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Research Article

Intracellular Traffic of Newly Synthesized Proteins

Current Understanding and Future Prospects

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Our survival depends on the specialized functions of our tissues and organs. These functions are carried out by specific proteins, i.e., enzymes, receptors, and channels, which reside in specific locations within the cell, separated by membranes. Indeed, most of the activities characteristic of a tissue can be traced back to individual gene products expressed in a particular membrane or membrane-delimited compartment. Mislocalization of such proteins is functionally equivalent to lack of expression, or worse.

Because almost all protein synthesis occurs in the cytosol, a complex system of targeting, sorting, and consignment must exist whereby the thousands of different newly synthesized proteins, both specialized for particular cell types and general to every cell, get to their correct location. These processes make up the intracellular traffic of newly synthesized proteins. Events in protein traffic involve not only processes of protein localization but also those of protein biosynthesis and assembly. A survey of current concepts and knowledge on protein traffic thus must address the disparate problems of targeting of proteins to particular membranes, translocation across and insertion into membranes, assembly into multiprotein complexes in or outside of a bilayer, transport from one compartment to another, secretion out of the cell, co- and posttranslational modifications of transported proteins, retention in and diversion to particular compartments, and regulation of all of these processes. In addition to establishing new tissue-specific functions, protein traffic is necessary for the constant repair and renewal that maintain the integrity of cell structure and function.

As specialized cellular functions depend on gene product expression in particular locations, it seems reasonable to suppose that some diseases should be traceable to lesions in protein trafficking. To date, several rare genetic lesions have indeed been described in which altered protein traffic appears to be the direct cause of disease, including α-1 antitrypsin deficiency (1), Zellweger's disease (2), and I cell disease (3). Understanding these lesions enhances our appreciation of the physiologic pathways, but is not of great clinical significance. However, recent progress in understanding the cell and molecular biology of protein traffic suggests that aberrations in protein traffic have fundamental implications for much more prevalent diseases that may be acquired (e.g., degenerative and malignant processes as well as those induced by the environment). Membranes not only define protein trafficking pathways but also serve as the interface between cells and the outside world. Hence an understanding of the structure, function, and dysfunction of biological membranes is likely to be of enormous significance for molecular medicine in the decades to come. In this Perspectives article, I will survey the range of processes of protein traffic with a focus on the pathway of protein secretion (e.g., as followed by polypeptide hormones and their receptors). Special emphasis has been placed on recent work elucidating events at the endoplasmic reticulum (ER)[1] membrane, as this is currently the best understood aspect of protein traffic. Transport of proteins to other organelles, such as mitochondria or the nuclear matrix will be addressed only briefly here, since I believe that the problems posed by protein secretion provide a suitable conceptual framework in which to situate the problem as a whole.

The secretory pathway is a paradigm for the study of protein traffic

More than a dozen membrane-delimited compartments can be identified in most eukaryotic cells (Fig. 1). Some of these, namely the ER, Golgi apparatus (G, including its *cis, medial,* and *trans* compartments), secretory vesicles and secretory granules (S) are related as components of the secretory pathway. The discipline of cell biology was established in part through the early efforts of Palade and others to define the traffic of newly synthesized secretory proteins from their initial synthesis on membrane-bound ribosome in the cytoplasm through this progression of membrane-delimited compartments (4). The content proteins of some organelles, such as lysosomes, are also recognized to traverse a subset of the compartments in the secretory pathway, as do various classes of integral membrane proteins.

A crucial realization was that the very earliest event in protein secretion, namely transport of the nascent chain across the ER membrane, was the only point in the process at which the polypeptide actually traversed a bilayer. All subsequent steps were accomplished by the fission, targeting, and fusion of membrane vesicles containing secretory proteins from one compartment to the next. It was also observed that synthesis of

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1. *Abbreviations used in this paper:* BiP, heavy-chain binding protein; ER, endoplasmic reticulum; G, Golgi apparatus; HBsAg, hepatitis B virus surface antigen; PI, phosphatidyl inositol; S, secretory vesicles and secretory granules; PrP, scapie prion protein; SRP, signal recognition particle; TGN, *trans* Golgi network.

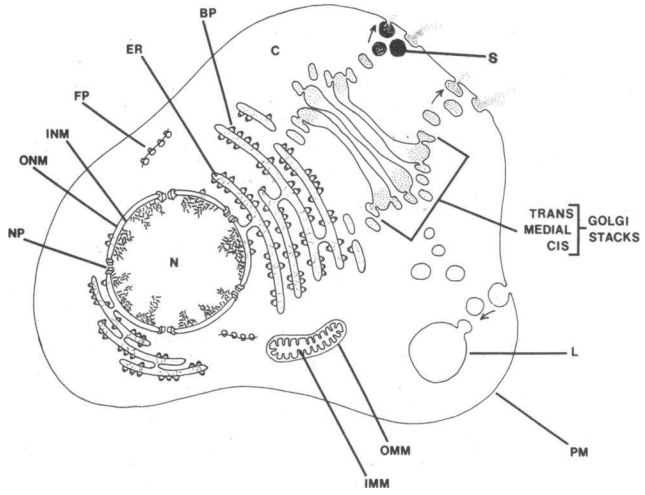

Figure 1. Schematic diagram of a typical eukaryotic cell indicating some of the distinct membrane: delimited compartments observed. C, cytosol; N, nucleus; INM, inner nuclear membrane; ONM, outer nuclear membrane; NP, nuclear pore; ER, endoplasmic reticulum; BP, membrane bound polyribosome (or polysome); FP, free polysome; IMM, inner mitochondrial membrane; OMM, outer mitochondrial membrane; *cis, medial, trans,* Golgi stacks; L, lysosome; S, secretory granule; PM, plasma membrane. Various endocytotic and exocytotic vesicles (e.g., trafficking to and from the plasma membrane to and from the Golgi stacks and the lysosome) are apparent (*arrows*). Note (*a*) the continuity between ER and the OMM; (*b*) the contrast between the grossly visible channel (nuclear pore complex) between the nucleus and the cytoplasm and the lack of a morphologic channel through the ER membrane involved in protein translocation; and (*c*) the fission and fusion of various vesicles involved in protein trafficking from the ER to the cell surface.

secretory proteins occurred on ribosomes bound exclusively to the membrane of the ER, and yet discrete channels, such as those that exist between the cytosol and the nucleus (nuclear pores), are not observed. Hence a specialized mechanism was suspected whereby (*a*) secretory protein synthesis was restricted to the ER (the problem of targetting) and (*b*) whereby the membrane barrier to protein diffusion was selectively and transiently overcome (the problem of translocation). Once translocated to the ER lumen, secretory proteins are never observed to reenter the cytosol.

