Chapter 4

Compartments of the Early Secretory Pathway

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1. INTRODUCTION

The secretory pathway consists of a set of compartments responsible for the assembly and modification of proteins destined for secretion, for transport to the plasma membrane, and to the other organelles of the cell. The classical description of the secretory pathway comprises the endoplasmic reticulum (ER) as the site of protein synthesis and first maturation steps, and the Golgi apparatus as the site of protein modification and sorting. Apart from synthesis and maturation of secreted proteins the pathway is also responsible for the production of its own components. Hence, the enzymes and factors involved in the functions of the pathway are continually renewed and are being transported to their positions within the pathway by the machinery that transports secretory proteins through it. It is this latter aspect of the pathway, its mechanism of self-renewal, that complicates both the definition and the description of the compartments of the secretory pathway. In this review we will adopt a functional approach to the description of the components of the early secretory pathway and focus on its dynamic aspects. Here the ER will be referred to as the aggregate of smooth ER (sER), rough ER

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(rER), and the nuclear envelope that is continuous with them. The Golgi is defined as the aggregate of cisterna and connected networks on both the *trans* (TGN, *trans*-Golgi network) and *cis* (CGN, *cis*-Golgi network) sides. We will focus on membrane traffic between the ER and Golgi compartment and will present a useful framework for understanding the membrane traffic that gives rise to these compartments.

1.1. Sorting in the Secretory Pathway

A consequence of the ability of the secretory pathway to replenish itself is that the pathway must be capable of sorting its own components. The past several years have brought a great deal of information and some consensus about the mechanisms and signals involved in sorting of proteins along the secretory pathway.

A basic premise is that of bulk flow. Early results studying ER exit of several secretory proteins (Lodish *et al.*, 1983; Scheele and Tartakoff, 1985) and transmembrane proteins (Rose and Bergmann, 1983; Williams *et al.*, 1985) suggested that a positive transport signal existed. These studies showed that transport was dramatically slowed down or stopped by mutations that changed regions of the protein. However, these results are now seen as reflections of differences in the overall structure that lead to different folding efficiencies (see for review Hurtley and Helenius, 1989; Rose and Doms, 1988). The lack of success in identifying positive signals for transport from the ER, coupled with an experiment using tripeptides to determine the bulk flow rate from the ER to the cell surface (Wieland *et al.*, 1987), informed the consensus that transport to the later stages of the exocytotic pathway is by default. Thus, proteins will be transported to the later elements of the pathway if they lack a specific signal for retention (Rothman, 1987).

Given bulk flow as the mechanism for the transport of most proteins, one can define three phenomenologically distinct sorting mechanisms utilized along the pathway (Warren, 1987). The first is retention in a compartment by direct and continuous interaction with its components. The ribophorins and components of the translocation machinery appear to exemplify this mechanism (Crimaldo *et al.*, 1987; Kreibich *et al.*, 1978). The second is selective signal mediated retrieval of components from downstream compartments. Retrieval of soluble proteins of the endoplasmic reticulum serves as the prototype for this mechanism (Dean and Pelham, 1990; Munro and Pelham, 1987; Pelham, 1989a). The third mechanism is the selective inclusion of components into a transport vesicle for transport downstream. This mechanism is used when there is a bifurcation in the pathway. For example, in the *trans*-Golgi network of a polarized cell, lysosomal, apical, and basolaterally directed proteins are separated (Griffiths and Simons, 1986;

Mellman and Simons, 1992). The first two mechanisms require that the interactions responsible for sorting continue for the life of the protein (Simons and Fuller, 1985). Hence, the signal must be a permanent part of the protein. This need not be true in the third case in which the signal can be recognized once and the protein diverted as a result.

Most of the identified sorting signals in the early secretory pathway are remarkably simple and well defined. The best-characterized is that of the carboxy-terminal four amino-acid (KDEL) signal for retention in the endoplasmic reticulum. Here, mutagenesis has clearly demarcated the functionally important sequence and demonstrated its function upon transfer to other proteins (Hardwick *et al.*, 1990; Munro and Pelham, 1987; Semenza *et al.*, 1990; Zagouras and Rose, 1989). Parsimony, the need for a limited number of receptors to recognize similar signals on a wide variety of different proteins, probably leads to a modular nature for most signals and hence their recognizability and apparent simplicity. Despite this apparent simplicity, closer examination of the evidence suggests that context is very important for the recognition and function of most signals. Further, the simplicity of the recognized signals has contributed to their identification, and other signals may be more complex. For example, three-dimensional structure is probably very important for signals that are responsible for static retention since interaction between distinct pairs of components is responsible for this phenomenon. This aspect makes these signals particularly difficult to characterize in terms of a defined sequence, and thus far none have been characterized completely.

2. COMPARTMENTS

What do we mean by the term "compartment," which we have been using so freely? Initially, compartments were defined morphologically. Electron microscopy reveals clear distinctions between smooth and rough ER, Golgi elements, and transport vesicles (Fawcett, 1981). This definition proved particularly appealing because biochemical work showed that the compartments associated with different morphologies had different components and functional activities (Palade, 1975). Unfortunately, the morphological definition or even the straightforward biochemical definition of a compartment is not useful for the description of compartments in terms of membrane traffic. For our task we need to adopt a very strong and restrictive definition that will give rise to predictable consequences in terms of membrane traffic. Such a definition will allow us to focus on the aspects of sorting and transport in the early secretory pathway. Many of the morphologically defined components of the secretory pathway, such as smooth

and rough ER, will fulfill neither this stronger definition of compartment nor some of its consequences.

2.1. Definition of a Compartment

Our definition is very simple. A compartment of the secretory pathway is defined as a collection of membrane-bound structures in which the membranes are physically discontinuous from other compartments and which has a unique set of proteins characteristic for the compartment (see Figure 1). These two aspects, physical discontinuity and unique components, are obviously selected with an eye toward describing features of membrane traffic between compartments. Physical discontinuity can be assayed by morphology and by studying the distribution of freely diffusing membrane or soluble proteins in the putative compartment. These two provide a check on each other. Verifying the uniqueness of components in a given compartment is complicated by the fact that the renewal of compartments requires that residents of one compartment are transiently present in others. The question of thresholds of detection must be considered. Because our interest is in transport, we will employ a threshold of detection that is relevant for transport. As a result, very sensitive measures are not needed to demonstrate either physical discontinuity or uniqueness of components. Two membrane structures are physically continuous when their degree of continuity obviates the need for membrane transport between them. It is very difficult to demonstrate that two compartments of complex geometry have no continuities between them. However, a degree of continuity that is so small that it is difficult to detect will also be relatively insignificant in terms of transport. In none of the cases that we will examine are the elements of the definition equivocal. Continuity is always extensive and obvious, and the localization of the bulk of components is always well defined in a given condition.

An important aspect of our operational definition of a compartment is the requirement for measuring several components simultaneously. If we wish to understand the membrane traffic between compartments, then we need to know the compartment size and composition accurately. Frequently, the description of a component of a putative compartment is performed in a very qualitative way. This leads to apparent contradictions. We will discuss the compartment(s) intermediate between the ER and the Golgi at some length below. Unfortunately, many different functions have been attributed to this portion of the secretory pathway and little dual labeling and quantitation is available so that even the existence of this (these) compartment(s) remains in dispute. Faced with such confusion it is tempting to simplify the early secretory pathway to a single compartment (Mellman and Simons, 1992). However, we feel it is better to take the confusion as a measure of the state of the field and focus our attention on the need for accurate descriptions.

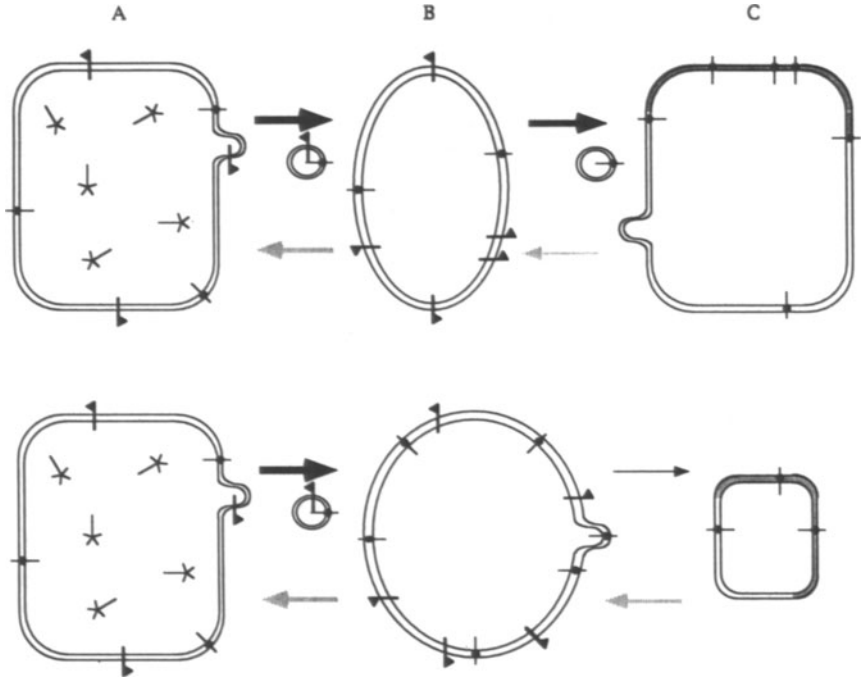

FIGURE 1. Schematic representation of our compartment definition and some of its consequences. A compartment is discontinuous from its upstream and downstream counterparts and contains unique components. Proteins ✳— and ▼— represent unique proteins of compartment (A) and (B), respectively. As depicted, compartment (B) contains none of the compartment (A) resident ✳— s; however, in addition to its own unique marker (▼—) it processes the protein —●— on transit to compartment (C). Owing to the existence of unique resident proteins, each compartment must have its sorting machinery. Traffic to and from the compartments is mediated by transport vesicles. Because a compartment has unique components, the upstream and downstream vesicles must be different. Differences in regulation of vesicular transport leading to and coming from a compartment render it dynamic. The dynamics can influence subdomains differently, as depicted in the lower panel of the figure.

2.2. Consequences of the Definition

2.2.1. Two Types of Transport Vesicles

Having established a definition, we can proceed to deduce consequences from it. The first consequence of our definition, and the one of most importance for a description of membrane traffic, is that there must be transport vesicles mediating the traffic to and from the compartment. This can be seen immediately as our definition allows no other communication between the compartment and the rest of the secretory pathway. The premise that the compartment has unique components leads to the conclusion that the vesicles mediating traffic to and from the compartment will be distinct. If this were not the case, the compartment would not be able to maintain a unique composition. If the transport vesicles are distinct then they can be regulated separately and as a result the compartment would be dynamic with respect to size and composition. This dynamism cannot be unconstrained; if otherwise, the compartment would lose its identity. Hence, there must be some components that are responsible for maintenance of size.

2.2.2. Uniqueness of Components

Three classes of components need to be distinguished. The first are the resident proteins, which are unique. These impart a unique function to the compartment (e.g., isomerization, glycosylation, etc.). As we are focusing on the functional significance of components, it is the location of the bulk of the marker that is important, not the location of all of the marker. The soluble proteins of the endoplasmic reticulum are a case in point (Dean and Pelham, 1990; Lewis and Pelham, 1992; Pelham, 1988). A second class that is characteristic but not unique are the proteins in transit to other compartments, such as the plasma membrane, endosomes, and lysosomes. Proteins involved in the vesicular transport machinery itself represent the third class of components. The rab (rat brain) proteins or the coatamer elements are examples of these (described in more detail later). These proteins can be better characterized as representing the (vesicular) connection between an upstream and its downstream compartment, rather than serving as a unique marker for an individual compartment.

