Mechanisms that determine the transmembrane disposition of proteins

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The final orientation that a protein assumes in the membrane of the endoplasmic reticulum is determined by a few types of signal sequences and their respective interactions with the membrane insertion complex. Membrane insertion occurs via a series of discrete steps, some of which are regulated by GTP- and ATP-binding proteins. Analysis of the protein components in proximity to nascent secretory and membrane proteins has revealed novel proteins in the endoplasmic reticulum that may form part of the membrane insertion complex.

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Introduction

The endoplasmic reticulum (ER) is a major site of membrane biogenesis in eukaryotic cells. A key feature of this biogenesis is the vectorial insertion of proteins into the lipid bilayer of the ER. After their synthesis, membrane proteins either remain in the ER or are transported to subcellular destinations throughout the exocytic and endocytic pathways, such as the Golgi complex and lysosomes, as well as the plasma membrane.

Different proteins assume different orientations within the membrane. Integral membrane proteins that span the membrane once can expose either the amino (type I) or carboxyl (type II) terminus on the exoplasmic side of the membrane. Proteins that span the membrane several times are referred to as multiple-spanning.

The orientation of a membrane protein is defined during its insertion into the ER membrane, and is maintained, whatever the destination of the protein. The orientation that a protein assumes depends on the type of signal sequence that it bears [1]. ER-specific signal sequences can be either cleaved or uncleaved, and are responsible for targeting proteins to the ER; they either initiate their membrane insertion, or, in the case of secreted proteins, their translocation across the membrane into the ER lumen [2]. All ER-targeting signal sequences contain a stretch of apolar amino acid residues. The signals are recognized by the signal recognition particle (SRP) [3•], which targets the nascent chain–ribosome–SRP complex to the ER membrane and initiates membrane insertion [2,4,5].

Membrane orientation

Single-spanning membrane proteins that have a cleavable amino-terminal signal sequence always have type I orientation (Fig. 1). A stop-transfer sequence [6] on the carboxyl-terminal side of the signal sequence aborts translocation of the nascent chain before it is complete and functions as a membrane anchor. In the absence of a stop-transfer sequence, the nascent chain is completely translocated across the membrane and enters the ER lumen (Fig. 1).

Membrane proteins with uncleaved 'signal-anchor' sequences of both type I and type II orientation (Fig. 1) have been identified [1]. The signal-anchor sequence mediates the ER targeting and insertion of the protein, and acts as the anchor sequence to retain the protein in the lipid bilayer. The final orientation that a signal-anchor protein assumes in the membrane depends on the nature of the hydrophilic amino acid residues that flank the hydrophobic core of the signal-anchor sequence [7].

The properties of signal-anchor sequences that determine topology have been determined by analyzing mutant proteins either expressed in cells or inserted into microsomal membranes *in vitro*. From such studies it has been deduced that the number and type of charged amino acid residues in the regions flanking the hydrophobic core of the signal-anchor sequence determine membrane orientation [8•,9•,10]. The more charged residues that a flanking segment contains, the more likely it is to be retained on the cytoplasmic side of the membrane.

Abbreviations

ER—endoplasmic reticulum; **SRP**—signal recognition particle; **TRAM**—translocating chain associating membrane protein.