Several early lines of evidence suggested that secretory proteins traversed the ER membrane during their synthesis. Perhaps the most compelling studies involved translation of mRNAs for secretory proteins in cell-free systems. Higher molecular weight precursor forms of these proteins were revealed. Generally, these were due to amino-terminal peptide extensions that were not observed in the mature proteins either secreted or stored in cells (5–7). In most cases, these amino-terminal extensions were cleaved to generate the authentic forms of these secretory proteins only if the cell-free system was supplemented with ER (in the form of microsomal membrane vesicles prepared by cell fractionation) during growth of the polypeptide chain.

Moreover, authentic forms so generated were demonstrated to be localized to the lumen of the microsomal membrane vesicles, thus indicating that a transport event had taken place in a fashion coupled to translation of the protein (8, 9). The development of such cell-free systems has had a powerful influence on progress in the field of protein traffic. A large body of work in these systems suggest that secretory protein targeting and translocation result from the interaction of these traffic-directing amino terminal extensions, termed signal sequences, with receptor proteins in and about the ER membrane (10). Below we shall see that the coupling of translocation across the ER to synthesis of the protein has an explanation that was not entirely expected.

The signal hypothesis, first proposed to explain translocation across the ER membrane (7, 8), is a useful framework for conceptualizing all other forms of protein traffic. Signal sequences can be viewed as only one example of a plethora of topogenic sequences (11). These are discrete peptide sequences, located in various positions within newly synthesized proteins, that are hypothesized to direct each of the various targeting, translocation and trafficking events that occur in the cell by interaction with specific receptor proteins (11). Identifying these receptor proteins and their ligands within newly synthesized proteins and understanding how they work is a major challenge of modern cell biology.

Targeting to the ER membrane

The molecular mechanism whereby targeting to the ER membrane takes place has been studied in considerable detail (Fig. 2 *A*). The signal sequences implicated in targeting to the ER membrane are discrete 15–30-residue regions of the nascent chain, which appear to act as ligands to direct the passenger, i.e., the rest of the protein, to and across the ER membrane. Typically, although not always (12–15), the signal sequence resides at the amino terminus, i.e., is encoded in the 5′ end of the mRNA for a secretory protein starting at the initial methionine codon, and is removed from the growing polypeptide chain during translocation.

After initiation of secretory protein synthesis on a free ribosome in the cytosol, the signal sequence emerges from the ribosomal large subunit as part of the growing (also called nascent) chain. It is rapidly recognized by a cytosolic ribonucleoprotein particle consisting of six proteins and a small RNA, termed signal recognition particle (SRP) (16–19). Upon binding of SRP to the signal sequence, further chain growth is considerably slowed. This arrest or pause in continued chain growth allows the SRP-nascent chain-ribosome-mRNA complex to be targeted specifically to the ER membrane by recognition of a receptor protein on the cytoplasmic face of the ER membrane, termed signal recognition particle receptor (SRPR) or docking protein (20–23). Docking of SRP with SRPR is a transient event that is followed rapidly by release of SRP from the nascent chain, allowing chain growth to resume at its normal rate, with recycling of SRP back to the cytosolic free pool (24). Only the mRNA of proteins destined for the ER lumen (e.g., secretory and integral transmembrane proteins) encode a signal sequence that recognizes SRP. Thus the SRP cycle (see Fig. 2 *A*) provides a means of discriminating these newly synthesized proteins from those destined for other compartments. Mitochondrial, nuclear, and peroxisomal proteins have alternate targeting systems (see below). Proteins destined to remain in the cytosol have no targeting information and hence can be directed into the secretory pathway when the coding region for a signal sequence is provided by molecular genetic engineering (25). Such molecular genetic manipulations have not only revealed that signal sequences are neces-