2.2.3. Sorting

The presence of transport vesicles gives rise to a need for sorting mechanisms. Two cases exist. If bulk flow operates in the compartment, then there must be retention signals to remove components from the flow and establish them in the compartment. The second case is one in which the pathway bifurcates and, hence, some components must be selectively diverted from the on-

ward bulk flow due to the presence of specific signals. (The above-mentioned features of a compartment are depicted in Figure 1.)

2.2.4. Viruses

The budding of membrane viruses serves as a useful indicator for the operation of the features of a compartment (Simons and Fullèr, 1987). This follows from several key features of enveloped virus budding. Enveloped viruses bud as a result of interaction of their envelope proteins with the internal components (Simons and Fuller, 1985). The attachment of internal components can only commence when sufficient glycoproteins have clustered to form an attachment site (Fuller, 1987). Often, clustering, and budding, will only occur when the glycoproteins are properly oligomerized or processed. The clustering also excludes the membrane proteins of the host, which leads to the lack of incorporation of host proteins into the budded virion (Simons and Fuller, 1987). After the attachment site has formed and internal structures interact with and stabilize the cluster, a reorganization must occur to allow the deformation of the envelope that accompanies budding. The initial clustering must represent some tendency of the envelope proteins to interact weakly since reorganization of the envelope proteins would be hindered by strong interactions with cellular proteins or each other (Fuller, 1987). To form such clusters by weak interaction, the membrane glycoproteins must be able to diffuse in the membrane. As a result, they cannot be concentrated in a compartment unless that compartment is physically discontinuous. Further, unless transport has been blocked, the localization of the bulk of the protein reflects the sorting mechanisms of the cell. In this way, the observation that a structure is the unique site of virus budding is a strong indicator that it is functioning as a compartment.

The use of virus budding as a compartment marker needs to be accompanied by the caveat that virus infections produce cytopathic effects. In some cases these can affect the composition of the compartments of the secretory pathway. Therefore, one needs to be sure that the distribution of other markers of the compartment is not disturbed by the infection itself.

2.2.5. Unique Environment

A consequence of the unique components of a compartment is that it is able to offer a unique environment for particular functions. Among the critical elements in such an environment are its pH, its reduction potential, its divalent ion concentration, and the concentration of substrates for enzymes resident in the compartment. For example, the exchange of disulfides in the ER is possible only because the ER contains an oxidizing environment, a non-acidic pH to allow exchange, and the enzyme protein disulfide isomerase that catalyzes the ex-

change so it can occur at a physiological time scale (Freedman, 1987; Freedman and Hillson, 1980; Freedman, 1984).

2.2.6. Degradation

A compartment as we have defined it must also have a pathway for degradation. This is a consequence of the need for retention mechanisms and the finite lifetimes of proteins (including the ones retained in a given compartment). One can imagine several mechanisms ranging from the selective recognition and sequestration of aberrant proteins to interference with the retention signals so that bulk flow brings proteins to another compartment for degradation. There is good evidence that degradation is under fine control and mediated by a variety of mechanisms (Bonifacino and Lippincott-Schwartz, 1991; Chau *et al.*, 1989; Klausner and Sitia, 1990).

2.3. Example of the Plasma Membrane

Before proceeding to describe the early secretory pathway in terms of compartments, we would like to show how our definition and its consequences apply to the less controversial case of the plasma membrane. In an epithelial cell there are two compartments called plasma membrane: the apical membrane and the basolateral membrane. Physical discontinuity is easily shown since these compartments are separated by the tight junction. The outer leaflet of the bilayer is broken by this structure although the inner leaflet is connected (Simons and van Meer, 1988; van Meer, 1989). The consequence of this discontinuity is that membrane proteins and outer membrane lipids must enter and leave these two compartments by vesicular transport. Indeed, transcytosis is well described for this system and a start toward the characterization of the vesicles involved in transport to the plasma membrane has been made (Sztul *et al.*, 1991; Wandinger-Ness *et al.*, 1990).

Subdomains can be found in the plasma membrane compartments, and they provide a useful example of structures that fail to meet our definition. A simple one is provided by the microvillar domains. Although these have unique components, these domains are incapable of confining freely diffusible components. It is worth pointing out that these subdomains can be isolated biochemically. Hence, biochemical isolation does not serve as a reliable indicator of compartmentalization.

Viruses provide an interesting illustration of the nature of the plasma membrane compartments. It is well established that vesicular stomatitis virus (VSV) buds from the basolateral compartment while influenza virus buds from the apical surface. This budding polarity reflects the sorting of the associated membrane glycoproteins: VSV-G protein is sorted to the basolateral surface while the

influenza haemagglutinin (HA) is directed to the apical surface (Hughson *et al.*, 1988; Simons and Fuller, 1985; Simons and Fuller, 1987). Hence, virus budding provides a reliable marker for compartmentalization. Both of these viruses are cytopathic and eventually cause a loss of integrity of the tight junctions separating the two compartments so that diffusible components mix. Under these conditions, virus budding loses its polarity so that the two viruses can be found budding from both surfaces (Roth and Compans, 1981).

3. ENDOPLASMIC RETICULUM

3.1. Morphology and Freely Diffusible Markers

The endoplasmic reticulum (ER) is the cell's largest compartment and typically constitutes more than half of the total cellular membrane (Weibel *et al.*, 1969). The ER provides the cell with a mechanism of segregating newly synthesized transmembrane, luminal, and secretory proteins from the cytoplasm (Palade, 1975; Walter and Lingappa, 1986). Although the ER can be divided in distinct regions (see below), morphological studies indicate that it is a continuous membrane structure (Fawcett, 1981), physically distinct from the downstream Golgi. These findings have been confirmed in experiments using the transmembrane G protein of VSV (Bergmann and Fusco, 1990) and the luminal heavy chain binding protein (BiP) (Ceriotti and Colman, 1988), which indicated that these proteins behaved as freely diffusible markers between the ER subdomains. Therefore, according to our definition of a compartment, these morphologically defined subdomains should be regarded as subcompartments, something that will be reflected in the consequences of the definition.

3.2. Subcompartments

The rough ER (rER), which is studded with ribosomes, is morphologically easily distinguishable from the smooth ER (sER), which is physically a portion of the same membrane but lacks attached ribosomes. Although many proteins are common to both subdomains, some such as ribophorin I and II (Amar-Costesec *et al.*, 1984), the docking protein (Hortsch and Meyer, 1985), or the originally designated signal sequence receptor subunits (Görlich *et al.*, 1990; Wiedmann *et al.*, 1987) are restricted to the rER, whereas one, epoxide hydrolase, has been reported to be restricted primarily to the sER in rat liver (Galteau *et al.*, 1985).

Apart from the rER and the sER, two other subdomains can be identified. The nuclear envelope (NE), which is continuous with the rER and also contains ribosomes, possesses unique structures such as the nuclear pore complexes as well as unique proteins that interact with nuclear lamins in the inner surface

(Gerace *et al.*, 1984). Another subdomain is the transitional elements (TE), which are smooth, vesicular-tubular structures facing the *cis*-Golgi and which are believed to be the ER exit sites (Palade, 1975).

The fact that there are components specific for ER subdomains indicates that some special restraining mechanisms must exist. Although the exact underlying mechanisms are still unclear, they might include complex formation between specific protein and/or lipid components in the bilayer, which exclude other proteins, and protein interactions on either side of the membrane.

3.3. Unique Proteins

In addition to the proteins mentioned above, which are enriched in specific subdomains, the ER lumen contains a set of resident proteins that catalyze the maturation events of a nascent polypeptide chain. The most abundant members are the *g*lucose-*r*egulated *p*roteins GRP78 (more commonly known as the heavy chain *b*inding *p*rotein, BiP), GRP94, and *p*rotein *d*isulfide *i*somerase (PDI). PDI is a multifunctional protein. Existing as a homodimer, it catalyzes the isomerization of intramolecular disulfide bridges, which is the rate-limiting step in the folding of many proteins (Freedman, 1984). But PDI is also the β-subunit of the $\alpha_2\beta_2$ tetramer of prolyl hydroxylase (Pihlajaniemi *et al.*, 1987) and has been reported to be an important component of the oligosaccharide transferase (Geetha-Habib *et al.*, 1988).

The binding protein BiP is not only involved in the translocation process of some proteins (Rose *et al.*, 1989), but also catalyzes folding by preventing aggregation of folding intermediates (Gething and Sambrook, 1990; Pelham, 1989a). Although BiP is not a heat-shock protein (hsp) in mammals, sequence homology indicates that it is a member of the hsp70 family of heat-shock proteins (Munro and Pelham, 1986), which are believed to mediate folding and stabilization of unfolded proteins in other compartments of the cell. Moreover, BiP was shown to be an ATPase that is released from its substrate by ATP hydrolysis (Flynn *et al.*, 1989; Kassenbrock and Kelly, 1989). Although the function of the calcium binding glycoprotein GRP94 has not yet been well defined, recent reports suggest that GRP94 also possesses protein-binding properties when malfolded proteins are retained in the ER (Navarro *et al.*, 1991).

3.4. Consequences of the Definition for ER

3.4.1. Upstream and Downstream Transport Vesicles

The most important consequence of our compartment definition is that membrane traffic between compartments must be mediated by transport vesicles. Furthermore, the upstream vesicles leading to the compartment must be different

form the downstream vesicles. Based on the experiments mentioned above, which utilized BiP and VSV-G as freely diffusible markers, it is clear that this consequence does not apply to the individual ER subdomains.

The ER is an obvious exception concerning vesicular transport as means of influx of an upstream compartment. However, transmembrane, luminal, and secretory proteins are selected from the cytoplasm and targeted to the ER membrane after formation of *s*ignal *r*ecognition *p*article (SRP)-ribosome-nascent chain complex (Meyer and Dobberstein, 1980a; Meyer and Dobberstein, 1980b; Walter and Blobel, 1982) . The targetting step is facilitated via a specific interaction of SRP with its receptor, the docking protein (Meyer *et al.*, 1982). After docking and release of the SRP, translocation of the polypeptide chain takes place. Although the translocation mechanism is still unknown, several proteins have been identified that are proposed to constitute a translocation complex (Görlich *et al.*, 1992; see for review High and Dobberstein, 1992; Nunnari and Walter, 1992).

Downstream traffic from the ER is mediated by transport vesicles (Palade, 1975). Accumulating evidence suggests that the transport from a donor to an acceptor compartment can be divided into the steps of budding, targetting, and fusion. By selecting for temperature-sensitive mutants blocked early in the yeast secretory pathway, Schekman and co-workers have identified groups of synthetic lethal *sec* (*sec*retory) genes (Kaiser and Schekman, 1990; Novick *et al.*, 1980). Utilization of the powerful yeast genetics system, in combination with *in vitro* reconstitution of protein traffic, led to the identification of at least 15 genes whose products are involved in trafficking between the ER and the Golgi apparatus (Schekman, 1992). The product of one of the genes, *sec*18p, seems to be required for fusion of the budded transport vesicles with the Golgi. In mutant *sec*18 yeast cells, glycosylated proteins destined for secretion remain EndoH (endoglycosidase H) sensitive and accumulate in transit vesicles during incubation at a restrictive temperature. Apart from identifying genes whose products are involved in vesicle formation or fusion, this approach also led to the demonstration that these proteins function in complexes with other (*sec*)proteins and helped to define the localization of sorting and enzymatic events. Additional components that function in ER to Golgi transport in conjunction with these *sec* proteins—for example, two *bet* mutants (*b*locked *e*arly in *t*ransport; Newman and Ferro-Novick, 1987) and *bos*1(*b*et *o*ne *s*uppresser; Shim *et al.*, 1991)—have now been isolated.

