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Intracellular targeting and sorting of newly synthesized proteins

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Liver and pancreas cells secrete large quantities of proteins. They have been used as model systems for studying secretion ever since the discovery of subcellular components in the 1950s and 1960s. At that time, the newly developed techniques of electron microscopy and cell fractionation yielded the first evidence that all eukaryotic cells are subdivided into membrane-enclosed compartments, the organelles. When *in vivo* pulse-labelling of newly synthesized proteins with radioactive amino acid analogues was introduced, the route followed by the labelled secretory proteins from their site of synthesis at the endoplasmic reticulum to their site of release at the plasma membrane was identified. The organelles of the secretory pathway are now well characterized. However, the detailed analysis of the steps and mechanisms involved in the distribution of newly synthesized secretory proteins to their final functional residence is still a major focus of attention in cell biology (for review see refs. 1 and 2).

Figure 1 schematically illustrates a liver cell and its intracellular compartments. These include the organelles of the secretory pathway, the nucleus, mitochondria and peroxisomes (microbodies). In addition to these, plant cells contain chloroplasts. The secretory pathway comprises the endoplasmic reticulum (ER), the Golgi apparatus, transport vesicles, lysosomes, secretory granules and the plasma membrane[1]. In epithelial cells, that perform vectorial functions and therefore are polarized, the plasma membrane is further compartmentalized into an apical and a basolateral domain (for review see ref. 3).

Cellular membranes are composed of lipids and proteins. Most lipids are synthesized in the endoplasmic reticulum. How they are transported to their final site is still an open question[4, 5]. Except for a few proteins whose mRNA is encoded in mitochondrial and chloroplast DNA, most proteins are translated from mRNA which is synthesized in the nucleus. Translation of all nuclear-encoded proteins is initiated on ribosomes in the cytoplasm.

One of the major issues in the biogenesis of organelles is how these proteins

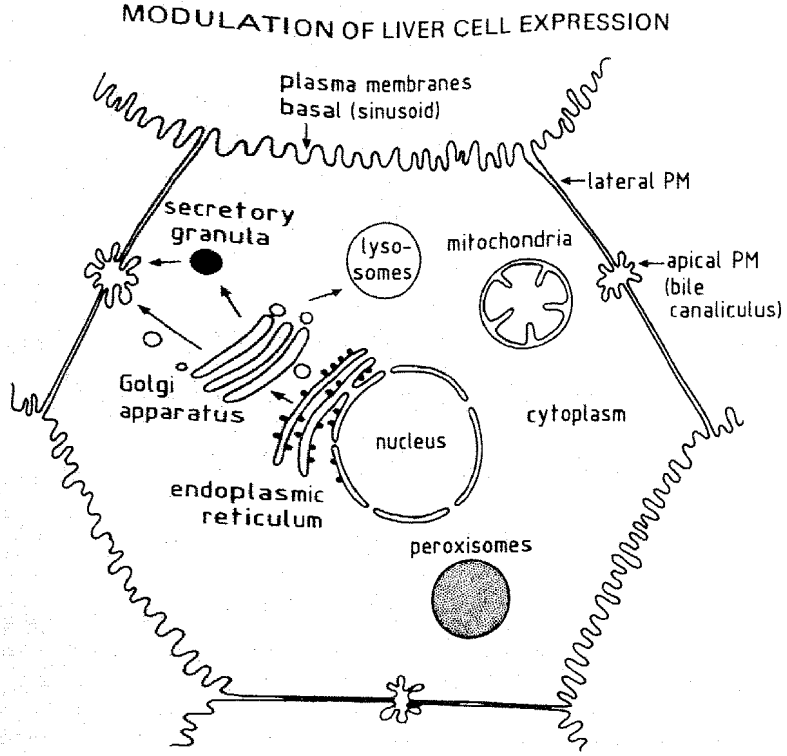


Figure 1 Schematic representation of a hepatocyte and its organelles. PM: plasma membrane

are directed to their correct target organelle. In this article we will mainly address the question how secretory and membrane proteins are targeted to the endoplasmic reticulum, translocated across or inserted into this membrane and finally sorted to their functional site within the secretory pathway.