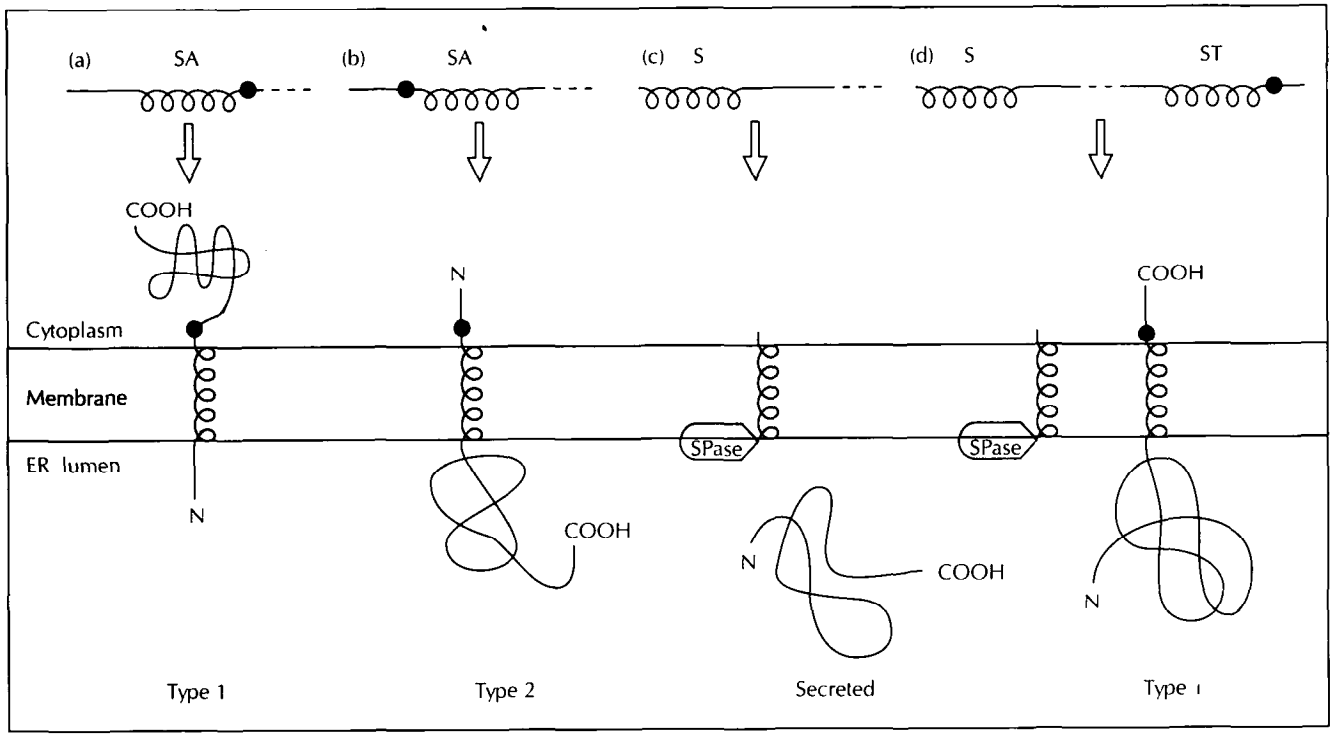

Fig. 1. Types of signal sequences. Membrane insertion in opposite orientations is mediated by (a) type I and (b) type II signal-anchor sequences (SA). Cleavable signal sequences (S) mediate (c) the membrane translocation of secretory proteins using signal peptidase (SPase) and (d) the membrane insertion in a type I orientation, which requires a stop-transfer sequence (ST). Hydrophobic membrane-spanning regions are indicated by helices. ●, Clusters of charged amino acid residues that often flank signal-anchor and stop-transfer sequences on the cytoplasmic side of the membrane-spanning domain. Broken lines indicate parts of the mature protein.

The correlation between the charge distribution of the regions proximal to the hydrophobic core of a signal-anchor sequence and the final orientation that the protein assumes in the membrane is strong enough to be used as the basis for predicting the membrane orientation of a protein from its amino acid sequence [11,12].

In addition to the effect of charge there is also, as might be expected, a minimum length required for the hydrophobic region of a signal-anchor sequence to remain functional [10]. It has been suggested that the balance between the length of the hydrophobic segment and the number of flanking charged amino acid residues determine whether a sequence functions as a signal-anchor sequence or a cleaved signal sequence [13•]. Introducing charged amino acids into a signal-anchor protein does not always result in it adopting only one orientation in the membrane. Often the same protein can be found in both orientations, and the addition or removal of charged residues alters the type I : type II ratio that the protein displays [8•,9•,14].

Membrane insertion as a loop

Following proposals that the initial insertion of secretory proteins into the membrane occurs as a loop, supporting experimental evidence has been obtained [15]. The topologies observed with mutated type I and type II signal-anchor proteins [9•,10,13•] are also consistent with this model (Fig. 2). The membrane insertion of signal-anchor proteins is predicted to occur via loops formed between the hydrophobic core of the signal-anchor sequence and the flanking hydrophilic region on its amino- (type I) or carboxyl-terminal (type II) side (Fig. 2). Upon membrane insertion the final orientation is determined by which of the two regions flanking the hydrophobic core of the signal-anchor sequence is retained on the cytoplasmic side of the membrane. The difference between a secreted protein and a type II signal-anchor protein is the presence of a suitable signal peptidase cleavage site exposed on the lumenal side of the ER membrane [15].

Multiple-spanning membrane proteins

It has been proposed that multiple-spanning membrane proteins achieve their final orientation by using successive signal-anchor and stop-transfer sequences [16]. There are good experimental data to support such a mechanism [17,18] and it still seems the most likely possibility [19]. Experiments with artificial chimeric proteins have shown that the hydrophilic regions between the signal-anchor and stop-transfer sequences can affect the final membrane topology of multiple-spanning membrane proteins [18]. This means that predictions of the orientation of multiple-spanning membrane proteins are always susceptible to errors and must be confirmed by suitable experimental approaches.