Although the *sec* genes were originally identified in yeast, the importance of these proteins for secretory pathway function has led to such sequence conservation that mammalian homologues for several have been identified. For example NSF (*N*-ethylmaleimide-*s*ensitive *f*actor) is the mammalian homologue of *sec*18p (Wilson *et al.*, 1989), and αSNAP (*s*oluble *N*SF *a*ttachment *p*rotein) has been identified as the *sec*17p counterpart (Clary *et al.*, 1990). These components of

the vesicular transport machinery are used extensively to manipulate traffic between the ER and Golgi (see below).

The consensus is that non-clathrin-coated vesicles (Fine, 1989) bud from the TE of the ER and are transported to a downstream acceptor compartment to which fusion takes place. If one regards, for matter of comparison, the SRP-ribosome-nascent chain complexes as the ER's equivalent of "upstream vesicles," then they are clearly distinct form the downstream non-clathrin-coated vesicles.

3.4.2. Regulation and Dynamics

Cell-free and permeabilized cell transport systems allowed a further dissection of the biochemical and molecular basis of individual steps in vesicular traffic, thereby gaining information concerning the regulation of vesicular transport from the ER. Transport in these systems requires cytosol and an ATP energy source (Baker *et al.*, 1988). ER to Golgi transport is also dependent upon physiological concentrations of Ca^{2+} (Beckers and Balch, 1989). These systems have also been used to investigate the role of GTP binding proteins in the regulation of transport (see below).

Indeed, the ER has been proven to be highly dynamic. For example, it can expand greatly upon an enlarged synthesis of the luminal enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase (Bergmann and Fusco, 1990) or upon increased load of transport-incompetent protein (J. Tooze *et al.*, 1989). Also, after exposure to drugs, such as phenobarbitol, the amount of smooth ER rapidly and reversibly increases (Jones and Fawcett, 1966). Furthermore, in response to the drug nocodazole, the ER membranes collapse, which suggests that dynamics of the ER are dependent upon intact microtubules (Terasaki, 1990).

3.4.3. Bulk Flow and Retention Signals

The consensus hypothesis of bulk flow requires that resident proteins possess a retention signal. The best-studied example of such a retention signal is that of the carboxy-terminal–KDEL signal for soluble resident ER proteins. These proteins include BiP and a whole family of glucose-regulated proteins, PDI, and a series of esterases (Pelham, 1989a; Pelham, 1990; Pelham, 1991). Many of these proteins are involved in the catalysis of protein folding and are present in the ER lumen at high concentrations. Protein disulfide isomerase, for example, is present in the ER at concentrations of nearly 1 mM (Freedman and Hillson, 1980; Freedman, 1984; Weibel *et al.*, 1969). The current retention model for soluble resident ER proteins envisages a recycling mechanism (reviewed in Pelham, 1989a) and will be discussed below.

Although a membrane protein has been identified in yeast that seems to be

sorted by the HDEL (histidyl-aspartyl-glutamyl-leucine) retention system (Sweet and Pelham, 1992) this does not seem to be the common mechanism for ER membrane protein retention. For many membrane proteins, residence appears to result from their tendency to form large aggregates (e.g., in the case of components of the translocation machinery) and/or by interactions between their membrane spanning/ectoplasmic domains and other, as yet unidentified, cellular structures (see below). Several transmembrane ER proteins contain a retention motif in their cytoplasmically exposed tails, consisting of two lysines positioned three and four or five residues from the C-terminus (Jackson *et al.*, 1990). This motif was first recognized in the adenoviral transmembrane E3/19K glycoprotein (Nilsson *et al.*, 1989), a protein that binds after synthesis immediately to human major histocompatibility complex class I antigens, thereby retaining them in the ER compartment. The retention does not seem to be based on simple charge interactions, since arginines or histidines cannot replace lysines. It is not yet clear how deeply proteins carrying this x-K-(x)-K-x-x signal can penetrate into the secretory pathway. The report that p53, a marker of intermediate compartment, contains this signal (Hauri and Schweizer, 1992) opens the possibility that these proteins are also retained by recycling.

Although retention signals have recently been identified in transmembrane domains, the exact signal determinants are less clear. Within the alpha chain of the multisubunit T-cell antigen receptor (TCR) complex, a transmembrane sequence containing two basic amino acid residues has been shown to act as a determinant for retention and rapid degradation in the ER. Furthermore, it was demonstrated that single basic or acidic amino acid residues can cause this targetting when placed in a context-dependent manner within the transmembrane domain of the Tac antigen, a membrane protein normally destined for the cell surface (Bonifacino *et al.*, 1991). The phenotypic changes induced by potentially charged transmembrane residues occur without apparent alterations of the global folding or transmembrane topology of the mutant proteins. These observations suggest the ability to induce protein-protein interaction by placing charged pairs within transmembrane domains, thereby masking specific retention and degradation signals once oligomerization occurred (Cosson *et al.*, 1991). Cloning and expression of two types of cDNAs encoding the H2 subunit of the human asialoglycoprotein receptor, differing only by the presence of a 15-bp (base pair) mini-exon encoding five amino acids (Glu-Gly-His-Arg-Gly) immediately exoplasmic to the single membrane-spanning segment, indicated the operation of a similar retention and degradation mechanism (Lederkremer and Lodish, 1991).

3.4.4. Unique Environment and Unique Functions

The ER is a highly dynamic structure possessing specific retention mechanisms to enable the preservation of its resident components. These unique com-

ponents create a unique environment in the ER lumen that is intermediate between the cytosol and the extracellular space. The pH is probably close to or somewhat above neutral, and the Ca^{2+} concentration is in the millimolar range (Suzuki *et al.*, 1991). The redox conditions in the lumen are more oxidizing than those in the cytosol (Hwang *et al.*, 1992). This is particularly important for disulfide bond formation and therefore for the folding conditions in the ER. The unique environment provides the means for specific ER-related functions.

3.4.4a. Protein Folding in the ER. As described before, the components of the targetting and translocation machinery make the ER a site of protein biogenesis. The folding of proteins translocated into the ER lumen usually begins on the nascent chain (Bergman and Kuehl, 1979). The number of proteins that have been characterized in detail, with respect to the *in vivo* process of protein folding within the ER, is small, but some interesting features of the process have emerged. The majority of studies concerning the role of protein conformation and ER exit rate are being performed using the viral proteins influenza virus hemagglutinin (HA) and VSV glycoprotein (VSV-G) as markers. Both HA and VSV-G are well-characterized type I transmembrane glycoproteins. Upon translocation into the ER the ectodomains become *N*-linked glycosylated. Both proteins form homotrimers, which are transported via the Golgi complex to the plasma membrane (for reviews see Burgess and Kelly, 1987; Simons and Fuller, 1985). In addition, they are stabilized by intrachain disulfide bonds.

Using very short radioactive pulses, Braakman and colleagues (Braakman *et al.*, 1991) analyzed the folding of the hemagglutinin precursor, HA0, in tissue culture cells. The folding rate was shown to be independent of expression level, but highly dependent on cell type and expression system, indicating that the folding kinetics were also determined by the cellular environment (Braakman *et al.*, 1991). The efficiency of folding and subsequent trimerization was not dependent on the rate of translation, nor on temperature between 37°C and 15°C. Trimerization itself was accelerated at higher expression levels and seems to be necessary for transport to the Golgi. However, evidence from various mutant VSV-G proteins suggests that trimerization alone is not sufficient for transport (Doms *et al.*, 1988).

The folding of wildtype HA0 was elegantly analyzed in more detail in a further set of studies. Reduction of HA0 monomers, by addition of the reducing agent dithiothreitol to the medium of living cells infected with influenza virus, suggests that the locally folded structures of the HA0 molecule are stabilized by disulfide bonds (Braakman *et al.*, 1992a). Proper folding of HA requires correct disulfide bond formation and glycosylation, and is energy dependent (Braakman *et al.*, 1992b). Experiments manipulating the cellular ATP level revealed that metabolic energy was required to secure the correct folding of newly synthesized HA0, to rescue misfolded HA0 molecules from disulfide cross-linked aggregates, and to maintain already folded monomeric HA0 in a folded state. Inter-

estingly, the rescue of a temperature-sensitive folding mutant of VSV-G, tsO45, from the aggregate after switch to the permissive temperature also depends on the presence of ATP (Doms *et al.*, 1987).

Although it is indisputable that the primary sequence determines the ultimate three-dimensional structure of a protein, it is clear that the folding process in the ER is facilitated by a set of resident proteins, the *chaperones*. Chaperones are proteins that enhance the folding or assembly efficiency but do not cause covalent modifications or become part of the final folded protein structure. The chaperones present in the ER lumen include BiP, GRP94, and various forms of *cis-trans* proline isomerase (Freedman, 1987). Association with the chaperones helps prevent aggregation of folding intermediates. In addition, association with chaperones might also contribute to the retention of improperly folded proteins (Pelham, 1989a).

3.4.4b. Glycosylation. Many proteins become glycosylated during their passage through the secretory pathway. Initial glycosylation steps start in the ER where necessary components are available. Here a lipid-linked oligosaccharide is synthesized and transferred en bloc to a nascent polypeptide chain (Kornfeld and Kornfeld, 1985). Subsequent trimming reactions of the oligosaccharide occur and may continue by further trimming reactions followed by addition of peripheral sugars in the Golgi apparatus (Roth, 1987). Various experimental observations suggest that glycosylation contributes to the folding efficiency of glycoproteins (Gallagher *et al.*, 1988; Hearing *et al.*, 1989), probably by stabilizing or shielding particular segments on the protein surface and/or by increasing the solubility properties of the folding intermediates (Marquardt and Helenius, 1992).

3.4.4c. Quality Control. Several thousand different newly synthesized proteins enter the lumen of the ER of a mammalian cell. Out of the pool of newly synthesized proteins, all functionally competent proteins have to find their appropriate localization, whereas misfolded or unassembled polypeptides need to be eliminated to prevent possible toxic effects. In the work on viral proteins described above, the unfolded or misfolded viral glycoproteins were not able to trimerize, nor to be transported to the *medial*-Golgi. This is consistent with a whole set of studies utilizing mutants of HA or VSV-G with alterations in the cytoplasmic tail (Doyle *et al.*, 1985; Gething *et al.*, 1986), in the transmembrane domain (Doyle *et al.*, 1986), or in the luminal portion (Doms *et al.*, 1988). In all cases, mutant proteins that fail to be transported to the Golgi apparatus remain in a partially unfolded state and/or are not efficiently assembled into native, trimeric structures. The misfolded proteins often form aggregates that generally contain aberrant disulfide cross-links and BiP as noncovalently associated component (Hurtley *et al.*, 1989; Marquardt and Helenius, 1992). The aggregation process itself probably reflects the poor solubility of incompletely folded polypeptide chains. A role for free thiol groups in preventing the unhindered transport

of proteins through the secretory pathway has also been proposed (Alberini *et al.*, 1990). Together, these observations suggest mechanisms for quality control; however, much more needs to be learned for a complete understanding.

3.4.4d. Degradation. Because the folding and maturation processes in the ER are not completely efficient there should be a degradation mechanism that allows for the elimination of misfolded or unassembled proteins. In a variety of studies a pre-Golgi proteolytic pathway has been characterized for rapid degradation of newly synthesized T-cell receptor (TCR) subunits, which is insensitive to drugs that block lysosomal proteolysis or ER to Golgi transport (Klausner *et al.*, 1990; Lippincott-Schwartz *et al.*, 1988). The site of degradation in this pathway is either part of, or closely related to, the ER. A similar pathway has been identified for the degradation of unassembled asialoglycoprotein receptor subunits (Amara *et al.*, 1989), of transport-impaired PiZ α_1-antitrypsin variants (Le *et al.*, 1990), and of carboxy terminally truncated forms of ribophorin I (Tsao *et al.*, 1992). This "ER" degradative pathway probably plays an important role in many cells in the removal of unassembled or incompletely assembled membrane protein complexes from the secretory pathway.