TARGETING FROM THE CYTOPLASM TO THE SITE OF MEMBRANE INSERTION

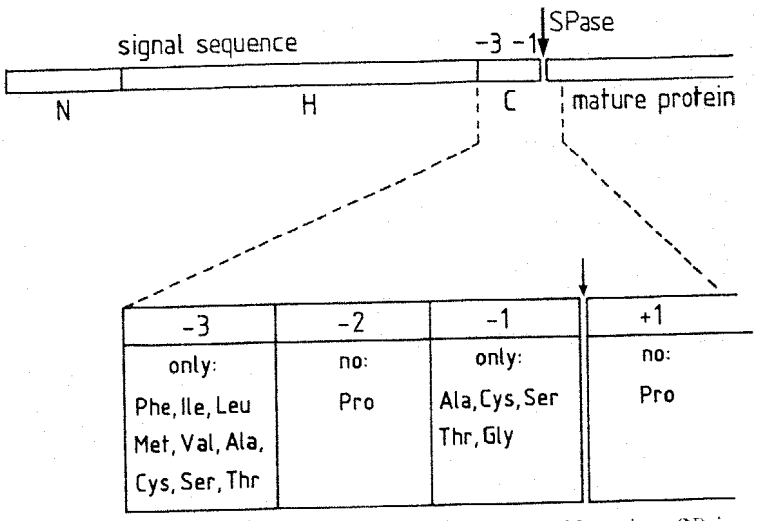
Conceptually, targeting of proteins from their site of synthesis in the cytoplasm to the site of translocation across the membrane of the correct compartment requires a signal which is contained in the protein sequence itself, and a receptor which is associated with the membrane of the target organelle.

Signal sequences

Targeting signals have been identified and characterized for proteins destined to the secretory pathway (for review see ref. 6), to mitochondria[7-9], chloroplasts[10-12] and to the nucleus[13-15]. Their information is encoded in a continuous stretch of amino acid sequences. In proteins targeted to the secretory pathway, to mitochondria and chloroplasts the signal sequences are located

at the N-terminus. Often, they are proteolytically removed after the targeting and translocation processes have been accomplished. In contrast, the internally located nuclear targeting signals are maintained throughout the proteins' life. Little is known about the signals responsible for targeting proteins to microbodies[16, 17].

There is no primary sequence homology among any of the signal sequences, but their targeting information appears to reside in a combination of physicochemical properties and secondary and/or tertiary structure. The N-



-3	-2	-1	+1
only: Phe, Ile, Leu Met, Val, Ala, Cys, Ser, Thr	no: Pro	only: Ala, Cys, Ser Thr, Gly	no: Pro

Figure 2 Characteristic features of an ER signal sequence. The N-terminus (N) is usually hydrophilic, the core of at least eight amino acid residues (H) is hydrophobic, and the C-terminal (C) three amino acids (position −1 to −3) specify the recognition site for cleavage by the signal peptidase (SPase). The table in the lower part of the figure shows which amino acid residues can occur at positions −3 to +1, as defined by von Heijne[6]

terminally located ER signal sequence (Figure 2) can be between 14 and over 70 amino acid residues long. Its N-terminus is usually hydrophilic with one to several basic amino acid residues. The core of at least eight residues is exclusively hydrophobic or uncharged. The C-terminal three amino acids (position −1 to −3) specify the recognition site for cleavage by the signal peptidase. They are uncharged and contain short side-chains[6]. The hydrophobic character is unique for ER signal sequences.

The most striking feature in signals responsible for targeting to mitochondria, chloroplasts and the nucleus is the clustering of positively charged and the absence of acidic amino acid residues. For mitochondrial signals it has been suggested that their targeting information might reside in their potential for forming amphiphilic helices[9]. Chloroplast and mitochondrial pre-sequences exhibit a striking structural similarity. In fact, Hurt *et al.*[18] showed that the pre-sequence of a chloroplast protein was capable of transporting a cytoplasmic passenger protein into yeast mitochondria. This result raises the question how specificity is provided for import of proteins into

mitochondria and chloroplasts of plant cells which contain both organelles (for review see ref. 19).