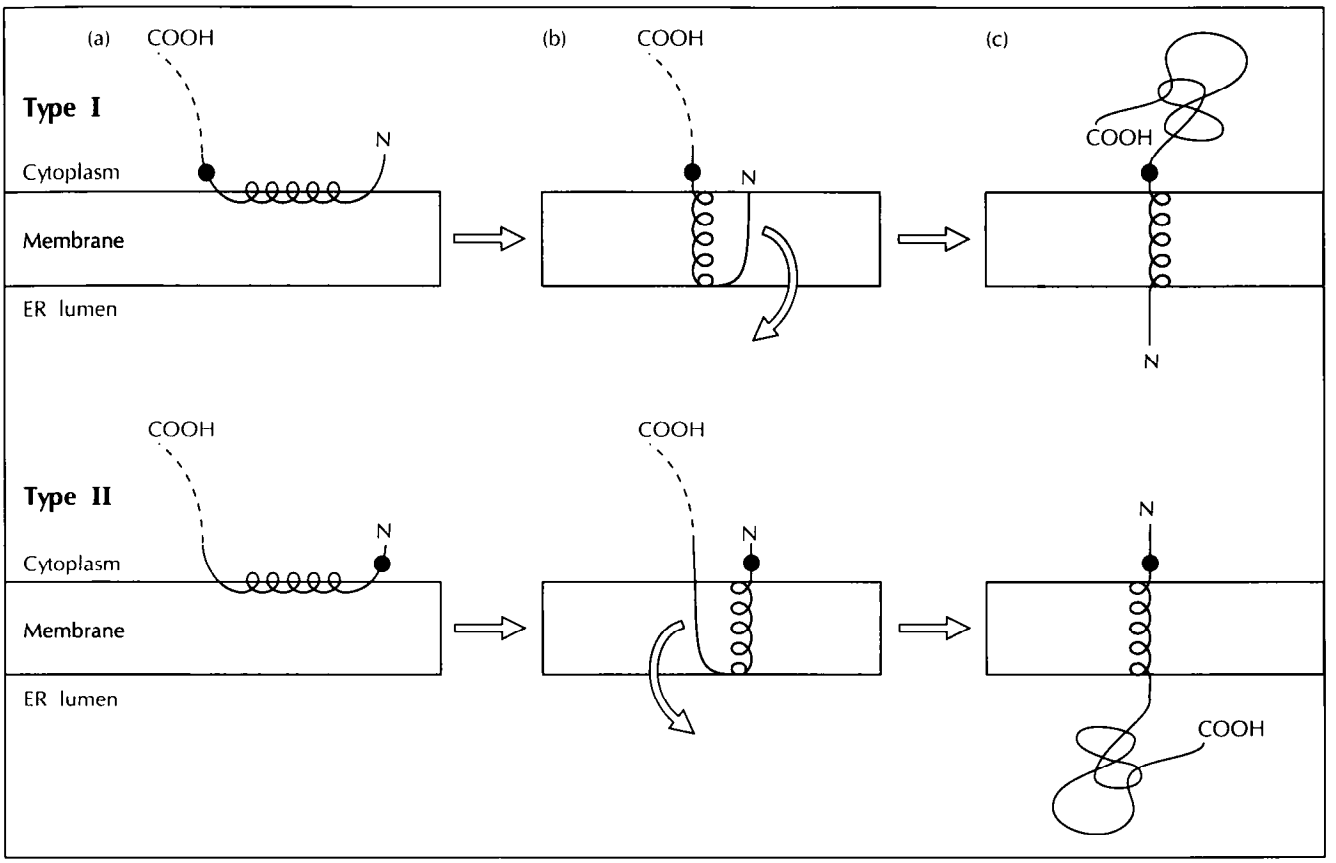

Fig. 2. Model of intermediate steps during the membrane insertion of signal-anchor proteins. The hydrophobic core of the signal-anchor sequence of a nascent protein (a) interacts with the endoplasmic reticulum (ER) membrane and (b) inserts into the membrane forming a loop with either the amino-terminal (type I) or the carboxyl-terminal (type II) flanking region of the protein. (c) The amino or carboxyl terminus of the protein is translocated into the ER lumen. ●, Clusters of charged amino acid residues that often flank signal-anchor and stop-transfer sequences on the cytoplasmic side of the membrane-spanning domain.

Targeting of nascent membrane proteins to the endoplasmic reticulum

The targeting of type I and type II signal-anchor proteins is, like secreted proteins, mediated by SRP. These ER-targeting signals all interact with the 54 kD subunit of SRP (SRP54) [3•]. Release of the signal sequence from the SRP54 protein requires the presence of microsomal membranes and GTP, although GTP hydrolysis is not required *in vitro* [20–22,23••].