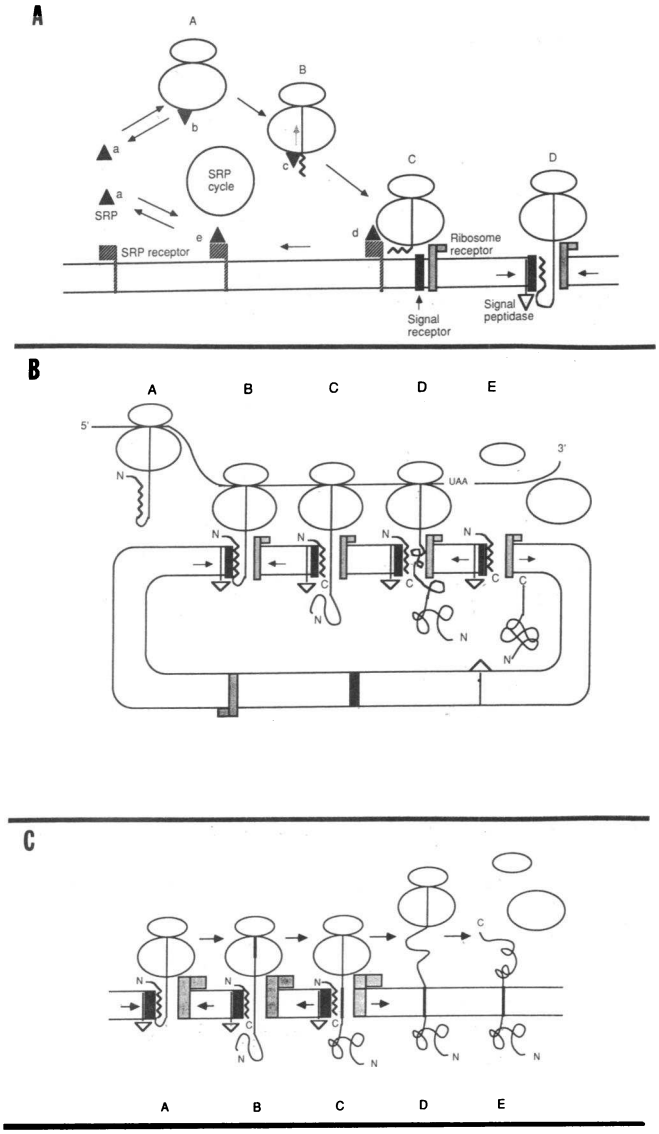

Figure 2. (*A*) Model of signal recognition particle (SRP) cycle for targeting nascent secretory and transmembrane proteins to the ER membrane. Soluble SRP (*a*) exists in equilibrium with a membrane-bound form, presumably bound to SRP receptor (*e*), and a ribosome-bound form (*b*). Upon translation of mRNA encoding a signal sequence for targeting to the ER membrane (*zigzag lines*), the affinity of SRP for the translating ribosome is enhanced (represented by dotted arrow, *B*) and SRP binds to the signal sequence directly (*C*), effecting elongation arrest (*B–C*). Upon interaction with ER membranes, elongation arrest is released and SRP and SRP-receptor are free to be recycled (SRP cycle, *A–E*), the synthesizing ribosome interacts with other transmembrane proteins, leading to formation of a functional ribosome membrane junction, translation resumes and translocation across the membrane occurs (*D*). (*B*) Steps in the process of translocation (subsequent to targeting) according to the receptor-mediated hypothesis. Synthesis begins on cytoplasmic ribosomes (*A*). Receptor-mediated targeting of the signal sequence-bearing ribosome to the ER membrane is described in the text and depicted in Fig. 2. Once targeted correctly, the signal sequence and the ribosome interact with their respective receptors in the ER membrane, resulting in the assembly of an aqueous, proteinaceous tunnel across the membrane (*B*). As protein synthesis continues, the chain passes through the tunnel to the lumen of the ER and the signal sequence is removed by signal peptidase (*C*). Translocation (possibly of partially folded polypeptide domains) continues concomitant with protein synthesis (*D*). Upon termination of protein synthesis, the ribosomal subunits dissociate, the carboxy terminus passes through the tunnel and the tunnel components disassemble, restoring the integrity of the lipid bilayer (*E*). (*C*) Receptor-mediated model for integral transmembrane protein assembly. Targeting of the nascent chain occurs as depicted in *A* and the signal sequence directs assembly of a tunnel for translocation across the membrane (*A*). Translocation occurs and the signal sequence is cleaved as depicted in *B*. Upon emergence of a stop-transfer sequence, components of the translocation machinery are disassembled (*C*) and the ribosome membrane junction is disrupted causing the remainder of the polypeptide to be synthesized in the cytoplasmic space (*D* and *E*). Reproduced from Protein Transfer and Organelle Biogenesis, 1988. Academic Press, Inc., published with permission.

sary and sufficient to direct a cytosolic protein into the secretory pathway, but also have provided insight into how signal sequences work (26, 27).

It appears that the various functions of SRP, namely signal recognition, slowing, or arrest of chain growth, and binding of SRPR, are mediated by distinct functional domains of this ribonucleoprotein particle (28). Essentially all of these conclusions regarding targeting of chains to the ER membrane are derived from studies in cell-free systems. Thus, while appealing in logic, the scheme described above awaits further confirmation by studies in vivo. Until such studies have been performed, it remains formally possible that aspects of our understanding of targeting and its machinery may need to be revised, and that experimental surprises are in store (see references 29 and 30). In this regard, recent studies have revealed a novel feature of SRPR biogenesis. This two-chain molecule appears to be targeted to the ER membrane by a multistep process involving a distinct targeting machinery all its own (31).

Translocation across the ER membrane

Our current understanding of the events in chain translocation subsequent to release of SRP are considerably more obscure. Some workers have suggested that translocation is a spontaneous process driven by the thermodynamics of protein-lipid interactions (32, 33). In this view, the signal sequence and its nascent passenger form a helical hairpin. The free energy gain achieved by burying the hydrophobic signal sequence in the bilayer more than offsets the free energy cost of pulling subsequent polar and charged sequences into the membrane as part of the hydrophilic limb of the hairpin. Continued chain growth results in extrusion of the unstable polar limb of the hairpin across the membrane as a consequence of either Brownian motion or the driving force of continued chain growth. Cleavage of the signal sequence by a specific protease on the lumenal side results in the passenger protein domain located in the lumen with the signal sequence retained in the membrane. Although elegant in its simplicity, several lines of evidence appear inconsistent with such a view of the mechanism of chain translocation. Instead, experiments support an alternative explanation (Fig. 2 *B*) in which translocation, like the preceding event of targeting, is directed by interactions between the signal sequence and proteins in the cytoplasm and the ER membrane serving as receptors. One powerful line of evidence in favor of this receptor-mediated model was the demonstration that chain translocation consumed energy in the form of nucleotide triphosphate hydrolysis distinct from

the requirements for protein synthesis (reviewed in reference 10). Because chain translocation appeared coupled to synthesis, some workers speculated that the driving force for translocation derived from the work done by the ribosome literally pushing the chain across the membrane (34). Instead it was demonstrated that a protein domain could be synthesized and then subsequently translocated even in the absence of further chain growth (35, 36). The translocation event was independent of protein synthesis but required a ribosome-dependent translocation competent state and nucleotide triphosphates (35). In contrast, studies on some proteins have suggested that the ribosome is not required for translocation of all newly synthesized proteins (37–40). In these cases it remains unclear whether ribosome-independent translocation is limited to cell-free systems (e.g., reflecting use of partially open translocation sites as a result of damage during cell fractionation), or whether the ribosome is involved in events (e.g., steps in unfolding) that may not be required for every newly synthesized translocation substrate. In any case, most nascent chains do require the ribosome for translocation competence (35, 41).

Together, these data implicate a protein machine in the ER membrane to drive chain translocation. One appealing view of this machinery is that its assembly or activation requires multiple recognition events involving the signal sequence, the ribosome and protein cofactors such as SRP. Such a scheme provides a failsafe mechanism to insure cellular integrity. The overall outcome appears exquisitely selective because cytoplasmic proteins are never secreted. The involvement of multiple recognition events may explain the paradox of an efficient recognition system that is both degenerate (e.g., the primary structure of signal sequences varies considerably within a particular cell, tissue, or species; see references 42 and 43) and universal (i.e., all signal sequences appear to recognize a common set of proteins that direct translocation of diverse protein domains).