The receptor system for ER signal sequences

A receptor system that interacts with signal sequences and targets export proteins to the correct organelle has only been characterized for the endoplasmic reticulum, the port of entry to the secretory pathway. This receptor system consists of two functional components, the soluble signal recognition particle (SRP)[20] and the docking protein which is associated with the membrane of the rough ER[21, 22].

In vitro reconstitution of the targeting of secretory proteins to the ER has

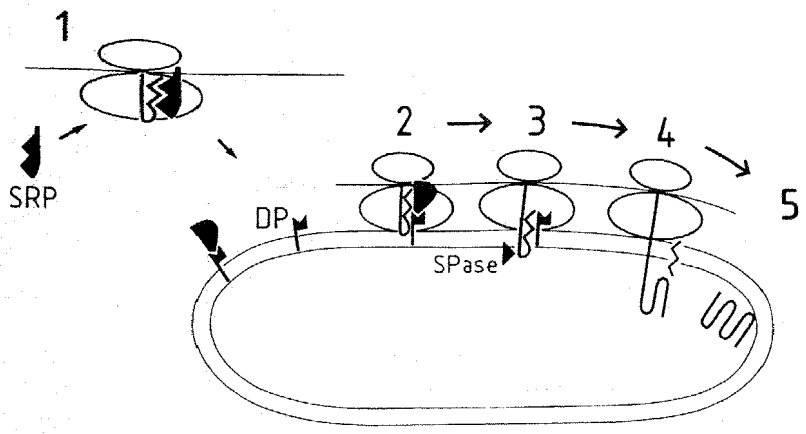


Figure 3 Model of targeting and translocation of secretory proteins. (1) Signal recognition particle (SRP) binds to the signal sequence and the ribosome and arrests further chain elongation. (2) SRP binds to the docking protein (DP) and thus targets the translational complex to the ER membrane. Upon binding to the docking protein, SRP presumably undergoes a conformational change which causes it to release the elongation arrest. (3) The signal sequence, in the form of a loop, initiates translocation. Signal peptidase (SPase) removes the signal sequence while the nascent chain is translocated. (4 and 5) The rest of the polypeptide chain traverses the membrane and is finally released into the ER lumen

allowed the identification of the following sequence of events (Figure 3; for review see refs. 23, 24). When the signal sequence, located at the N-terminus of a secretory protein, extrudes from the ribosome, it binds to SRP. By an as yet unknown mechanism, the interaction between signal sequence, SRP and the ribosome causes an arrest in further chain elongation[20, 22] until the translational complex is docked to the ER membrane by the additional binding of the SRP to the docking protein. This latter interaction causes the arrest in nascent chain elongation to be released. The nascent protein can now be segregated into the ER.

Six heterologous protein subunits of 72, 68, 54, 19, 14 and 9 kDa bound to one molecule of 7SL RNA form the signal recognition particle. The soluble ribonucleoprotein complex can bind to the ER signal sequences, to the large ribosomal subunits and to docking protein. Interaction of SRP with a signal

sequence and the ribosome causes an arrest in nascent chain elongation, while additional binding of the SRP to docking protein induces the ribonucleoprotein to dissociate from the translational complex and thus to release the arrest[20-22]. This switch in binding to different ligands might be facilitated by a dynamic property of the SRP: its RNA backbone, which by intramolecular basepairing forms a defined secondary structure, was shown to have the potential of undergoing conformational changes[25].

Docking protein, which is also termed SRP-receptor[21] is a 70 kDa integral membrane protein of the rough ER. Its main portion of approximately 52 kDa is exposed at the cytoplasmic face of the membrane[22, 26]. The amino acid sequence of docking protein has been deduced from its cDNA sequence and exhibits clusters of mixed-charge amino acids. Based on this finding, it was proposed that docking protein might interact directly with the 7SL RNA of SRP[26].