Membrane insertion

While it is now well established that signal-anchor sequences consist of a hydrophobic core and flanking hydrophilic regions, little is known about the mechanism by which they insert into the ER membrane and attain a particular orientation across it. There is good evidence that protein components of the ER membrane are directly involved in mediating the insertion of proteins into the membrane. Thus, the translocation of secretory proteins across the ER membrane is prevented by pre-treatment of the membranes with N-ethylmaleimide [24], which modifies the cysteine residues of proteins, or 8-azido-ATP [25•,26•], which is thought to block the function of ATP-binding proteins.

Nascent secretory proteins appear to be in a proteinaceous environment in the membrane since they are released from the membrane by agents that disrupt protein–protein interactions [27].

It is likely that the interaction of the nascent chain with these membrane components is also responsible for determining the final orientation of a membrane protein. The different orientations of membrane proteins could arise in at least two ways: different proteins may mediate the insertion of type I and type II signal-anchor proteins and proteins with cleaved signal sequences; or the same proteins mediate all membrane insertion and translocation events, and the details of the molecular interactions are influenced by the properties of the nascent chain.

Proteins that may mediate membrane insertion

To determine which ER proteins make up the membrane insertion machinery, cross-linking experiments have been used to define the nearest neighbours of different types of proteins during their membrane insertion. A ribosome–nascent chain–SRP complex is formed *in vitro* and allowed to interact with the ER membrane

to generate a stable translocation intermediate [2,28]. After activation of a photocross-linking reagent incorporated into the nascent chain, or cross-linking with homobifunctional reagents, the nearest neighbours can be determined. The results from a number of different laboratories [29••–31••] show that only a few ER proteins are close to the nascent chain during membrane insertion. The proteins identified by cross-linking approaches can be divided into two groups [28]: non-glycoproteins (34 to 37 kD) and glycoproteins (35 to 39 kD). At least one of these components, a 37 kD non-glycoprotein (P37), is next to nascent chains with type I and type II signal-anchor sequences as well as those with cleaved signal sequences ([30••]; S High, B Dobberstein, unpublished data). The glycoproteins consist of at least two distinct proteins: the so-called signal sequence receptor (SSRα) and the translocating chain associating membrane protein (TRAM; [32••].

Membrane insertion complex

The fact that at least three ER proteins (TRAM, P37 and SSRα) are in close proximity to membrane-inserting nascent chains suggests that a protein complex may mediate membrane insertion. In yeast, three proteins that are involved in the translocation of secreted proteins across the ER, Sec61p, Sec62p and Sec63p [33–35], form part of a complex in the ER membrane [36••]. Mutations in the *SEC61*, *SEC62* and *SEC63* genes also affect the insertion of some membrane proteins [37•,38•], suggesting that the same complex is involved in membrane protein insertion. Although no homologues of Sec61p, Sec62p or Sec63p have yet been identified in mammalian ER, it is tempting to speculate that the non-glycoproteins identified by cross-linking are the mammalian counterparts of at least some of these proteins [4].

Mechanisms

While it remains to be established that the proteins identified by cross-linking are involved in the insertion of membrane proteins into the ER, the results described above suggest that a common machinery mediates membrane insertion. How could such a membrane insertion complex facilitate insertion in a type I or type II orientation? A charged region of the membrane insertion complex, present at the cytoplasmic face, could retain a charged region of the nascent chain adjacent to the hydrophobic core of the signal-anchor sequence (Fig. 2). The other end of the nascent chain would then be translo-

lective retention of one of the two hydrophilic regions flanking the hydrophobic core of the signal-anchor sequence may determine the membrane orientation.

The observation that one signal-anchor protein can assume two orientations in the membrane [8•,9•,14] suggests that kinetic or thermodynamic competition occurs between the translocation of the amino and carboxyl termini. This is consistent with the suggestion that the same machinery is responsible for the membrane insertion of type I and type II signal-anchor proteins, and that the way in which a nascent chain interacts with components of this machinery determines its final orientation in the membrane.

An interaction between a charged region of the membrane insertion complex and the cluster of charged residues that normally follows the apolar region of a stop-transfer sequence would also account for the ability of a stop-transfer sequence to integrate into the lipid bilayer in a stable fashion [19]. Thus, one translocation site would mediate the insertion of all types of membrane proteins in a manner determined by the properties of the nascent chain itself.

Conclusion

The principal features of signal-anchor sequences, cleaved signal sequences and stop-transfer sequences have been elucidated recently. In each case it has been found that a hydrophobic core region combined with flanking hydrophilic sequences is important for function. How these different sequences function in the process of membrane insertion is not known. An attractive possibility is that their interaction with components of the membrane insertion complex determines the final orientation that a protein assumes in the membrane. With the prospect that the components of this complex will be identified in the near future we can look forward to understanding the molecular interactions that determine membrane protein orientation in the ER.

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