Translocation of the passenger domain appears to occur through an aqueous channel (44). Whether actual translocation takes place passively, e.g., through Brownian motion, or actively, is unknown since the precise role of nucleotide triphosphates remain to be established (45–47). In either case, the vectorial character of chain translocation might be maintained by recognition of higher affinity signal sequence receptors on the lumenal face of the ER membrane. Such a hypothetical gradient of higher affinity receptors would direct the signal sequence to the lumen. Dissembly or closure of the transmembrane channel would happen concomitant with termination of chain synthesis. Perhaps signal cleavage serves to reduce the signal sequence's affinity for the terminal (highest affinity?) receptor protein in the ER lumen and activate degradation and clearance of the signal peptide. Several recent observations are consistent with this view, including the discovery of a protein in the ER membrane that appears to bind signal sequences after their release from SRP (48); the discovery of translocation mutants in which the passenger sequence is translocated but the signal sequence remains bound to the cytoplasmic face of the ER membrane (49, unpublished observations); and the demonstration in engineered translocation substrates that the entire signal sequence can be found translocated to the lumenal space and not integrated into the lipid bilayer (26, unpublished observations). Identification of these receptor proteins and the reconstitution of their functions in vitro will be required to prove this hypothesis.

Recently, a member of the HSP 70 heat-shock gene product family has been shown to facilitate chain translocation (50, 51). Earlier studies had implicated members of this family in ATP-dependent protein unfolding and possibly, refolding (52). It thus is possible that the more recent findings regarding HSP 70 stimulation of chain translocation reflects a role for protein unfolding in this process (53, 54). Alternatively, HSP 70 may simply maintain nascent chains (or their signal sequences) in a soluble, translocation-competent form, and is not actually involved in translocation per se. Regardless of its precise role, the possible involvement of this member of the family of stress response proteins suggests that chain translocation can be influenced by stress and impaired in disease (55).

Transmembrane integration as a variation on the theme of secretory protein translocation

Just as secretory proteins, synthesized on ribosomes in the cytosol, need to translocate across the ER membrane, so also the biogenesis of integral transmembrane proteins requires translocation of particular domains, whereas other sequences of the protein are prevented from being translocated. Any hypothesis on how membrane protein topology is achieved must account for the observation that every copy of a given transmembrane protein appears to have the identical orientation with respect to the bilayer (56). Yet different transmembrane proteins display quite distinct topology, not only with respect to disposition of amino and carboxy termini, but also in the number of transmembrane spanning regions and in the size of the various domains disposed on one side of the membrane or the other (Fig. 3).

It has been proposed that all of these aspects of membrane protein biogenesis can be accounted for by a variation on the theme of the receptor-mediated hypothesis of protein secretion (Fig. 2 *C*). In this view, the translocation of integral trans-

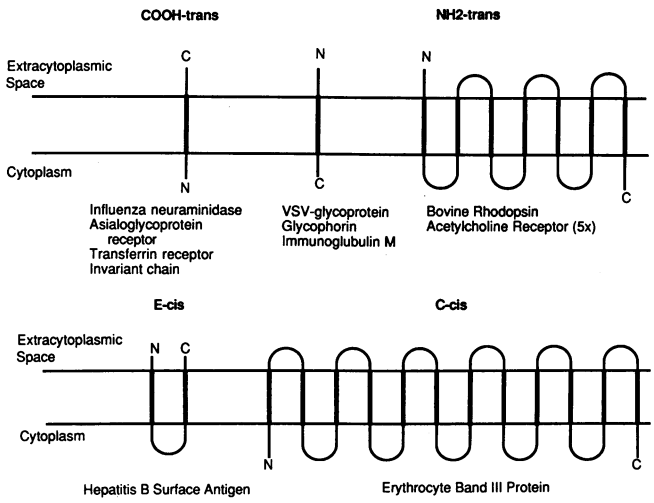

Figure 3. Examples of the diversity of integral transmembrane protein orientations. Membrane spanning segments are represented by heavy black lines. References for sequence and/or experimental orientation data are as follows: influenza neuraminidase (15), asialoglycoprotein receptor (157), transferrin receptor (158), invariant chain (159), VSV glycoprotein, (56, 57), glycophorin (160), immunoglobulin M (161, 162), bovine rhodopsin (163), acetylcholine receptor (66), hepatitis B surface antigen (152), and erythrocyte band III protein (164).

membrane protein domains is initiated by a signal sequence engaging the same protein machinery as in the case of protein secretion. However, the reason these proteins are not completely translocated is that they contain another discrete coding region, termed a stop-transfer sequence, which is responsible for termination of chain translocation across the ER membrane (11, 57, 58). The mechanism whereby stop transfer sequences act remains a point of controversy. As with protein secretion, hypotheses emphasizing both receptor-mediated (protein-protein interactions) and spontaneous/thermodynamic (protein-lipid interactions) processes have been proposed (49 vs. 59). The former envisions the stop-transfer sequence directing the dissembly or inactivation of the protein channel or machinery whose assembly or activation was originally catalysed by the signal sequence. In this view, stop-transfer sequence interaction with a receptor protein results in termination of translocation. At the same time the membrane bound ribosome is converted into the functional equivalent of a free ribosome (although it still remains tethered to the membrane by the nascent chain, Fig. 2 *C*). Hence further chain growth now occurs into the cytoplasm rather than into the ER lumen, with subsequent protein-lipid interactions serving to stabilize a transmembrane disposition.

In contrast to the receptor-mediated view, the alternative perspective proposes that simple hydrophobic interactions between transmembrane regions and the lipid bilayer are sufficient to stop a protein on its way through the membrane, without invoking receptor-ligand interactions with other polypeptides (33). Evidence for and against each view suggests that this issue remains to be resolved and each model may be an accurate description of a subset of cases (49, 59–61). More recent studies have clearly demonstrated that although signal and stop-transfer sequences share some similar physical and functional properties, they are distinct, noninterchangeable determinants of protein topology (69). Conversely, it appears that in at least some cases topologies based on analysis of hydrophobicity alone have resulted in incorrect transmembrane orientations. In some cases such analyses have erroneously predicted that a peptide sequence would span the membrane (63 vs. 68, 64). In other cases hydrophobicity indices have overlooked regions which appear experimentally to span a membrane (68, 65, 66 and 63, 67).