3.4.5. Virus Budding and Protein Localization

Although many of the best-characterized enveloped viruses bud through the plasma membrane, some viral families bud into intracellular compartments. The issue of the precise site of budding is complicated by the cytopathic effects of virus infection as by the fact that individual virus proteins may change their localization when expressed separately.

3.4.5a. Rotavirus. The Rotaviruses are a family of nonenveloped, double-stranded RNA viruses. Their site of budding is the ER. This budding can occur through ribosome-free regions of the ER, as well as into the nuclear envelope (Estes, 1990). The crucial event for this process seems to be an interaction between the structural viral glycoprotein (VP6) and the cytoplasmic domain of the nonstructural protein NS28 (Meyer *et al.*, 1989). After budding into the ER, the immature virus is enclosed by a lipid bilayer in which the glycoproteins NS28 and VP7 are embedded. Both these proteins behave as typical ER resident membrane glycoproteins, something that is reflected in the nature of their *N*-linked oligosaccharides (Kabcenell and Atkinson, 1985; Kabcenell *et al.*, 1988). Thus, NS28 and VP7 must contain targetting information that retains them after synthesis in the ER, allowing accumulation of these proteins above a threshold level for budding. Indeed, for NS28 it was shown that the second of the three hydrophobic domains is responsible for its retention in the ER (Bergmann *et al.*, 1989). In the case of VP7, the available evidence suggests that the signal sequence and presumably the extreme N-terminus of the mature protein (Griffiths and Rottier, 1992; Stirzaker and Both, 1989) are essential for its retention in the

ER membrane. Although rotavirus particles bud into the ER, they do not enter the secretory pathway and are only released following cell lysis. After budding into the ER the lipid bilayer as well as the NS28 is lost from the virus surface, leading to mature, infectious virus particles (for reviews see Estes, 1990; Estes and Cohen, 1989).

3.4.5b. Rubella Virus. Rubella virus (RV) is an enveloped, positive-stranded RNA virus. The RV virions contain two membrane envelope glycoproteins, E1 and E2, which form a heterodimeric complex. RV budding has been reported to occur preferentially from internal membranes at the Golgi region in baby hamster kidney (BHK) cells and at the plasma membrane in an African green monkey kidney (Vero) cell line (Bardeletti *et al.*, 1979; von Bonsdorff and Vaheri, 1969). Studies utilizing Chinese hamster ovary (CHO) cells expressing RV glycoproteins reveal that transport of E1 to the Golgi complex and plasma membrane is dependent upon interaction with the E2 glycoprotein (Hobman *et al.*, 1990; Hobman *et al.*, 1992). Unassembled subunits of RV E1 accumulate in a tubular network of smooth membranes that are in continuity with the rER. Furthermore, although this structure was shown to possess distinct properties from either the rER and the Golgi, luminal ER proteins bearing the KDEL signal and the transmembrane tsO45 VSV-G mutant accumulated at the nonpermissive temperature have access to it (Hobman *et al.*, 1992), indicating that it is a subcompartment of the ER rather than a separate entity. The site of E1 arrest appeared distal to or at the site where palmitoylation occurs and proximal to the low temperature 15°C block. These findings suggest that the site of E1 arrest corresponds to, or is located close to, the exit site from the ER, although the site of rubella budding is later in the secretory pathway.

3.4.5c. Coronavirus. Coronaviruses are enveloped animal viruses with a single positive-stranded RNA genome, possessing two viral membrane glycoproteins, E1 and E2. Several features of coronavirus make the identification of the budding site equivocal, although some early work ascribes an ER localization for its maturation. The budding of coronavirus at early times in infection has been described to occur in a smooth-surfaced, tubulo-vesicular structure, whose membranes were associated with rough ER cisternae, including apparent transitional elements, and were also present close to the *cis* face of the Golgi stack (Tooze *et al.*, 1984; Tooze *et al.*, 1988). At later stages of infection, coronavirus buds into the rough ER and nuclear envelope. Although this process clearly can occur in the ER, the exact budding site is controversial. In part that results from the lack of available intermediate compartment markers in the early studies, a lack of consensus between results using different coronavirus strains in studies expressing individual (mutated) viral proteins, and the possible cytopathic effect of the infection on the secretory pathway. The budding process involves an interaction between the nucleocapsid and the viral glycoprotein E1 (Spaan *et al.*, 1988). This protein contains three membrane-spanning regions, each of which

can individually insert and anchor the polypeptide in the membrane (Krijnse-Locker *et al.*, 1992). Most likely E1 forms a complex with a second membrane glycoprotein, the E2 protein. The restricted intracellular localization of the E1 protein is believed to play a major role of directing virus assembly at intracellular membranes (Tooze *et al.*, 1984). In the case of avian infectious bronchitis virus (IBV), this protein, expressed by itself, localizes to the tubulo-vesicular/*cis*-Golgi region (Machamer *et al.*, 1990). However, in the case of the mouse hepatitis virus (MHV) the E1 protein accumulates in the Golgi complex (Rottier and Rose, 1987). Possibly the E1 and E2 MHV proteins form a complex that is retained at the site of budding. For IBV, the domain in the E1 protein responsible for its intracellular retention was clearly shown to be contained in the first transmembrane spanning segment (Swift and Machamer, 1991). However, MHV E1 mutants that possess only the first transmembrane domain are retained in the ER (Armstrong *et al.*, 1990). Once budded into the secretory pathway, coronavirus particles are transported through the Golgi complex to the plasma membrane and secreted into the medium.

3.4.5d. Hepatitis B Virus. Hepatitis B is an enveloped, partially double-stranded DNA virus that is usually described as budding in the endoplasmic reticulum (Ganem, 1991; Ganem and Varmus, 1987). The viral surface antigen (HBsAg) is a multiple-spanning protein capable of assembling to form lipoprotein particles when expressed separately. This antigen assembly and budding process was also assigned an ER localization; however, this was based on early studies in which the existence of compartments between the ER and the Golgi was not considered. More recent work (Huovila *et al.*, 1992; Huovila and Fuller, 1993) shows that the HBsAg assembly process occurs in the intermediate compartment, as will be discussed below. It is possible that hepatitis virion formation also occurs in the intermediate compartment, but this issue needs to be re-examined.

3.4.5e. Vaccinia Virus. Vaccinia is a large DNA virus with several intracellular forms. The budding of this virus was originally believed to occur via a *de novo* membrane synthesis pathway. However, more careful recent work (Sodeik *et al.*, 1993) shows that it, like hepatitis B, utilizes an intermediate compartment (discussed below).

4. INTERMEDIATE COMPARTMENTS

The presence of compartments intermediate between the ER and the Golgi is not a novel idea. The past few years have brought a set of new tools and new markers that have begun to allow the characterization of this region of the secretory pathway. Markers such as p58 and p53 and functions such as the recapture of escaped ER proteins have been attributed to the intermediate com-

partments. Further, several treatments have been developed that appear capable of affecting membrane traffic through this region.

The nature of the compartments between the ER and the Golgi and even their existence as independent compartments remain extremely controversial. In light of this controversy about the existence and nature of intermediate compartments, it is worthwhile examining the importance of the following question: Does the introduction of further compartments between the ER and the Golgi simply obscure the underlying simplicity of the secretory pathway or is it necessary to understand the biology of the system? We will attempt to address this question in a functional way. There are important biological functions such as the assembly of disulfide-linked complexes that are inconstant with the environment of the ER and yet appear to occur at a pre-Golgi stage. These processes can be understood in light of an intermediate compartment, and hence the added complexity is a reasonable price to pay for an expanded understanding of the biology.

One advantage of our definition is that it is recursive; a collection of (morphologically indistinguishable) compartments is a compartment. We will consider the evidence and functions ascribed to the intermediate compartment between the ER and the Golgi first, without trying to decide whether they all occur in the same structures. This is analogous to referring to the plasma membrane of an epithelial cell as a compartment for purposes of describing transport from the Golgi, although it clearly contains two compartments.

Several reviews have been published recently that summarize work in this field (Hauri and Schweizer, 1992; Klausner *et al.*, 1992; Mellman and Simons, 1992; Saraste and Kuismanen, 1992). Each emphasizes particular characteristics of this region and advocates a simplifying view of the components and their distribution. Unfortunately, the dynamic nature of the distribution of markers that have been used make the question of localization a complex one. Furthermore, relatively few of the markers have been used for co-localization studies, so that one is left with many open questions and the possibility that different authors are describing different compartments. Our approach will be first to describe the tools and markers used to study this compartment and then to address each of the proposed descriptions of intermediate compartments separately in terms of our definition of a compartment. This will allow us to highlight some of the open questions remaining in the field and allow the reader a better perception of the limits of our knowledge in this area. A summary of the responses of the compartments of the early secretory pathway to drug and other treatments is given in Table I and Figure 2.

4.1. Recycling and Traffic between the ER and the Golgi

Evidence indicates that recycling of proteins occurs between the Golgi apparatus and the ER. The best evidence is available for the soluble proteins of

Table I
Summary of the Responses of the Compartments of the Early Secretory Pathway to Drug Treatments[a]

	ER	IC	CGN
GTPγS	x	?	x
$AlF_{(3-5)}/\beta\gamma$	x	(x)	?
Mastoparan	x	?	?
α-rab1	x	?	x
α-NSF	-	?	x
CA^{2+}	-	?	x
Caffeine/20°C	-	?	x

[a]The positions of the blocks indicated in the table refer to anterograde transport. In most cases a block at an early stage obscures the effects of the same agent at a later stage. the effects of GTPγS, $AlF_{(3-5)}$, βγ subunits of a trimeric G protein, mastoporan, anti-rab1, anti-NSF, and Ca^{2+} deprivation are all assayed in permeablized cell systems. Caffeine treatments were performed in intact cells, IC, intermediate compartment; CGN, *cis*-Golgi network.

the ER that bear the KDEL retention signal (Lewis and Pelham, 1992; Pelham, 1988; Pelham, 1989b; Pelham, 1991). The lysosomal protease, cathepsin D, is modified by a series of Golgi enzymes so that it bears the mannose-6-phosphate signal required for its post-Golgi routing to the lysosomes (Kornfeld and Kornfeld, 1985). By attaching a KDEL signal to cathepsin D, Pelham and co-workers generated an elegant probe for the exposure of an ER resident protein to the enzymes of the Golgi (Dean and Pelham, 1990; Pelham, 1988). During a prolonged time course the glycans of cathepsin D–KDEL hybrid were modified by addition of GlcNAc-phosphate, although the bulk of the protein was always found in the ER. This modification indicates that KDEL-terminated proteins encounter the early Golgi, although the bulk of the protein is localized to the ER. The question of whether the modification could be accomplished by Golgi enzymes present in the ER was addressed by showing that modification did not occur in yeast that had a mutation in *sec*18, resulting in a transport block to the Golgi. Similar modifications have been demonstrated on a number of other proteins localized to the ER, indicating that this recycling is a general feature of ER proteins (Mazzarella and Green, 1987)

4.1.1. 15°C Treatment

One of the most striking and useful treatments affecting ER to Golgi transport is that of low temperature. Saraste and co-workers used immunocytochemistry to show that incubation of BHK cells at 15°C blocked the transport of viral glycoproteins to the Golgi (Saraste and Kuismanen, 1984). By taking advantage