TRANSLOCATION OF PROTEINS ACROSS OR INSERTION INTO THE ER MEMBRANE

Translocation

How hydrophilic proteins traverse the hydrophobic lipid bilayer is still an unsolved puzzle. As depicted in Figure 3, the signal sequence is thought to initiate translocation by dipping into the 'translocation site' in a loop-like conformation[27, 28]. In mammalian cells, nascent secretory proteins were found to move across the membrane while they were still being elongated. In the ER lumen, the signal sequence is proteolytically removed by the signal peptidase. This modification as well as Asn-linked core glycosylation can occur before the nascent chain is fully polymerized[29, 30]. Translocation of secretory proteins across ER membranes thus appears to be tightly coupled to nascent chain elongation. Recent evidence suggests that secretory and membrane proteins, although less efficiently, can also cross the ER membrane after large domains or even the entire protein has been synthesized. Such post-translational translocation across membranes appears to be the rule rather than the exception for those proteins destined to all other organelles[31].

Removal of the signal sequence by signal peptidase is not required for translocation across ER membranes to be productive[32]. However, it might be necessary for the protein to fold into its mature structure and become competent for transport through the secretory pathway. By comparing the cleavage site for many secretory proteins, von Heijne[6] deduced some of the rules which might define the recognition site for signal peptidase ($-1, -3$ rule, see Figure 2). Recent experiments revealed that charged amino acid residues at both ends of the hydrophobic core of a signal sequence might be crucial for positioning the cleavage recognition site during passage through the membrane, so that it becomes accessible to the signal peptidase[33].

Embedding of proteins into the ER membrane

Insertion of integral membrane proteins has been shown to depend on the presence of SRP. Therefore, initiation of their translocation is thought to be identical to that of secretory proteins. However, additional signals must be required which cause these proteins to be retained in their correct topology in the membrane[34, 35]. Three types of membrane proteins can be defined with respect to their orientation in the lipid bilayer (Figure 4). They can span the membrane either one (type I and II) or several times (type III), and their N-terminus can be exposed either to the luminal (type I) or the cytoplasmic (type II) face of the membrane. Examples of type I membrane proteins are the G protein of the vesicular stomatitis virus[36], the α and β subunits of class II histocompatibility antigens[37] and the membrane-associated form of the IgM heavy chain[38]. Their common features are an N-terminally located cleavable signal sequence and a continuous stretch of at least 20 hydrophobic amino acid residues usually close to the C-terminus. Type II membrane proteins do not contain a cleavable signal sequence and have a single stretch of hydrophobic residues located internal to the protein sequence but usually close to the N-terminus. Examples thereof are the invariant chain of the class II histocompatibility antigens[39], the transferrin receptor[40] and the asialoglycoprotein receptor[41]. Type III proteins such as opsin[42] or the facilitated glucose transporter[43] contain many stretches of hydrophobic sequences and they may or may not have a cleavable signal sequence. Either by fusing such segments of 20 or more hydrophobic amino acid residues to secretory or to cytoplasmic proteins or by deleting them from a membrane protein, it was clearly shown that they are responsible for correctly embedding proteins in the membrane[33, 40, 44, 45]. However, we do not yet understand how the translocation machinery recognizes and decodes the topological signals.

The model in Figure 5 depicts how hydrophobic segments are envisaged to retain and orient a protein in the membrane. The N-terminal signal sequence of a type I protein binds to SRP and is thereby targeted via the docking protein to the ER membrane. Translocation is initiated and the signal peptide cleaved as for secretory proteins. However, when the hydrophobic segment arrives in the lipid bilayer during translocation, further passage is stopped. Thus the N-terminus of the protein is released in the ER lumen while the C-terminus remains in the cytoplasm. The hydrophobic sequence constitutes the transmembrane segment.