How can we account for the topology of polytopic integral transmembrane proteins, i.e., those that span the membrane multiple times? One hypothesis was that alternating signal and stop transfer sequences in register might serve to direct intervening passenger domains into such a fate (12). Studies from several approaches have suggested that this view is correct (69–71), although the molecular mechanism by which the NH_2- versus COOH-termini of a protein is translocated by a signal sequence remains to be resolved. This specificity could result from features of the particular signal sequences, flanking sequences or global properties of the protein domains to be (or not) translocated. The molecular genetic approach described earlier should allow resolution of these issues.

Multisubunit assembly and posttranslation modifications

One of the most striking characteristics of membrane-delimited compartments is the asymmetry across their membranes (72). It thus should not be surprising that many activities take place in lumenal compartments that cannot occur in the cytosol. Upon appearance of a nascent chain in the lumen of the ER some modifications take place cotranslationally, that is, before completion of synthesis. These include cleavage of signal sequences, addition of core N-linked carbohydrates, and formation of disulfide bonds (73, 74). Other modifications take place in the ER but shortly after chain completion. Among these ER lumenal events is assembly of multisubunit complexes (75). There appears to be a machinery in the ER to prevent unassembled monomers from being transported out of the ER (76, 77). In some cases such molecules appear to be bound to a heat-shock gene product, HSP-78, also known as heavy-chain binding protein or BiP (77, 110). For example, expression of immunoglobulin heavy chain in the absence of light chain results in its retention in the ER complexed with BiP (110, 135). Secretion of the molecule can be rescued by expression of the missing immunoglobulin light chain (76). With assembly of the complex, release from BiP and transport out of the ER happen in an apparently concerted fashion (77). In other cases, such as trimerization of the viral envelope glycoprotein influenza hemagglutinin, monomers do not leave the ER, but are not found in association with BiP (79) although it appears that BiP is involved in binding monomers of mutant HAs that are misfolded or denatured (80). It thus is not yet clear if BiP's role is to recognize misfolded proteins, to prevent monomer transport out of the ER, or to promote correct refolding and assembly. The latter process might explain the observed ATP dependent release of BiP from some proteins (52). In addition to the BiP-mediated pathway, others have recently characterized a pathway for rapid degradation of incompletely assembled polymers (81). The relationship of either of these mechanisms to pathways of intracellular quality control remains to be elucidated. As in the case of HSP-70, the involvement of HSP-78, in intracellular transport suggests the importance placed on faithful protein trafficking by the cell and indicates a very likely point of intersection between profitable investigations into cell biology and the molecular basis of disease.

Post-ER events in secretion: transport to and through the Golgi apparatus

As stated earlier, protein transport through the secretory pathway beyond the lumen of the ER occurs by a process of vesicles budding from one compartment and fusing to the next compartment in the pathway. The number of distinguishable compartments within the secretory pathway has grown in recent years with the increased sophistication of techniques and probes with which to study cells and cell fractions and the transport events in which they are involved (82–84). The number of compartments is likely to continue to increase as further regional specialization within compartments is recognized (85).

A consideration of the problem of vesicle-mediated protein sorting suggests the need for two recognition systems. One, operating in the lumenal space or on the lumenal side of membranes, must identify different classes of proteins with different fates and divert them to laterally specialized regions of the membrane, i.e., from which fission of the correct vesicle will take place (82, 84). As a result of this recognition system, proteins are sorted into the correct vesicle containers. Another recognition system must operate on the cytoplasmic face of these membrane vesicles. The latter system directs vesicles to interact with one particular target membrane but not with others. Because these recognition systems must operate be-

tween completed, folded proteins and because protein folding is poorly understood, the identification of the regions within proteins that serve as ligands for these classes of sorting receptors is problematic and remains as a major experimental challenge for the coming period.

During the process of intracellular transport, various compartment-specific posttranslational modifications may occur. For example, as a result of such modifications, different cell types in the anterior pituitary are able to generate distinct cleavage products from a common precursor polypeptide, proopiomelanocortin (86). Moreover, catecholamines, glucocorticoids, and acute or chronic stress appear to regulate processing of this precursor to the mature polypeptides adrenocorticotrophic hormone versus β-endorphin (87, 88).

It has also been observed that the internal pH of the compartments becomes progressively more acidic (89, 90). The pH gradient and the cascade of posttranslational modifications may both serve to provide a vectorial character to lumenal recognition systems of intracellular transport. For example, recognition of one sorting receptor may occur at a particular pH and be lost as the pH falls. The new pH in turn, may allow recognition of a subsequent sorting receptor. The dependency of receptor recognition on pH could occur through posttranslational modifications occurring in one but not another pH or ionic environment (see reference 91).

Recently, an important clue as to the control of intracellular sorting events on the cytoplasmic face of vesicles has been established. A number of GTP binding proteins have been implicated in the control of vesicle traffic from compartment to compartment (92, 93). Whether these proteins mediate some manner of transmembrane signaling from lumenal to cytosolic aspects of vesicles or instead serve as a recognition system on the cytosolic face of membranes for correct docking and fusion of vesicles remains to be established, although the latter seems most plausible (94). Some of the most exciting current research in the field of protein trafficking involves development of systems in which these recognition events are reconstituted in vitro (95–98), and studies directed towards identification of the molecular components involved in protein and vesicle recognition (92, 99). Combined with the generation of mutants in yeast that define genetic complementation groups in the secretory pathway, a powerful set of tools are in hand for the dissection, reconstitution, and analysis of molecular events in post-ER sorting (100, 101).