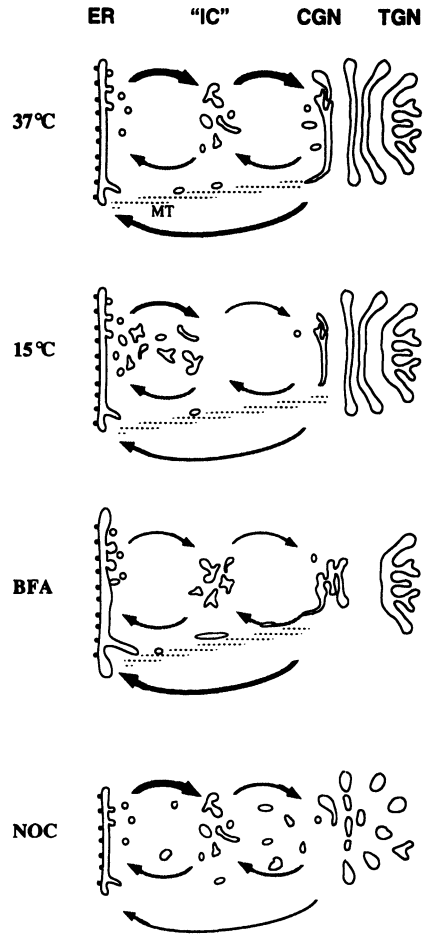

FIGURE 2. Schematic view of the responses of the compartments of the early secretory pathway. Major changes in the cellular localization and compartment size, introduced by a low temperature block and drug treatments, are depicted. IC, intermediate compartment; CGN, *cis*-Golgi network; TGN, *trans*-Golgi network; MT, microtubule.

of a temperature-sensitive mutation in the Semliki Forest virus (SFV) glycoprotein to accumulate a pulse of protein in the ER before inhibiting transport at 15°C, these researchers could characterize the morphology of the structures in which the blocked protein accumulated (Kuismanen and Saraste, 1989). They observed a striking assembly of pleimorphic vacuolar and tubulo-vesicular structures near the Golgi region as well as in the peripheral regions of the cell. The blocked proteins were present throughout both types of structures. Return of the cells to 31°C (the permissive temperature for transport of the mutant glycoprotein) reversed the block causing accumulation of protein in the tubular regions of the structures and allowed its transport to the Golgi.

The use of 15°C treatment has become quite standard for the characterization of the transport between ER and Golgi. The state of the blocked proteins can

be characterized by pulse-chase analysis using labeled proteins for biochemistry or by taking advantage of the use of mutant viral glycoproteins such as the ts1 mutation of SFV or the tsO45 mutant of the G protein of VSV for immunocytochemistry (Kuismanen and Saraste, 1989). These proteins are blocked in the ER because of a folding defect at high tempreatures (39.5°C), and they exit the ER at lower temperature. Incubation at the restrictive temperature allows synthesis of protein to continue while its transport is blocked in the ER and the later stages of the secretory pathway are cleared. Switching to the permissive temperature allows the protein to fold, to exit the ER, and to define the position of the block by immunocytochemistry.

A detailed characterization of the morphology of the structures containing the VSV-G protein at 15°C allowed Bonnatti and co-workers (Lotti *et al.*, 1992) to address the question of whether this treatment generates a novel compartment or rather blocks transport in a pre-existing one. Comparison of electron microscopic immunocytochemistry and immunofluorescence microscopy allowed these authors to identify all of the structures containing G protein at 15°C. The labeled structures were localized in circular areas characterized by numerous small vesicles with an average diameter of 80 nm, which were near short tubules. The circular appearance of the areas and the close clustering of the vesicles and tubules allowed them to be distinguished from the ER and Golgi stacks. After the 1-hour incubation at 15°C, weak labeling of ER and negligible labeling of the Golgi cisternae showed that transport of VSV-G protein was slowed between these two compartments. These structures were also labeled with rab2 (Lotti *et al.*, 1992). Furthermore, the authors examined noninfected cells and cells incubated at 31°C and 37°C. Comparison with the 15°C incubated cells revealed that the same circular areas could be found in all three conditions, indicating that they were not induced either by temperature block or viral infection. The G protein was found in structures that were both peripheral and perinuclear, but it rapidly localized to the Golgi complex after release of the block by very short (<10 min) incubations of cells at 31°C.

Several important caveats should be stated when trying to interpret the results of 15°C treatment. The most important is that the block is not absolute. Hence, protein blocked for 3 hr may have advanced further along the pathway than protein blocked for only an hour. Moreover, the precise temperature of the block is quite critical, and there is no guarantee that all mammalian cell lines will behave identically with respect to 15°C incubation. These three points explain the inconsistency of the results described above with the finding (Schweizer *et al.*, 1990) that VSV-G protein could reach the *cis*-Golgi during 3-hr incubation at 15°C. Finally, one needs to remember that because some components are continually recycling, the co-localization of a component with a newly synthesized one that has blocked at 15°C may simply represent the fact that a recycling component was blocked there.

4.1.2. Brefeldin A

Brefeldin A (BFA) is a macrocyclic fungal antibiotic that blocks the transport of proteins through the secretory pathway. Takatsuki and co-workers introduced BFA as a tool when they reported (Takusuki and Tamura, 1985) that BFA treatment blocked the transport of newly synthesized VSV-G protein to the cell surface and caused it to accumulate in an endoglycosidase H-sensitive form, indicating that the glycoprotein had not encountered the *medial*-Golgi. Morphological work showed that BFA treatment caused the Golgi complex to disappear (Fujiwara *et al.*, 1988). Later work by several groups showed that established markers of the *cis*- and *medial*-Golgi return to the ER upon disassembly of the Golgi during BFA treatment (Doms *et al.*, 1989; Lippincott-Schwartz *et al.*, 1989). An exception is that of sialyl transferase, a marker of the *trans*-Golgi network (TGN). Although one group (Ivessa *et al.*, 1992) has reported that ribophorins I and II are sialylated during BFA treatment, immunocytochemistry indicates that the bulk of sialyltransferase does not return to the ER (Chege and Pfeffer, 1990). This confirms that the TGN is a functionally distinct structure from the Golgi and defines the limits of BFA-induced recycling to the ER to the early secretory pathway. The action of BFA appears to be reversible; washing out the drug causes the rapid return of the Golgi markers to their original location (Doms *et al.*, 1989; Hsu *et al.*, 1991; Klausner *et al.*, 1992; Lippincott-Schwartz *et al.*, 1990; Lippincott-Schwartz *et al.*, 1989).

Although the mechanism of action of BFA is not yet understood, the first clue has come from the identification of a peripheral Golgi protein whose redistribution to the cytoplasm is a very rapid consequence of BFA treatment. The β-coatamer protein (β-COP) has partial homology to the clathrin-coated, vesicle-associated protein β-adaptin (Duden *et al.*, 1991a; Duden *et al.*, 1991b). The β-COP has been shown to be a component of the non-clathrin-coated vesicles, which are believed to mediate transport between the successive cisternae of the Golgi. A model has been suggested in which BFA treatment prevents the formation of the nonclathrin coats of the forward (anterograde) transport vesicles while leaving unaffected the recycling (retrograde) transport that appears to be mediated by noncoated tubules along microtubules (Klausner *et al.*, 1992; Lippincott-Schwartz *et al.*, 1990). By blocking anterograde transport, BFA would allow the effect of the retrograde pathway to be manifested in the striking return of Golgi components to the ER. The effect of BFA on the endosomal system and the TGN suggests that its mechanism of action could be a quite general one at the level of inhibition of the formation of transport vesicles for several steps in intracellular traffic (Klausner *et al.*, 1992).

Two recent papers present a possible explanation for the actual mechanism of BFA action on the assembly of nonclathrin coats (Donaldson *et al.*, 1992; Helms and Rothman, 1992). The low molecular weight GTP binding protein

ADP ribosylation factor (ARF) is required for coatamer binding to membranes and probably mediates the control of guanine nucleotides of the coatomer membrane interaction (Serafini *et al.*, 1991). The new results indicate that BFA inhibits the GTP dependent interaction of ARF with the Golgi membrane by inhibiting the exchange of GTP onto ARF, which is normally catalyzed by Golgi membranes (Donaldson *et al.*, 1992; Helms and Rothman, 1992). A simple inference from these results is that BFA accomplishes its effect on the Golgi by blocking GTP-GDP exchange in ARF. This blocks the activation of ARF for membrane binding and coat assembly and hence prevents the budding of transport vesicles. Because only the GTP form of myristoylated ARF but not the GDP form can insert into membranes (Serafini *et al.*, 1991), the location of the exchange enzyme may control the localization of the ARF proteins. It is unclear whether BFA acts directly or indirectly on the exchange protein, for the protein has not yet been purified but only localized to Golgi fractions.

Whatever its mode of action, BFA has become a widely applied reagent for the dissection of the secretory pathway (Klausner *et al.*, 1992). During the redistribution of Golgi markers, tubular elements can be seen extending toward the ER, apparently showing the organization of transport along microtubules. The redistribution seen for Golgi markers does not extend to the entire early secretory pathway; several markers of intermediate compartment (see below) are unaffected by BFA treatment. Interestingly, these markers are transiently returned to the ER after BFA has been removed (Chavrier *et al.*, 1990; Hauri and Schweizer, 1992; Lippincott-Schwartz *et al.*, 1990; Vaux *et al.*, 1990). This later redistribution reflects the changes in membrane traffic as the Golgi is reassembled and provides an indication that there is no direct retrograde transport from these intermediate compartments.

4.1.3. Microtubule Agents

Microtubule disrupting agents have been useful in dissecting the retrograde and anterograde pathways of transport between the ER and the Golgi. Retrograde transport of Golgi proteins to the ER can be visualized as a series of transient tubulo-vesicular projections from the Golgi membrane (Lippincott-Schwartz *et al.*, 1990). These necklace-like Golgi processes are often seen to localize along microtubules. As treatment of cells with nocodazole results in disruption of the microtubule network, it could have an effect on this microtubule-associated retrograde transport. The Golgi is dispersed into vesicles by nocodazole treatment of normal cells. Application of BFA to nocodazole-treated cells results in no further dispersion of the Golgi and no apparent return of Golgi markers to the reticular ER. Hence, microtubules appear to be involved in the retrograde direction of the ER to Golgi transport.

4.1.4. Caffeine

Kuismanen and co-workers (Kuismanen *et al.*, 1992) have introduced caffeine as a blocker of ER to Golgi transport using a similar protocol to that employed by Saraste in the first work on the 15°C block. The effect of caffeine on SFV glycoprotein transport is temperature sensitive. At physiological temperatures, the drug affects the later stages of the secretory pathway, appearing to cause accumulation of protein in the TGN as shown by the proteolytic processing of the glycoprotein precursor p62. Treatment of cells with 10 mM caffeine at 20°C appears to block the transport of the glycoprotein prior to the acquisition of EndoH sensitivity and hence before its entrance to the *medial*-Golgi. Caffeine is known to have multiple effects on cells, including the inhibition of the breakdown of cyclic AMP, the premature triggering of events in the mitotic program, and the release of calcium from intracellular membranes (Burgoyne *et al.*, 1989; Downes *et al.*, 1990; Schlegel and Pardee, 1986). Because neither stimulation of adenylate cyclase with forskolin nor treatment with the inhibitor 8-bromo-cyclic AMP mimicked the effect of caffeine, this raises the interesting possibility that either calcium fluxes or the link between intracellular transport and mitotic events may be the basis of this effect.

4.1.5. The Use of Semiintact Cells

The use of permeabilized cell systems has allowed precise manipulation of ER to Golgi transport and demonstrated the role for a number of components. A fundamental finding was that energy is required for the exit of VSV-G protein from the ER (Balch *et al.*, 1986). Both GTPγS and Ca^{2+} depletion inhibited entrance of G protein to the *cis*-Golgi (Beckers and Balch, 1989) as defined by the acquisition of EndoD sensitivity in CHO 15B cells. The Ca^{2+} effect was shown to be fully reversible and distal to the GTPγS effect.