Since type II membrane proteins do not contain a cleavable signal sequence at their N-terminus, it was postulated and later shown[33, 40, 41] that their single internal segment of hydrophobic residues contains both a targeting and a membrane-anchor signal. If the signal sequence forms a loop when it initiates membrane translocation[27, 28] and no cleavage by signal peptidase occurs, the hydrophilic N-terminus of a type II membrane protein will remain in the cytoplasm, the combined signal-anchor sequence stabilizes the protein in the membrane and the nascent C-terminal sequences move across the membrane and are released in the ER lumen (see Figure 5).

For the generation of type III membrane proteins, two different mechanisms have been proposed. Blobel[34] suggested that the hydrophobic segments

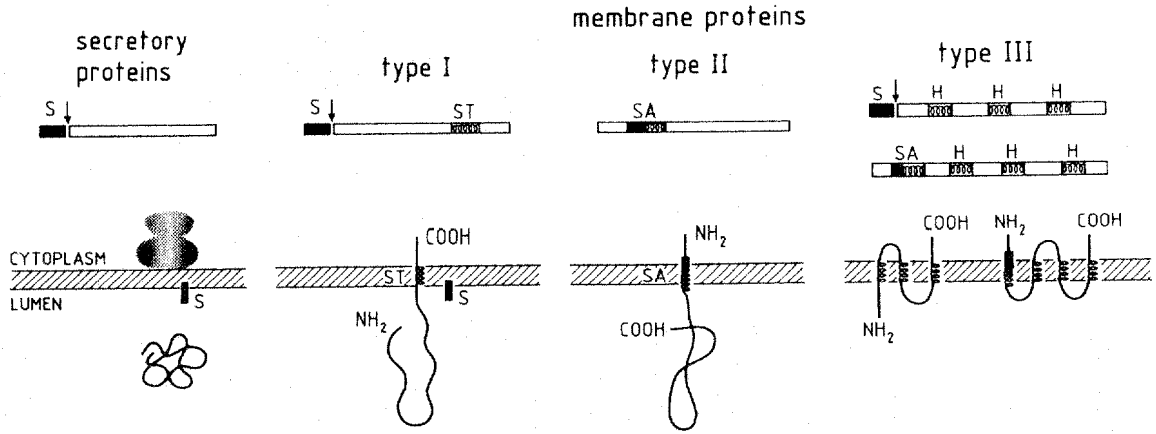


Figure 4 Signals which are involved in generating either secretory proteins or membrane proteins with different topologies. The definition of type I, II and III membrane proteins is given in the text. The upper part of the figure shows a linear representation of the polypeptides and their signals, with the N-termini to the left. The signals are: signal sequence (S), stop-transfer sequence (ST), signal-anchor sequence (SA), and hydrophobic sequence (H) with as yet undefined signal function. The lower part of the figure shows the different orientations of type I, II and III proteins in the membrane

might contain alternating signal-anchor and stop-transfer signals. A type III membrane protein would then be threaded through the membrane, whereby the first signal-anchor sequence initiates translocation, the following stop-transfer segment arrests further passage of the nascent chain through the membrane, the next signal sequence initiates it again. Alternatively, it was proposed that a single signal sequence might be sufficient to initiate insertion. The subsequent hydrophobic segments would fold into loop structures and spontaneously insert into the membrane in a zipper-like fashion[43]. There is not yet sufficient experimental evidence available which would favour one or the other of these models.

SORTING OF PROTEINS IN THE SECRETORY PATHWAY

From the endoplasmic reticulum, membrane and secretory proteins need to be sorted to their final location in the secretory pathway, i.e. many of them have to pass through several organelles. For this purpose, however, they no longer cross membranes. Instead, they use small vesicles as a transport vehicle which pinch off from the donor and fuse with the next target organelle. Except for those polypeptides whose functional site resides in the ER itself, all proteins are transported to the Golgi apparatus. The Golgi stacks are the major factory for post-translational modifications such as the processing of N-linked sugars, O-linked glycosylation and sulphation. These modifications provide a convenient experimental tool for identifying the route followed by export proteins. From the Golgi apparatus the pathway diverges either to lysosomes or to secretory granules and the plasma membrane (see Figure 1). Different proteins can be transported along the same pathway at different rates. The rate-limiting step appears to be the passage from the ER to the Golgi apparatus[46, 47].