From the ER, it appears possible to identify a special class of vesicles involved in traffic to the *cis* stack of the Golgi apparatus, a distinct cluster of membrane cysternae found in all cells (4, 102). The component membrane stacks of the Golgi apparatus were originally defined on morphologic grounds as *cis, medial,* and *trans.* Several lines of evidence, including fractionation of membranes, electron microscopic immunocytochemistry, and demonstration of intercompartmental protein transfer have suggested strongly that Golgi stacks are both structurally and functionally distinct (reviewed in reference 102). Thus, products deposited in the *cis* stack are transported by another cycle of vesicle fission and fusion to the *medial* stack and similarly from *medial* to *trans* stacks. One characteristic of these transport vesicles is that they are coated, a morphologic term referring to the presence of proteins on the cytoplasmic face that are suspected to play a role in some aspect(s) of targeting and transport (103). In this case the protein coat is distinct from the protein clathrin observed to serve as a coat for vesicles internalized from the plasma membrane by receptor-mediated endocytosis (104).

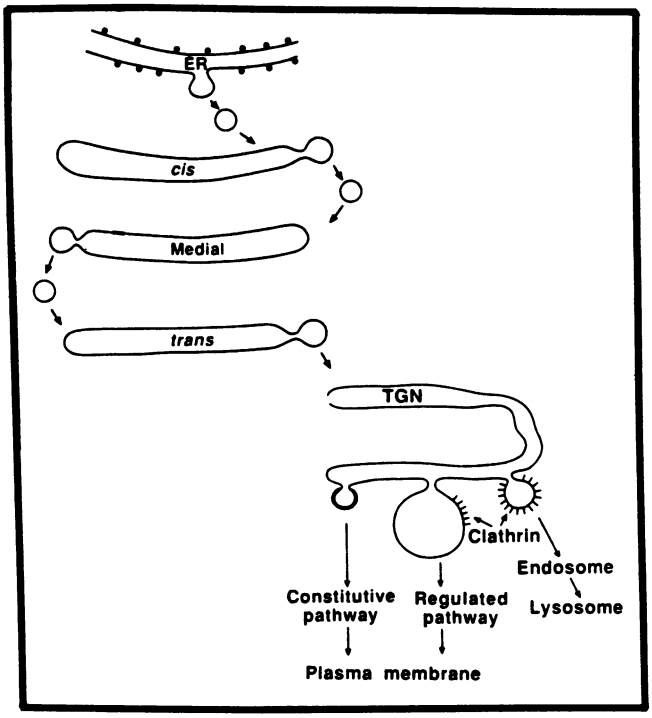

Figure 4. Compartmental organization of the secretory and sorting pathways. ER, endoplasmic reticulum; *cis, medial,* and *trans* refer to Golgi stacks; TGN, *trans* Golgi network; *arrows* refer to pathways of protein traffic. Reproduced from Griffiths, G., and K. Simons. 1986. *Science (Wash. DC).* 234:438–443, 24 October 1986. Copyright 1986 by the AAAS.

A number of studies suggest that the *trans*-most stack of the Golgi apparatus, also termed the *trans* Golgi network (TGN) is responsible for a qualitatively different sorting event than occurs in earlier compartments (Fig. 4) (82). It appears that vesicles derived from the TGN target separately to lysosomes and to regulated and constitutive secretory pathways that will be discussed below (82).

An interesting observation that may have profound implications for the nature of membrane traffic is that the pathways of vesicle transport in secretion and receptor-mediated endocytosis intersect at discrete points. Thus, endocytosed markers can intermix with secretory products in transit through the intracellular pathway at the level of the TGN (105). Whether this simply reflects the need to recycle receptor proteins involved in biosynthetic protein traffic or has more profound implications for common pathways and feature of vesicle dynamics (see reference 106), remains to be elucidated. As indicated above, some of these vesicles are coated with clathrin, whereas others are not. Yet another member of the heat-shock protein family has been shown to serve as an uncoating ATPase involved in clathrin cage dissembly (78).

Lysosomal enzyme sorting

Best understood of the sorting events that direct newly synthesized proteins from the TGN is the targeting of specific hydrolases to lysosomes. One pathway by which this occurs is through a recognition system mediated by mannose-6-phos-

phate (M-6-P) (107). Phosphomannosyl transferase is a *cis* Golgi enzyme that appears to recognize lysosomal enzymes upon their arrival to the Golgi apparatus and selectively tags them by the addition of phosphate residues. Upon arrival in the *trans* Golgi, these phosphomannosylated proteins are recognized and bound by a specific receptor, the M-6-P receptor, that is localized to the *trans* Golgi stacks. After budding from the TGN, these vesicles appear to undergo a process of progressive acidification resulting in receptor-ligand dissociation. Because the M-6-P receptor is not found in lysosomes, it is believed that an intermediate compartment must exist where ligand is released and from which free receptor recycles back to the TGN (108). Upon fusion of this vesicle to lysosomes, the content protein is released into the lysosome. This molecular mechanism is believed to be one means of sorting lysosomal enzymes out of the secretory pathway, and provides an example of how both protein recognition and posttranslational modifications may be used as sorting signals. In the case of I cell disease, a deficiency in the phosphotransferase results in failure to tag lysosomal enzymes with the M-6-P recognition marker (3). As a result, some cells in affected individuals secrete their lysosomal enzymes. These patients display skeletal abnormalities and psychomotor retardation and a rapidly fatal course (165). Despite the logical appeal of this model for lysosomal sorting, as in the case of targeting to the ER membrane, a word of caution is in order: although phosphotransferase is deficient in various cells of I cell patients, including fibroblasts, hepatocytes, and leukocytes, lysosomal enzyme levels are found in the latter two cell types, indicating the presence of a non-M-6-P mechanism of lysosomal targeting. The existence of two distinct M-6-P receptors, one of which is identical to the receptor for insulin-like growth factor II (111) indicates the complexity of these receptor proteins and the ligands that they recognize and how poorly we understand their role in sorting. Moreover, in yeast, which are lower eukaryotes, a topogenic sequence is used directly for targeting to the lysosome equivalent, apparently bypassing the modification recognition steps (112, 113).