The observation that GTPγS inhibited transport to the *cis*-Golgi is consistent with the previous demonstration that members of both the rab and ARF families of small GTP binding proteins are necessary for ER to Golgi transport in both mammalian and yeast cells (Balch, 1992). More recent work in digitonin-permeablized cells clarified the involvement of multiple GTP binding proteins in ER to Golgi transport. Use of specific antibodies and experiments with mutant proteins (Plutner *et al.*, 1991; Schwaninger *et al.*, 1992; Tilsdale *et al.*, 1992) have confirmed that rab1A, rab1B, and rab2 are all required. The involvement of a member of the ARF family was shown by inhibition with a specific N-terminal peptide (Schwaninger *et al.*, 1992). The role of heterotrimeric G proteins was shown both by the inhibition of export from the ER by treatment with mastoporan, a G-protein activator peptide, and by the addition of purified βγ sub-

units (Schwaninger *et al.*, 1992). The authors suggest that the role of ARF is in the formation of the coat of the transport vesicle and that heterotrimeric G proteins may function by defining the site of assembly of the vesicles (Schwaninger *et al.*, 1992). Further work from the same group defined the morphology of the compartments after these blocks (Plutner *et al.*, 1992). The effect of incubation at 15°C on the transport of G protein was similar to that seen by Bonnatti and co-workers (Lotti *et al.*, 1992) in intact cells. Morphological studies revealed that removal of calcium, or addition of antibody to the transport component NSF, blocked VSV-G protein in tubulo-vesicular structures containing the intermediate compartment marker p58 (Plutner *et al.*, 1992). Although the use of a permeablized cell system is very powerful in terms of the manipulations that are possible owing to the accessibility, it is important to note that the cytoskeleton is affected by this treatment. In particular, the microtubule network is disrupted gradually, and the authors suggest that the spread in localization of components after long times of incubation could be a reflection of this effect (Plutner *et al.*, 1992).

4.2. Markers of the Intermediate Compartment

One major problem with defining the intermediate compartment is the paucity of static markers for it. The accepted markers ascribed to the intermediate compartment are described below. These markers are usually characterized in terms of the treatments already described. However, it is important to remember that their localization can be affected by these treatments.

4.2.1. p53

Hauri and co-workers have used a monoclonal antibody directed against a protein of 53 kDa as a marker of intermediate compartment (Hauri and Schweizer, 1992; Schweizer *et al.*, 1988). The monoclonal antibody labels a collection of tubulo-vesicular membranes on the *cis* side of the Golgi. The distribution of these structures is similar to that described by Lotti *et al* (1992) after release of the 15°C block in that they are both peripheral and perinuclear (Lippincott-Schwartz *et al.*, 1990). A low level of p53 labeling is also found in the *cis*-Golgi cisterna. Biochemical characterization of the antigen shows that it is a non-glycosylated, oligomeric, transmembrane protein (Hauri and Schweizer, 1992; Schweizer *et al.*, 1988). Sequence data show that this protein contains a -x-K-(x)-K-x-x retention signal at its carboxyterminus, suggesting its function in the localization of the protein (cited in Hauri and Schweizer, 1992).

The localization of the protein overlaps with that of VSV-G protein, blocked at 15°C for 3 hours, and is found concentrated close to the Golgi (Schweizer *et al.*, 1990). Rewarming the cells causes extension of p53 positive tubules and an

ER localization of p53 for up to 1 hour before its normal distribution is recovered. BFA treatment causes a coarsening of the p53-positive structures but not the return of p53 to the ER (Lippincott-Schwartz *et al.*, 1990). Upon washing out the BFA, p53 returns transiently to the ER. Together, these results suggest that p53 is not a static marker but, rather, undergoes continual recycling between the ER and the Golgi.

Although p53 is a very useful marker, the fact that its detection has been limited to the use of a monoclonal antibody, which only reacts with the antigen in primate cells, has made it difficult to generalize to other systems. The availability of the sequence should change this situation.

4.2.2. p58

Saraste and co-workers have characterized a protein of 58 kDa that shares many properties with p53 (Lahtinen *et al.*, 1992; Saraste and Kuismanen, 1984; Saraste and Svensson, 1991). Antibodies against p58 are available that react with a variety of cell types, and so its localization to tubulo-vesicular structures adjacent to the *cis*-Golgi is well established (Lahtinen *et al.*, 1992; Saraste and Kuismanen, 1984; Saraste and Svensson, 1991). In some cell types, p58 appears to be more extensively localized to the *cis*-Golgi than p53 (Lahtinen *et al.*, 1992). The protein bears immature *N*- and *O*-linked glycans, which indicate that it is never processed by the enzymes of the *medial*- or *trans*-Golgi. The behavior of p58 upon 15°C treatment and BFA treatment is similar to that of p53, suggesting that it too recycles between the ER and the Golgi. Studies of permeablized cells indicate that treatment at 15°C, with GTPγS or with anti-rab1B antibody to block transport, causes co-localization of p58 with VSV-G protein (Plutner *et al.*, 1992) although one has to consider the possibility that this is not its normal localization but one induced by the block. An obvious question is whether p53 and p58 are homologous; however, the lack of cross-reactivity of the antibodies means that this question will only be resolved when the sequence of p58 is available and that of p53 is published.

4.2.3. p63

The fact that p53 and p58 recycle between the ER and the Golgi complicates their use as intermediate compartment markers (as discussed above). For this reason the identification of another intermediate compartment marker, p63, by a panel of monoclonal antibodies raised against a fraction enriched in p53 is very important (Hauri and Schweizer, 1992). Also, p63 is a membrane protein that overlaps in distribution with p53 and with VSV-G protein at 15°C. Distribution of p63 is unchanged by treatments with BFA or low temperature. It also has been reported to be palmitoylated even when traffic between the ER and Golgi is

blocked by BFA (cited in Hauri and Schweizer, 1992). Hence, p63 represents a good candidate for a marker of the stable portion of intermediate compartment.

4.2.4. The rab Proteins

The rab (rat brain) proteins have been used as markers of intermediate compartment. Recent functional data show that rab2, rab1B, and rab1A are all required for ER to Golgi transport (Plutner *et al.*, 1991; Schwaninger *et al.*, 1992; Tilsdale *et al.*, 1992) and are consistent with the localization of these rab proteins, and in particular rab2 (Chavrier *et al.*, 1990), to the early secretory pathway. The proposed function of rab proteins in transport must be considered when they are referred to as markers of a compartment (Goud, 1992). Rab proteins are believed to function in a cyclic fashion and hence be present on both the acceptor and donor compartments involved in a transport event. Hence, the observation that rab2 is found on tubulo-vesicular structures and on the *cis*-Golgi can also be interpreted as demonstrating the connection of these two structures by transport vesicles rather than indicating that they represent the same compartment.

4.3. Functions Ascribed to the Intermediate Compartment

The paucity of static markers for the intermediate compartment is more than compensated by the large number of functions from salvage to protein modification that have been attributed to it. These functions have been extensively reviewed elsewhere (Hauri and Schweizer, 1992; Klausner *et al.*, 1992; Mellman and Simons, 1992; Saraste and Kuismanen, 1992). Here we will mention them only to point out the implications of having these functions localized to an intermediate compartment.

A recurring problem in discussing the intermediate compartment functions is that we know relatively little about the functions of the *cis*-Golgi. The two functions usually ascribed to the *cis*-Golgi are the phosphorylation, which generates the mannose-6-phosphate signal on newly synthesized lysosomal proteins, and the trimming of high mannose residues (Kornfeld and Kornfeld, 1985). Unfortunately, the localization data for these activities derive from a time at which the intermediate compartment was not considered.

4.3.1. KDEL-Mediated Retention

The key observation in understanding the retention of soluble ER proteins was made by Munro and Pelham (1986, 1987), who noticed upon determining the sequence of rat BiP that the carboxy-terminal four residues matched the terminal residues of the previously established sequence of PDI (protein disulfide

isomerase). They went on to show that transfer of this sequence to the carboxy-terminus of lysozyme caused this normally secreted, soluble protein to be retained in the ER. This result was interpreted as evidence that the last four residues, KDEL, comprised a necessary and sufficient signal for the retention of soluble proteins in the endoplasmic reticulum (Munro and Pelham, 1987).

Although the original work showed the modular nature of the signal, by transfer of the last six residues from BiP to lysozyme, the signal was preceded by a sequence from the *myc* protein as an upstream tag for the protein. It appears that this upstream region is necessary for KDEL function in retaining secreted proteins. Work from several groups confirms some requirement for an immediate upstream region (Buonocore and Rose, 1990; Pelham, 1989b; Pelham, 1990). The sequences of a large number of soluble ER proteins are now known, and the recognized signal varies in different organisms. The HDEL is the recognized signal in the yeast *Saccharomyces cerevisea,* whereas DDEL is recognized in the yeast *Kluyveromyces lactis* and ADEL is found to be functional in the *Schizosaccharomyces pombe,* although none of these appear to function in mammals (Hardwick *et al.*, 1990; Lewis *et al.*, 1990; Pelham, 1989a; Pelham, 1990; Pidoux and Armstrong, 1992; Semenza *et al.*, 1990). In mammalian systems, KDEL appears to be able to be substituted by RDEL and KEEL and still retain function. Our attempts at modeling these sequences suggests that the upstream region may be important in modulating the tendency of the KDEL itself to assume the recognized conformation on the protein (Fuller *et al.*, 1993).

Addition of residues after the signal (−KDEL → −KDELGL) destroyed the ability of the signal to function in retaining lysozyme (Munro and Pelham, 1987). Further, although lysozyme is secreted from the ER with a half-time of roughly 10 min, BiP without its KDEL signal is only secreted slowly with a half time of 3 hr. This suggests that the body of an ER protein as well as the signal may have a function in the retention. An elegant experiment using *Xenopus* oocytes (Ceriotti and Colman, 1988) provided clear evidence of this by showing that both BiP terminated with KDEL and with the nonfunctional signal KDELGL were free to diffuse throughout the ER but did so with a diffusion rate intermediate between that of a membrane protein and a secreted protein. Their results are consistent with a model of retention in which interactions between the walls of the ER and the body of the protein lower the effective concentration of KDEL tails, which must be recognized by the specific retention system.

4.3.1a. Retention Is Environment Dependent. The specific recognition of the KDEL signal was hypothesized to be accomplished by a receptor (Warren, 1987). The high concentration of the proteins, which must be controlled by this recognition system, and the lack of any ER membrane protein present in sufficient abundance to interact with all of them led to the hypothesis that retention was accomplished by a recycling mechanism (Munro and Pelham, 1987). Pelham and co-workers (Munro and Pelham, 1987) proposed the existence of a

salvage compartment in which escaped ER proteins would be recognized on the basis of their KDEL tails and recycled to the ER. For this mechanism to function, the binding of KDEL tails by the putative receptor must be subject to modulation. Binding must be tight in the post-ER salvage compartment but weak in the ER where the proteins are released. A simple way to accomplish this modulation would be to make the interaction of the KDEL tail with the receptor dependent on the environment of the compartment. This would be analogous to the recycling of ligands during endocytosis, in which the change in pH between the endosome and the extracellular environment modifies the binding of ligand to receptor.