Two conceptually different mechanisms could be involved in sorting proteins to their final functional site. Active sorting signals encoded in the sequence of the transported proteins could direct their passage from the ER to their target site in the secretory pathway. Alternatively, all proteins could passively flow from the ER through the Golgi to the plasma membrane and only those destined either to remain in any of the transit organelles (ER, Golgi) or to be diverted to lysosomes and secretory granules, would contain either 'retention' or 'diversion' signals. In addition, specific receptors would be necessary to decipher such sorting signals (for reviews see refs. 1, 34, 35, 47–9). Recent experimental evidence indicates that secretion out of the cell does not require any signal. A bacterial secretory protein which, by virtue of its origin, could not contain a sorting signal, was shown to be secreted from Xenopus oocytes[50]. On the other hand, a 'diversion' signal and its receptor have been identified: lysosomal hydrolases are tagged in the Golgi apparatus by the addition of mannose-6-phosphate to their carbohydrate moiety[51]. Two specific receptors have been isolated that recognize the mannose-6-phosphate marker and route the hydrolases to the lysosomes[52–55].

The identification and characterization of signal sequences that direct

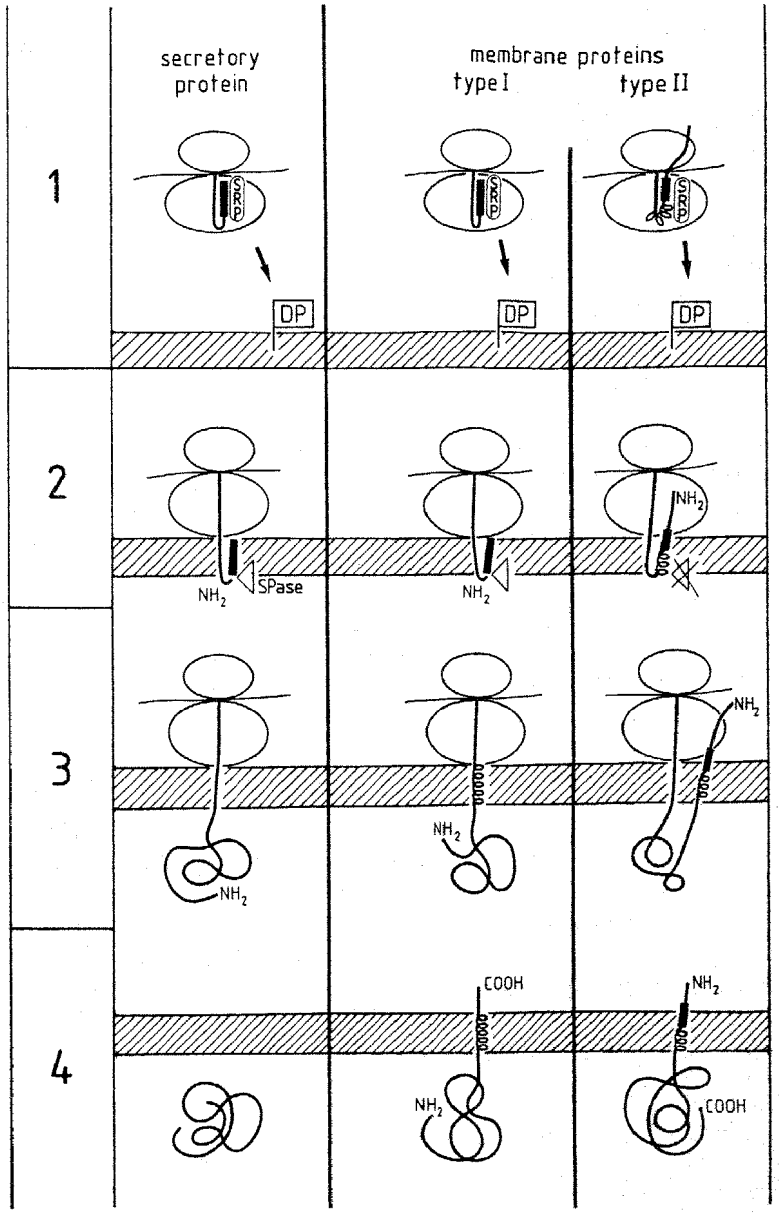


Figure 5 The membrane translocation of a secretory protein is compared to the membrane insertion of type I and II proteins. (1) Targeting to the ER membrane is similar for all polypeptides. (2) The signal or signal-anchor sequence initiates translocation. The signal peptidase (SPase) removes the signal sequences of secretory and type I proteins, but not of type II proteins. (3) The remainder of the nascent chain of secretory and type II proteins traverses the membrane unhindered, while translocation of the type I nascent chain is interrupted when the hydrophobic stop-transfer sequence arrives at the membrane. (4) Secretory proteins are released into the ER lumen. The stop-transfer sequence of type I and the signal-anchor sequence of type II proteins are embedded in the membrane and thus retain these polypeptides in their respective orientations

newly synthesized proteins from the cytoplasm to their target organelle and of signals that are responsible for membrane embedding were greatly facilitated by the fact that they are encoded in continuous and functionally independent protein segments. In addition, their information is presumably decoded as these signals emerge from the translating ribosome. Upon release into the ER lumen, the proteins fold into three-dimensional structures that are further stabilized by the formation of disulphide bonds. Two considerations are therefore important for efficient passive luminal flow of secretory and membrane proteins through the secretory pathway and for 'retention' and 'diversion' signals to become effective. The proteins have to remain in a soluble conformation and sorting signals, whether contained in a continuous