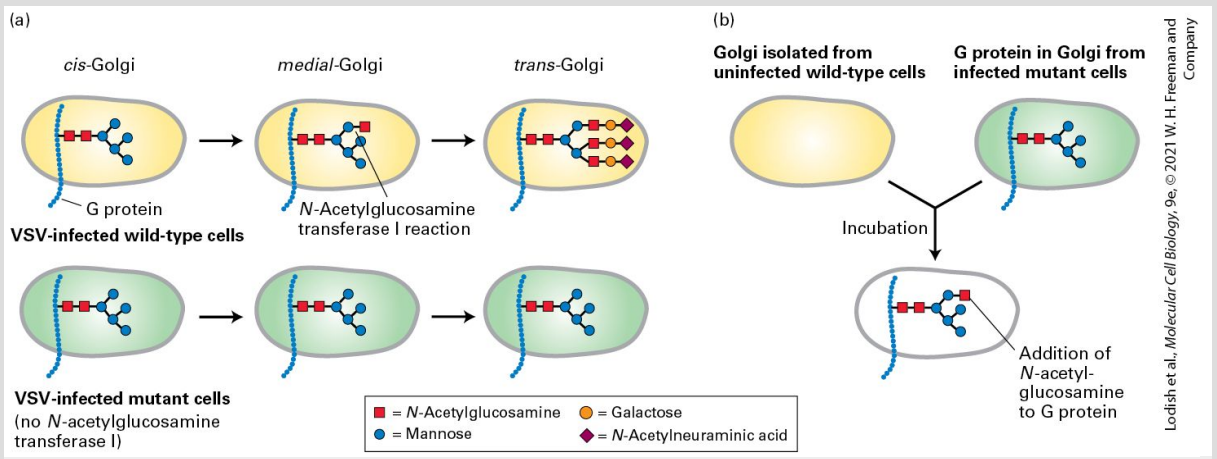
To determine the order of the steps in the pathway, researchers analyzed double *sec* mutants. For instance, when yeast cells contain mutations in both class B and class D functions, proteins accumulate in the rough ER, not in the Golgi cisternae. Because proteins accumulate at the earliest blocked step, 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 to the rough ER, to ER-to-Golgi transport vesicles, to Golgi cisternae, to secretory vesicles, and finally is exocytosed.

Most importantly, the yeast *sec* mutants define many of the genes and encoded proteins that are required for vesicle budding and fusion at each of the major steps of the secretory pathway. Each of the individual steps in the secretory pathway is currently being studied in mechanistic detail, and biochemical assays and protein structural studies are being used to understand each of these steps in terms of the structure and function of individual protein molecules.

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* mutants 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, and the fate of the VSV G protein is followed. 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 14-6a). 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 14-6b). This modification is the consequence of vesicular transport of *N*-

acetylglucosamine transferase I from the wild-type *medial*-Golgi to the *cis*-Golgi isolated from virally infected mutant cells.



EXPERIMENTAL FIGURE 14-6 A cell-free assay demonstrates protein transport from one Golgi cisterna to another. (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 (see step **2** in Figure 14-15). 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 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.

Description

The illustration labeled (a) shows the transport of protein from cis-Golgi to trans-Golgi in both V S V infected wild-type and mutant cells. The illustration shows two fibroblast cultures. One culture contains (V S V)-infected wild-type fibroblasts, while the other contains V S V-infected mutant cells. In the wild-type cells, A V S V G protein in the

cis-Golgi, labeled with a five mannose, two N-acetyl glucosamine oligosaccharide is transported to the medial Golgi. There, N-acetyl glucosamine transferase 1 adds additional N-acetyl glucosamine to the oligosaccharide. On further transport to the trans-Golgi, reactions add additional N-acetyl glucosamine, galactose, and N-acetylneuraminic acid groups the oligosaccharide. The mutant cells do not undergo an N-acetyl glucosamine transferase 1 reaction. Hence, there is no growth of the oligosaccharide chain during the transport of the protein from cis-Golgi to trans-Golgi.

The illustration labeled (b) shows Golgi isolated from uninfected wild-type cells and protein in Golgi from infected mutant cells incubated to form a hybrid Golgi. The hybrid Golgi contains the glycoprotein. The oligosaccharide is found to contain an additional N-Acetylglucosamine in the oligosaccharide attached to the G-protein.

Proteins that play a role in Golgi transport have been identified by fractionation methods. Under appropriate conditions, a uniform population of the transport vesicles that move *N*-acetylglucosamine transferase I from the *medial*- to *cis*-Golgi can be separated 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 membranes. In vitro assays similar in general design to the one shown in Figure 14-6 have been used to study the proteins that play a role at various transport steps in the secretory pathway.

KEY CONCEPTS OF SECTION 14.1

Techniques for Studying the Secretory Pathway

- All assays for following the trafficking of proteins through the secretory pathway require a way to label a cohort of secretory proteins and a way to identify the compartments where the labeled proteins are subsequently located.
- Transport of a fluorescently labeled protein along the secretory pathway can be observed by microscopy (see Figure 14-3). A temperature-sensitive mutant protein that is retained in the ER due to misfolding at the nonpermissive temperature will be released as a cohort for transport when cells are shifted to the permissive temperature.
- Alternatively, pulse labeling with radioactive amino acids can specifically label a cohort of newly made proteins in the ER and the transport of a radiolabeled protein can be 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 14-5).
- 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.