The M-6-P-mediated sorting events of lysosomal enzyme biogenesis in higher organisms require phosphomannosyl transferase to remain in the *cis* Golgi, whereas the M-6-P receptor recycles from TGN to endosomes and back, but apparently never appearing in lysosomes (114). How these examples of selective retention are achieved remains to be established. However, in the case of BiP in the ER lumen, where a similar retention event must take place, the peptide sequence lysine-aspartic acid-glutamic acid-leucine (K-D-E-L) has been demonstrated to confer retention in the ER (115). Perhaps other compartment-specific recognition systems will be discovered. Of course, implicating the involvement of another class of asymmetrically distributed receptors, simply defers the problem of how such protein distribution is achieved to another level of targeting, sorting and maintenance: that of the biogenesis of the receptors for compartmentalized proteins. It remains unclear whether even secretory proteins proceed through the pathway passively, i.e., through bulk flow (116), or whether they are actively or passively directed or retained from compartment to compartment (117, 118). Here also, both genetic and environmental bases exist for a connection between protein traffic and disease. In alpha 1-antitrypsin deficiency, a point mutation renders the molecule unable to leave the ER, thereby conferring the deficient phenotype on individuals with the disease. Expression of the mutant protein in heterologous systems such as in *Xenopus* oocytes reproduces the trafficking defect (119). Likewise, in some cases altered protein transport, rather than simply expression of a new gene product, appears to be responsible for neoplastic transformation (120).

Regulated vs. constitutive secretion

Just as sorting systems must achieve diversion of lysosomal enzymes from the secretory pathway, so also secretory proteins must be separated into those that are constitutively released, i.e., in a continuous fashion commensurate to the rate of synthesis and turn over, and those that are secreted in a regulated fashion (121). These latter proteins are packaged in concentrated form into secretory granules and are released only upon stimulation of that particular cell (122, 99). Most integral membrane proteins follow the constitutive pathway, i.e., probably travel to the plasma membrane in the same vesicles that deliver continually secreted proteins. A topogenic sequence or post translational modification directing regulated versus constitutive sorting has not yet been identified. Thus, the mechanism by which proteins destined for the regulated pathway are diverted to vesicles that will mature into secretory granules remains an extremely important unsolved problem. Possibly recognition is mediated by three-dimensional features not discernible at the level of linear protein sequence. In recent years the importance of pulsatile release of hormones for endocrine (123, 124), exocrine (125), and perhaps also paracrine and autocrine actions (126) has been recognized. It seems possible that this variation on the theme of regulated secretion or related phenomena are involved in the pathophysiology of subsets of a variety of common but poorly understood diseases including diabetes mellitus and hypertension (127, 128).

Apical vs. basolateral localization

Newly synthesized protein traffic can be controlled not only with respect to consignment in regulated vs. constitutive pathways, but also with regards to which side of a cell proteins are delivered. This kind of protein sorting results in specialized functions of various plasma membrane domains. For example, in a liver cell, it is the basis for bile secretion exclusively from the apical face and serum protein secretion exclusively basolaterally. In some cells it appears that this sorting event, e.g., apical vs. basolateral localization of proteins, occurs at the TGN (129). However, recent studies in hepatocytes suggest there must exist more than one way to achieve plasma membrane polarity. Here, it appears that all newly synthesized plasma membrane proteins are first localized to the basolateral surface. Subsequently, vesicles depart for the apical plasma membrane carrying those transmembrane proteins destined for that location (130). This may be a variant of the pathway of protein transcytosis by which, for example, immunoglobulin A is transferred across cells to enter the secretions in the glandular lumen of various epithelia (131). Finally, in some systems, populations of vesicles bearing a particular cell surface protein appear to be inserted into, or endocytosed from, domains of the plasma membrane in response to specific physiological stimuli. In the case of the H^+/ATPase, the choice of apical versus basolateral plasma membrane of renal tubule cells may regulate metabolic correction of acidosis and alkalosis (132). Likewise, some evidence suggests that vasopressin-sensitive water channels (133) and insulin-dependent glucose transporters (109) are recruited to, and removed from, the

plasma membrane through a population of intracellular vesicles. All of these processes serve further evidence for a relationship of vesicle dynamics in the secretory and endocytotic pathways whose implications remain unexplored.

Protein traffic to other compartments

As mentioned earlier, protein traffic from the cytosol into mitochondria (134, 140), chloroplasts (136), nuclei (137, 138), and peroxisomes (organelles containing enzymes involved in various pathways including those of peroxide metabolism, see reference 139) are directed by mechanisms distinct from that of protein secretion. However, some common themes have emerged: each destination is reached by interaction of a distinct class of topogenic sequence within the newly synthesized protein with receptor proteins on the surface of the particular organelle; unfolding activity and heat shock gene products have been implicated in a fashion similar to their proposed role in protein translocation across the ER membrane (50, 122); translocation appears to be an energy-consuming process. Beyond these broad conceptual similarities, are found many differences in the details: whereas signal sequences targeting proteins to the secretory pathway generally act in a cotranslational fashion, i.e., while the chain is still being synthesized, those of proteins targeted to the other organelles appear to act exclusively posttranslationally, i.e., after completion of synthesis. In the case of targeting to and translocation across membranes of mitochondria and chloroplasts, the topogenic signal sequence is characterized by a striking lack of acidic amino acid residues, less hydrophobicity than the ER signal sequences, and more basic amino acids (140, 141). Moreover, in addition to nucleoside triphosphate hydrolysis, membrane potential has been shown to provide a necessary source of energy to drive mitochondrial, but not ER, translocation (121, 142). The receptor proteins involved in these organellar targeting and translocation processes remain largely uncharacterized (with a few exceptions, e.g., reference 136).

In the case of nuclear transport small positively charged signal sequences have been identified which appear to direct transport from the cytosol through the nuclear pore (137, 138). Again, receptors have been implicated (138) but their identity and mechanism of action remains unknown.

One rare genetic disease, Zellweger's syndrome, appears to result from inability of peroxisomal enzymes to be localized to the matrix of that organelle, whose content proteins are normally taken up by yet another topogenic sequence mediated process (2). These patients display metabolic disturbances, central nervous system, hepatic, renal, and skeletal abnormalities and typically die within 6 months of birth (142).