sequence or in dispersed segments, need to be expressed in surface domains. The main experimental approach that has been chosen in recent years to identify the mechanism of secretion and, in particular, sorting signals, involved the introduction of mutations in those domains of secretory and membrane proteins that seemed most likely to contain sorting markers. However, it proved difficult to distinguish between experimentally introduced changes that specifically affect a signal and those that simply cause denaturation and/or aggregation of the protein in question. Denaturation might lead modified proteins to precipitate in the ER or to interact non-specifically with organellar components, as is suggested by several studies on mutant proteins that were routed either incorrectly or at significantly slowed rates[56–58]. In one case defective transport could be salvaged by introduction of consensus glycosylation sites into the cDNA[59]. However, absence of glycosylation does not affect transport as a general rule[60].

Many mature secretory and most plasma membrane proteins are oligomeric complexes. Such proteins are properly routed only after oligomerization has occurred in the ER. This observation also points to the fact that only correctly folded proteins are efficiently sorted to their destination. In the absence of their partners, the single subunits probably cannot form the intermolecular bonds necessary for their proper folding. For heterodimeric proteins it was observed that one of the two subunits is synthesized in excess over its partner. Residual free subunits either remain in the ER membrane until they are joined by the heterologous subunit and transport can begin, or they are sorted to the lysosomes and degraded. Experimentally documented examples are the immunoglobulins where either soluble or membrane-bound heavy chains are only secreted or expressed at the cell surface in cells that equally express light chains[61, 62]. Class I (consisting of a membrane-bound and a soluble subunit) and class II antigens (a heterodimer of two membrane-bound subunits transiently associated with an invariant chain) of the major histocompatibility complex are exported from the ER and transported to the cell surface only after assembly of all the subunits[37, 63, 64].

The integrity of the three-dimensional structure of secretory and membrane proteins thus appears to play a crucial role for their proper passage through the secretory pathway. Analysis of the export of model proteins with enzymatic activity might to some extent allow to test for conformational integrity[58]. Another promising approach for the identification of sorting signals might be to study model proteins, the structure of which is well characterized.

The conformational implications of any modifications might then be predicted and their effect on sorting experimentally determined.

ACKNOWLEDGEMENTS

We thank David Meyer for critical reading of the manuscript, Annie Steiner for typing it, and Petra Riedinger for the art work.

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