Although we know very little about the environment of the ER and even less about the environment of the putative salvage compartment, the available evidence suggests that the pH is higher in the ER than in the TGN, while the concentration of free calcium goes up between these two points (Suzuki *et al.*, 1991). Koch and co-workers (Booth and Koch, 1989; Koch *et al.*, 1988) have provided further evidence for environmental effects on retention by showing that modifying the concentration of calcium along the secretory pathway led to secretion of KDEL-terminated proteins in a particular clone of 3T3 cells, although it appears that their results are difficult to repeat in other cell lines. Either pH or Ca^{2+} concentration or both could then be candidates for the environmental switch that modulates affinity to the receptor.

4.3.1b. Identification of the Receptor for ER Retention. Identification of the KDEL receptor, the protein that recognizes the signal for retention, would be an important step toward understanding the retention system.

One approach (Vaux *et al.*, 1990) has been the generation of anti-idiotypic antibodies that should mimic the signal structure. These antibodies identified an antigen localized between the ER and the Golgi by immunocytochemistry and Western-blotted a 72-kDa protein. This protein apparently had the appropriate properties to be the receptor. Our further work, however, now indicates that the monoclonal anti-idiotypic antibodies recognize a different protein by immunofluorescence than by Western blotting. This fact invalidates the biochemical characterization of the putative receptor and, together with other evidence that the 72-kDa protein is cytoplasmic, suggests that it is not involved in KDEL retention (Vaux *et al.*, 1992).

Pelham and co-workers have used a fundamentally different approach taking advantage of yeast genetics to identify a protein involved in the retention of HDEL-terminated proteins in the ER (Hardwick *et al.*, 1990; Lewis *et al.*, 1990; Semenza *et al.*, 1990). They have identified a protein, ERD2 (for ER *r*etention *d*eficient, which affects the retention of HDEL-terminated proteins. The evidence that this is the functional receptor is threefold: First, mutations in ERD2 cause proteins with an HDEL retention signal to be secreted (Semenza *et al.*, 1990). Second, overexpression of erd2 increases the capacity of yeast cells to retain HDEL-terminated proteins (Semenza *et al.*, 1990). Finally, transfer of the *K.*

lactis ERD2 into *S. cerevisea* alters the ligand specificity of retention (Lewis *et al.*, 1990).

In addition, these authors have identified a mammalian homologue of ERD2 (hERD2) and have shown that overexpression of hERD2 allows retention of DDEL terminated proteins, which are normally poorly recognized in the mammalian system (Lewis and Pelham, 1990; Lewis and Pelham, 1992). Mutation in the putative binding site of the hERD2 modifies this effect, suggesting its interaction with the ligand (Lewis and Pelham, 1992). Unfortunately, antibodies are not available against the normal ERD2 protein, so that localization and expression studies have all utilized a tagged version of the protein to allow its detection. The tagged protein is found throughout the Golgi in cells that express large amounts of ERD2 as well as in cells that stably express smaller amounts of the protein (Lewis and Pelham, 1992). By monitoring the tagged protein, Lewis and Pelham showed that overexpression of KDEL- or DDEL-terminated proteins results in the redistribution of ERD2 to the ER. A second human erd2 homologue, elp1 (*erd-l*ike *p*rotein), has been identified (Hsu *et al.*, 1992). It has been shown that overexpression of either erd2 or elp1 causes a profound change in the organization of the Golgi similar to that seen in BFA-treated cells (Hsu *et al.*, 1992). The relationship between this effect and the effect of ERD2 on retention remains unclear. The fact that ER retention is not essential for yeast although the ERD2 gene is essential (Semenza *et al.*, 1990) suggests that ERD2 must also have a function other than ER retention.

The localization of the ERD2 protein to the Golgi argues against an intermediate compartment function. As only the tagged protein has been localized, it is possible that the Golgi localization results from the introduction of the tag and/or reflects expression level rather than function. Until this is resolved, the connection between ER retention and the intermediate compartment will remain unclear.

4.3.2. Degradation

There is good evidence for at least two mechanisms of protein degradation in the early secretory pathway. For some proteins, such as the T-cell receptor subunits, degradation appears to occur in the ER (Lippincott-Schwartz *et al.*, 1988). Indeed, these subunits can be degraded in permeablized cells that have been deprived of the components necessary for exit from the ER (Wikstroem and Lodish, 1991). For others, such as an unassembled subunit of the asialoglycoprotein receptor (Amara *et al.*, 1989) and the truncated ribophorin I (Tsao *et al.*, 1992), degradation appears to require transport. Intermediates for degradation can be identified in the ER; however, degradation of these intermediates can be blocked with energy poisons. In the case of a truncated form of ribophorin I, the degradation can be seen to occur with biphasic kinetics. If it can be shown that

this second phase occurs prior to the Golgi, this function would be associated with the intermediate compartment.

4.3.3. Complex S–S Bond Formation

Study of the assembly of hepatitis B surface antigen (HBsAg) reveals an unusual activity of the early secretory pathway. Surface antigen is a transmembrane glycoprotein that assembles into large oligomeric lipoprotein complexes which are extensively linked by disulfide bonds. Examination of the kinetics of oligosaccharide modification indicates that the protein is blocked at a pre-Golgi stage for much of its time in the cell and then is rapidly secreted (Ganem, 1991; Ganem and Varmus, 1987). For this reason, HBsAg has been believed to be assembled in the ER. The presence of a large number of disulfide bonds in the assembled particle argues against this site of assembly, since PDI, which is present in the ER in large amounts, should resolve these cross-links. Indeed, mature HBsAg particles are not stable in the presence of PDI but are broken down to dimers (Huovila *et al.*, 1992).

Huovila *et al* (1992) showed that the formation of the disulfide links in HBsAg occurs in two stages. The first, the formation of disulfide-linked dimers, occurs rapidly and appears catalyzed by PDI. The second occurs more slowly and appears to match the rate-limiting step in assembly. This second step occurs in a compartment that excludes PDI and is insensitive to BFA treatment. The treatment with BFA also appeared to separate the two stages of disulfide bond formation since newly synthesized HBsAg only progressed as far as dimers in the presence of BFA (Huovila *et al.*, 1992). Because this function is pre-Golgi and relies on a non-ER environment, it is a strong candidate for localization to the intermediate compartment.

4.3.4. Antigen Attachment to Class I

Most antigens must be processed intracellularly before they can be presented at the cell surface in association with major histocompatibility complex molecules (MHC). It is this complex that is recognized by the antigen-specific receptor of T cells and can lead to specific killing of the presenting cell (Klausner *et al.*, 1990). Processing of antigen involves cleavage of protein antigens to smaller peptides, which then interact with MHC molecules. Class I restricted antigens include viral antigens and other cytoplasmic proteins synthesized within the presenting cell. The mechanism by which processed fragments of these antigens enter the secretory pathway and bind to the luminal domain of class I MHC molecules remains one of the important questions in understanding antigen presentation.

Two groups have shown that application of BFA to cells abolishes their ability to present endogenously synthesized antigens to class I restricted cytotoxic T cells (Nuchtern *et al.*, 1989; Yewdell and Bennink, 1989). Both groups showed that this effect was specific for presentation of endogenous antigens by demonstrating that BFA had no effect on the presentation of exogenously added peptides at the cell surface in fixed cells. The inhibition indicates that the association of antigen with class I MHC requires transport of the molecule from the ER, and because of the previous work on the action of BFA, suggests that this interaction cannot occur later than *trans*-Golgi. The effect of the E19 protein on the presentation of antigens in adenovirus-infected cells has the same basis since E19 forms a complex with MHC molecules restricting them to the early portion of the secretory pathway (Nilsson *et al.*, 1989; Paabo *et al.*, 1987). Recent evidence that the site of interaction of processed antigen with MHC class I molecules occurs early in the secretory pathway comes from the localization of TAP1, a peptide transporter encoded in the class II region of the MHC. Specific antisera showed that the transporter was found only in the ER region and the *cis*-Golgi (Kleijmeer *et al.*, 1992). Together with the evidence that MHC class I molecules circulate between the ER and the Golgi (Hsu *et al.*, 1991), this implicates either the early Golgi or an intermediate compartment in the association of these molecules with antigen.

4.3.5. Palmitoylation

A number of viral and cell surface proteins are palmitoylated in a post-translational event, occurring before glycoproteins acquire complex-type carbohydrates (Sefton and Buss, 1987). The exact intracellular localization of palmitoylation, however, is not well defined. Combining a biochemical and kinetic study with the utilization of the 15°C block, Bonatti and co-workers (Bonatti *et al.*, 1989) showed that quantitative palmitoylation of VSV-G and SFV E1 membrane glycoproteins seems to take place between the sites defined by low temperature and before exposure to the *cis*-Golgi enzyme 1,2-mannosidase I (Balch *et al.*, 1986). Similar results were obtained previously working with VSV-infected CHO 15B cells (Dunphy *et al.*, 1981). These results are consistent with a post-ER localization. In contrast, some workers suggest that the fatty acetyltransferase is located in the ER (Berger and Schmidt, 1985; Hobman *et al.*, 1992); however, the extent of palmitoylation of the followed marker proteins in these cases is only low or not clear. Consistent with all these findings, work in the yeast *Saccharomyces cerevisea* indicates that palmitoylation can occur in a *sec*18-independent manner (Wen and Schlesinger, 1984) and hence prior to entrance to the Golgi. Therefore, this is a good candidate for an intermediate compartment function.

4.3.6. Virus Budding

Although virus budding may seem an unusual function for a compartment, the fact that the maturation and the assembly of viral proteins require specialized functions makes it a reasonable assay for specific compartmental functions.

Two virus-budding events have been proposed to occur in the intermediate compartment. The first is the formation of the HBsAg particle. Immunocytochemistry and morphology show that the maturation occurs in a compartment disconnected from the ER and, hence, this process has been localized to a pre-Golgi intermediate compartment (Huovila *et al.*, 1992; Huovila and Fuller, 1993). Localization of the budding of hepatitis B virus itself is not known, although evidence suggests that it occurs prior to the Golgi (Ganem, 1991; Ganem and Varmus, 1987). The second budding event now believed to be in the intermediate compartment is the budding of the intracellular infectious form of vaccinia. Originally this event was believed to occur by a process of *de novo* membrane formation; however, more recent work shows that budding occurs through the membranes of the intermediate compartment. Evidence for this localization is a combination of immunocytochemistry and morphology (Sodeik *et al.*, 1993). The fact that the initial budding event is restricted to this compartment strongly suggests that structures between the ER and the Golgi form a compartment by our restrictive definition, although continuities are seen with the ER at later times in infection.

4.4. Proposed Intermediate Compartments

In this section we will examine the evidence that various proposed intermediate compartments do in fact represent compartments in terms of intracellular transport. We will use the definitions and names provided by the original authors; thus, the names should not be considered as a description of the structure but rather as a convenient reference for it.

4.4.1. Transport Vesicles

The simplest description of the structures lying between the ER and the Golgi is that they comprise transport vesicles. This would not satisfy our definition of a compartment. Obviously, transport vesicles are physically discontinuous from either the ER or the Golgi; however, they lack unique components. This is an important functional distinction. Because the entire content of a transport vesicle is delivered to its acceptor, there can be no sorting or monitoring of the degree of completion of modification. Hence, when specific functions requiring unique components must be attributed to intermediate compartments, they cannot be accomplished in transport vesicles.

4.4.2. The CGN

There are two definitions of CGN, the *cis*-Golgi network. The more restrictive definition refers to the elaborate system of tubules that are in direct connection with the *cis* cisterna of the Golgi. This complex network is reminiscent of endosomal tubules believed to function in the separation of ligand and receptor during endocytosis (Tooze and Hollinshead, 1991). This definition of CGN is an apt description of morphology; however, it is not a compartment as it is not disconnected from the *cis*-Golgi itself. The CGN and the *cis*-Golgi are analogous to the smooth ER and rough ER.