Novel pathways of protein traffic and some speculation

Thus far, our focus has been the conventional mechanisms by which proteins are translocated across, anchored to, or sorted between membranes. In addition, a number of unusual processes of protein traffic have been only recently recognized. These appear to represent novel means by which a protein domain can be either presented as a surface marker on a membrane or be secreted out of the cell in which it was synthesized. Conventionally, two mechanisms have been recognized by which a protein domain can achieve both secreted and membrane bound forms. One of these is RNA splicing to generate separate mRNAs, e.g., with and without coding regions for a stop-transfer sequence, as in the case of the heavy chain of membrane and secretory IgM (143). The other is proteolytic cleavage to release a domain from a transmembrane protein (144). Conversion of the IgA receptor into secretory component is a good example. Some studies suggest this mechanism may also apply to the biogenesis of epidermal growth factor, proposed to be synthesized initially as a much larger transmembrane precursor which is proteolytically cleaved (145, 146). In the last few years other very different mechanisms by which certain proteins can be directed into either secreted or membrane bound forms have become apparent. Some of these are discussed briefly below.

Glycolipid linkage of proteins to membranes

A novel mechanism whereby proteins can be anchored to membranes through a glycolipid linkage has been elucidated in recent years (147). Addition of this phosphatidyl inositol (PI)-linked glycan appears to be directed by a short stretch of hydrophobic amino acids at the extreme carboxy terminus of these proteins. Observed first for the variant surface antigens of the trypanosome coat, it has now been demonstrated that a growing family of functionally diverse cellular surface proteins are anchored to the plasma membrane by a heterogeneous set of such glycolipid linkages. The linkage can be broken by specific phospholipases thereby releasing a secretory form of the protein (148). Moreover, a PI-linked glycan that is quite similar in structure to this class of membrane anchor has been implicated as a possible second messenger in the action of insulin (148, 149). Are these roles of PI-linked glycans in protein anchoring and signal transduction related? In the case of lipoprotein lipase, metabolism of the glycolipid linkage is stimulated by insulin (149, 150). The details and full significance of glycolipid addition, hydrolysis, and function remain to be elucidated. If the relationship between these phenomena prove to be of general significance, this posttranslational modification may prove to be an important point of intersection between protein traffic and other dimensions of cellular metabolism.

Posttranslational particle formation from transmembrane intermediates

The surface antigen of hepatitis B virus (HBsAg), has provided a model for another remarkable cell biological process (151). HBsAg exists in two forms, as the coat protein of the virus and in the form of 20-nm soluble particles in the bloodstream of infected individuals (151). The particles consist of approximately one hundred HBsAg monomers in association with lipid but lacking a discernable bilayer. Particles are found in up to one million-fold excess over virions, but their precise role in virus survival, (e.g. immune modulation), remains to be elucidated. Studies of particle biogenesis reveal an unusual mechanism by which transmembrane monomers of surface antigen are posttranslationally converted into secretory particles (152, 153). Discrete steps in this process must include transmembrane assembly, aggregation of monomers in the plane of the membrane, lipid rearrangement with exclusion of host proteins, and finally extrusion of the assembled, heavily disulfide-linked protein particle into the ER lumen. Signal sequences and stop-transfer sequences are implicated in achieving the initial transmembrane disposition (154). Whether additional topogenic sequences and compartment-specific receptor proteins are involved in the subsequent unconventional steps of this process, is unknown. However, it is notable that surface antigen is the only viral protein necessary for this process. If

cellular machinery is involved in particle formation, the mechanism may reflect a more general cellular solution to the problem of how to maintain solubility of hydrophobic protein monomers during biogenesis of complex, highly posttranslationally modified protein polymers. From this perspective the HBsAg model may be relevant to assembly of other complex particulate biological macromolecules, such as mucins and lipoproteins, aberrations in whose metabolism are involved in common human diseases such as chronic bronchitis and atherosclerosis, respectively.

Alternate cotranslational secretory and complex transmembrane fates

The scrapie prion protein (PrP) is an unusual developmentally expressed molecule found in normal brain (155). An altered form of this protein has been implicated in a transmissible form of progressive neuronal cell death in animals (scrapie) that is very similar to some human diseases (Creutzfeld-Jacob disease and Gerstmann-Straussler syndrome) (156). Studies on the biogenesis of PrP involving expression of a cloned cDNA in cell-free systems have revealed that it can display either an integral transmembrane or a secretory fate (67, 68). The choice of transmembrane versus secreted fates appears to be made while the chain is growing (68) and seems to be dependent on protein machinery in the cytosol engaging novel topogenic sequences (68, Yost, C. S., et al.; Lopez, C. D., et al., manuscripts in preparation). Thus, in this case protein phenotype appears to be regulated cotranslationally at the level of the ER membrane, representing a novel form of control over gene expression. Perhaps this unusual pathway of biogenesis is related to the (unknown) physiologic function of this protein or to the bizarre disease process in which it is implicated.

These mechanisms appear to represent unusual variations on the more conventional processes described earlier. Their study has and likely will continue to provide insight into more general mechanism by which cellular components are built. Also, precisely because they are not general mechanisms they represent potential points at which either acquired or genetic lesions might result in an aberrant but viable phenotype relevant to human disease. Certainly other mechanisms of protein traffic remain to be discovered. Whereas the significance for cell biology and medicine of these and other mechanisms of protein traffic remain to be established, their existence indicates new links in the chain of biological regulation. It is precisely in such details of biological regulation that the connections between basic science and medicine will be forged.

Future directions for the study of intracellular protein traffic and its implications for medicine

At the outset of this Perspectives article, a distinction was drawn between congenital lesions of protein traffic and those aberrations of protein biogenesis that may be acquired as a result of degenerative diseases and environmental stress. The former are mechanistically revealing but rare and hence of limited clinical importance. The latter are much more difficult to recognize or study and currently are poorly understood, but are likely to be much more common and hence clinically important. In this review, I have emphasized avenues of basic investigation that seem likely to yield new insight into the latter class of clinical disorders. It should be clear from this discussion that fundamental unanswered questions remain before conventional mechanisms of protein traffic are well understood. Until these conventional mechanisms are clear, the full significance of the unconventional mechanisms discussed will remain unresolved, as will the implications of both conventional and unconventional processes for acquired disease. The answers to these questions are thus likely to change our thinking on processes as diverse as oncogene activation, autoimmunity, and organ failure. Progress in understanding the cell and molecular biology of protein traffic over the last decade has brought us to the point where these potential connections between basic cell biology and medicine can be appreciated conceptually. The challenge of the next decade is to make these connections experimentally.

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