A more liberal definition combines this elaborate network with the *cis*-Golgi itself (Mellman and Simons, 1992). Defined in this way the CGN is clearly a compartment, and it is this definition that we will use below. The various features we attribute to compartments all apply. There are clearly transport vesicles, and there is evidence that the compartment can change in size upon alteration of the amount of membrane traffic (Griffiths *et al.*, 1989). Functions that have been assigned to this compartment include all of the early functions of the Golgi such as the attachment of the lysosomal sorting signal, irreversible palmitoylation, and initial *O*-glycosylation and the trimming of high mannose residues (Hauri and Schweizer, 1992; Kornfeld and Kornfeld, 1985; Mellman and Simons, 1992; Schweizer *et al.*, 1991).

4.4.3. ERGIC

Schweitzer and Hauri (Hauri and Schweizer, 1992) have defined ERGIC (ER–Golgi intermediate compartment) by use of the marker p53. ERGIC refers to the disconnected tubulo-vesicular elements between the ER and the Golgi. Immunocytochemistry shows that the bulk of p53 is in this compartment under normal conditions. The p53 is not a unique marker, because it can also be found in the CGN; however, this can easily be explained by its recycling. A more useful marker is p63, which does not appear to recycle (cited in Hauri and Schweizer, 1992).

A cell fractionation procedure has been developed that enriches for p53 (Schweizer *et al.*, 1991). Unfortunately, this procedure is not a purification and cannot be used in many other cell types. It shows clearly that some components are not enriched with p53 and that the functions described above for the CGN are not found in ERGIC. Reversible palmitoylation appears to be localized here since both p62 (which co-localizes with p58) and p63 are palmitoylated (Hauri and Schweizer, 1992; Mundy and Warren, 1992).

ERGIC remains the best characterized of the putative intermediate compartments; however, much remains to be done. Although a large amount of ER proteins including both PDI and the membrane protein ribophorin is found in the

p53-enriched fractions, we assume that this reflects contamination. Because we have assumed that the functionally significant continuities will be extensive, the lack of observed continuities fulfills our definition's requirement. The size of ERGIC appears to fluctuate with incubations at 15°C and during BFA treatment, although the marker, p53, being used to follow these events can relocalize. A full characterization of ERGIC as a compartment will require exploiting markers such as p63 to define the boundaries and components more precisely.

4.4.4. The Pre-Golgi Compartment

Saraste and Kuismanen (Saraste and Kuismanen, 1992) have used the pre-Golgi as marked by p58 to describe the tubulo-vesicular compartments between the ER and the Golgi. It is not clear whether this protein marks the same structures as p53; indeed, the distribution of p58 is skewed toward the CGN. In describing the pre-Golgi, the authors draw the analogy with a maturation model of the elements of the endocytotic pathway (Saraste and Kuismanen, 1992). Hence, traffic between the ER and the Golgi pass through a series of intermediates whose properties more and more closely approximate that of the CGN until they reach the CGN. One aspect of this model is that the localization of the elements of this portion of the secretory pathway changes from peripheral to perinuclear during transport. This is, indeed, observed by several groups. If we follow the authors' suggestion that the pre-Golgi represents a continuum of structures that mature from an ER character to that of the *cis*-Golgi (Saraste and Kuismanen, 1992) then the pre-Golgi does not fit our definition of a compartment. We would not expect to be able to attribute control of membrane traffic and the other features we suggest for compartments to such structures. Indeed, transport vesicles would be unnecessary. It is also very difficult to test their maturation model with the markers presently available.

4.4.5. The 15°C Compartment

The careful work of Bonnatti and co-workers (Lotti *et al.*, 1992) have established that the collection of vesicles and tubules that they see marked with VSV-G protein during short incubations at 15°C is a separate and discontinuous structure from the ER. The morphology of this structure seems different from those labeled with p53; hence, it is presumably distinct from ERGIC, although double labeling has not been performed. Unfortunately, we are lacking any unique components of this compartment, although these structures are labeled with rab2 (Lotti *et al.*, 1992). The *in vitro* studies of Balch and co-workers suggest that p58 accumulates in similar structures at 15°C. Their work also indicates control by GTP-binding proteins over the exit and entrance. We do not know, however, whether the structures identified by Lotti *et al* (1992) are identi-

cal with those for which functional data are available. An interesting, and still unresolved, question is whether the KDEL-terminated proteins have been excluded from this very early portion of the intermediate compartment. Until unique components of this compartment can be identified, the 15°C compartment cannot be considered a compartment by our definition.

4.4.6. The Salvage Compartment

As more is learned about the mechanism of ER protein retention, evidence for the existence of an intermediate compartment involved in salvage of ER proteins becomes less convincing (Pelham, 1991; Warren, 1987). The only remaining candidates for the receptor that marks this compartment do not localize to the pre-Golgi region of the cell and, in fact, appear to be present in the late Golgi (Lewis and Pelham, 1992; Hsu *et al.*, 1992). This could reflect the fact that these proteins cannot be detected unless they are expressed with a tag allowing their immunolocalization. Hence, they may be mis-localized owing to overexpression or the addition of the tag although they still remain functional. Other evidence still supports the existence of such a compartment. In most cells, one cannot detect KDEL proteins in the Golgi or indeed in most of the post-ER region by immunocytochemistry (Huovila *et al.*, 1992; Huovila and Fuller, 1993; Tooze *et al.*, 1989). Together with the fact that KDEL-terminated proteins contain only early Golgi modifications, this suggests that salvage occurs at an early stage in the secretory pathway. The need for modulation of the interaction of KDEL tail with receptor argues for the existence of a separate compartment. However, until comprehensive morphological studies can be combined with the biochemistry and genetics, this prototype of the intermediate compartment will remain enigmatic.

4.4.7. The Rubella E1 Compartment

Recent work (Hobman *et al.*, 1992) has suggested that the E1 of rubella marks a unique intermediate compartment when it is expressed in the absence of the other viral proteins. This structure does not meet our definition of a compartment because it is both continuous with the ER and freely accessible to ER markers. Consistent with our definition, this structure is not the site of rubella virus budding. The other envelope glycoprotein of the virion, E2, forms a complex with E1, which allows it to exit the ER during normal infection (Oker-Blom *et al.*, 1983).

4.4.8. The HBs Assembly Compartment

The HBsAg matures in a structure that is discontinuous from the ER and the Golgi (Huovila *et al.*, 1992). This structure appears to fulfill the requirements of a

compartment. Morphometry shows that it can expand to become almost twice the size of the ER and has a tubulo-vesicular structure (Huovila *et al.*, 1992; Huovila and Fuller, 1993). The only ascribed function of this compartment is that of formation of complex disulfide links. For this purpose it is necessary that it maintains a distinct environment from the ER. Further, exit from this compartment awaits the completion of assembly of the particle so that sorting must occur here. Rab2 has been found to co-localize with this compartment (Huovila and Fuller, 1993). A great deal more work needs to be done to define this structure adequately. In particular, we need to show its relationship to the other markers of this region.

5. CONCLUSIONS

This chapter addressed a basic question concerning the early compartments of the secretory pathway. The first phase of the characterization of these compartments is drawing to a close; a number of markers have been defined and a series of manipulations have been established modulating traffic in this region. This would have been adequate as a characterization of the more classical and less dynamic compartments of the cell, but is clearly not sufficient for an understanding of ER to Golgi traffic. The next phase, in which the separate identities of the compartments in this region will be established, is just beginning. It is hoped that such functions as reversible palmitoylation and complex S–S bond formation will become better characterized as a result. A complete understanding must rest on a careful and quantitative synthesis of morphological, immunocytochemical, and biochemical work on this region. It will also require a combined effort to compare compartments identified in different ways using different markers. We have tried to show here that the biology of this region of the cell is still obscured by the lack of such comparisons. Until we can develop coherent quantitative models for these compartments, their interesting biology will remain hidden.

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NOTE ADDED IN PROOF: Hong and coworkers (Tang *et al.*, 1993) have recently localized the endogenous bovine ERD2 homologue to the *cis*-side of the Golgi apparatus and to a spotty intermediate compound.

ABBREVIATIONS USED IN THIS MANUSCRIPT

-x-K-(x)-K-x-x:	retention motif identified in several transmembrane ER proteins, consisting of two lysines positioned 3 and 4 or 5 residues from the C-terminus
3T3:	a mouse fibroblast cell line
ADEL:	alanyl-aspartyl-glutamyl-leucine; retention signal used for *S. pombe* BiP
ARF:	ADP-ribosylating factor; a small GTP-binding protein
β-COP:	β-coatamer protein
bet:	blocked early in transport
BFA:	Brefeldin A; a macrocyclic fungal antibiotic
BHK:	cell line derived from baby hamster kidney
BiP:	heavy chain binding protein or GRP78
bos:	bet one suppresser
CGN:	*cis*-Golgi network
CHO:	cell line derived from Chinese hamster ovary
DDEL:	aspartyl-aspartyl-glutamyl-leucine; retention signal functional in *K. lactis*
E1/E2:	viral envelope transmembrane glycoprotein 1 or 2
EndoD:	endoglycosidase D; sensitivity to this enzyme indicates that the glycans of a protein have been exposed to the *cis*-Golgi, but were not processed by *medial*-Golgi glycosidases.
EndoH:	endoglycosidase H; acquisition of resistance to this enzyme indicates that the glycans of a protein have been exposed to the *medial*-Golgi.
ER:	endoplasmic reticulum
ERD:	ER-retention defective
ERGIC:	ER-Golgi intermediate compartment
GDP:	guanosine diphosphate
GRP:	glucose-regulated protein
GTP:	guanosine triphosphate
HA:	influenza virus hemagglutinin
HBsAg:	hepatitis B surface antigen
HDEL:	histidyl-aspartyl-glutamyl-leucine; retention signal used in *S. cerevisea* and *K. lactis*
hsp:	heat-shock protein; members belonging to this group are stress-inducible proteins
IBV:	avian infectious bronchitis virus, a member of the coronavirus family
KDEL:	lysyl-aspartyl-glutamyl-leucine; retention signal used in mammalian cells

KDELGL:	lysyl-aspartyl-glutamyl-leucyl-glycyl-leucine; C-terminal extension to retention signal rendering it nonfunctional
KEEL:	lysyl-glutamyl-glutamyl-leucine; retention signal functional in mammalian cells
MHC:	major histocompatibility complex
MHV:	mouse hepatitis virus, a member of the coronavirus family
NE:	nuclear envelope, a subcompartment of the ER
NS:	nonstructural; NS proteins are not part of the mature virus
NSF:	*N*-ethylmaleimide-sensitive factor
PDI:	protein disulfide isomerase
PiZ:	naturally occurring variant of the Z type α_1-antitrypsin gene
rab:	rat brain; *ras*-like small GTP binding protein, first cloned as mammalian copy from rat brain
RDEL:	arginyl-aspartyl-glutamyl-leucine; retention signal functional in mammalian cells
rER:	rough ER, a subcompartment of the ER
RV:	rubella virus
sec:	secretory
sER:	smooth ER, a subcompartment of the ER
SFV:	Semliki Forest virus
SNAP:	soluble NSF attachment protein, the mammalian homologue of *sec*17p
SRP:	signal recognition particle
Tac:	interleukin-2 receptor α-chain
TAP:	translocator of antigenic peptides, also called RING4
TCR:	T-cell receptor
TE:	transitional elements, a subcompartment of the ER
TGN:	*trans*-Golgi network
tsO45/ts1:	temperature-sensitive mutants of VSV (tsO45), or SFV (ts1)
Vero:	cell line derived from African green monkey kidney
VP:	viral protein that is eventually integrated in the mature virus
VSV:	vesicular stomatitis virus
VSV-G:	vesicular stomatitis virus transmembrane G-protein

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