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Minireview

Heads or tails – what determines the orientation of proteins in the membrane

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Abstract The same translocation machinery in the endoplasmic reticulum translocates either the N- or the C-terminal domain of signal-anchor proteins across the membranes. Charged residues flanking the signal sequence are important to determine which end is translocated, but are not sufficient to generate a uniform topology. The folding state of the N-terminal segment, which is to be translocated posttranslationally, and the length or hydrophobicity of the signal sequence are additional criteria to determine protein orientation in the membrane.

Key words: Membrane insertion; Protein topology; Endoplasmic reticulum; Protein folding

1. Four ways to insert single-spanning proteins into the ER membrane

Four classes of single-spanning membrane proteins can be distinguished based on their orientation in the bilayer and on the topogenic sequences that direct their insertion into the endoplasmic reticulum (ER) membrane (illustrated in Fig. 1). Type I membrane proteins are initially targeted to the ER by a cleavable, N-terminal signal sequence and then anchored in the membrane by a subsequent stop-transfer sequence. In proteins of types II and III, a single topogenic sequence, a signal-anchor, is responsible for both insertion and anchoring. Like cleaved signals, signal-anchor sequences may initiate translocation of the C-terminal portion of the polypeptide to the ER lumen, resulting in a type II orientation. Alternatively, the N-terminal portion may be translocated to yield type III proteins with the opposite orientation in the membrane. Proteins that traverse the membrane more than once may be similarly classified as type I, II, or III multi-spanning proteins based on the characteristics of the first transmembrane segment responsible for targeting to the ER and for the initial insertion process (Fig. 1). All these proteins use the same targeting and insertion machinery which includes signal recognition particle (SRP), SRP receptor, and the Sec61 complex [1,2]. In contrast, type IV membrane proteins, which are cytoplasmically exposed and anchored by a transmembrane segment very close to the C-terminus, have recently been shown to be inserted by a different, as yet uncharacterized machinery into the ER membrane [3,4].

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2. Charged residues are important but not sufficient to define the topology

What determines whether the N- or the C-terminal sequence (the 'head' or the 'tail') of a signal-anchor protein is translocated across the membrane to generate type III or type II topology, respectively? Statistical analysis of membrane proteins, from both prokaryotes and eukaryotes, revealed an enrichment of positive charges in cytoplasmic sequences and a depletion from exoplasmic ones. In prokaryotes, this observation was initially formulated as the 'positive-inside-rule' [5]. In eukaryotes, the charge difference between the two flanking segments, rather than the positive charge *per se*, correlates with the orientation of a signal-anchor: the cytoplasmic sequence generally carries a more positive charge than the exoplasmic one [6]. The charge rule is also reflected in the structure of cleaved signals: the positively charged N-terminus stays in the cytoplasm, whereas the C-terminal end, which is depleted of positive charges, is translocated [7]. The statistics thus suggested that the flanking charges of cleaved and uncleaved signals determine the orientation in the translocation apparatus.

A causal role of flanking charges in topogenesis was confirmed by site-directed mutagenesis. Cytochrome *P*450, a type III protein, was converted to a type II protein by insertion of positively charged residues into the short polar N-terminal domain [8–10]. Similarly, deletion of a positively charged cytoplasmic loop segment of bacterial leader peptidase, a type III twofold membrane-spanning protein, resulted in the reversed orientation [11]. The opposite, conversion of type II to type III proteins, could also be accomplished, but not efficiently. Inversion of the charge difference between the segments flanking the type II signal-anchor of the asialoglycoprotein (ASGP) receptor subunit H1 and of the paramyxovirus hemagglutinin-neuraminidase (HN) yielded up to 75% type III insertion [12–14]. However, the rest of the molecules inserted with the original type II orientation. N-terminal charges proved to be more important for the topology than C-terminal ones, and the closer they were to the transmembrane segment, the more they affected the insertion behavior. In addition, the topological effect of charge alterations was enhanced in the context of a truncated N-terminus.

Although the charge difference rule has proven useful in predicting the topology of natural proteins, this criterion is apparently not sufficient to generate uniform type III topology in mutant and recombinant proteins (e.g. [15]). Additional requirements besides a negative charge difference Δ(C–N) must be met for efficient type III insertion.

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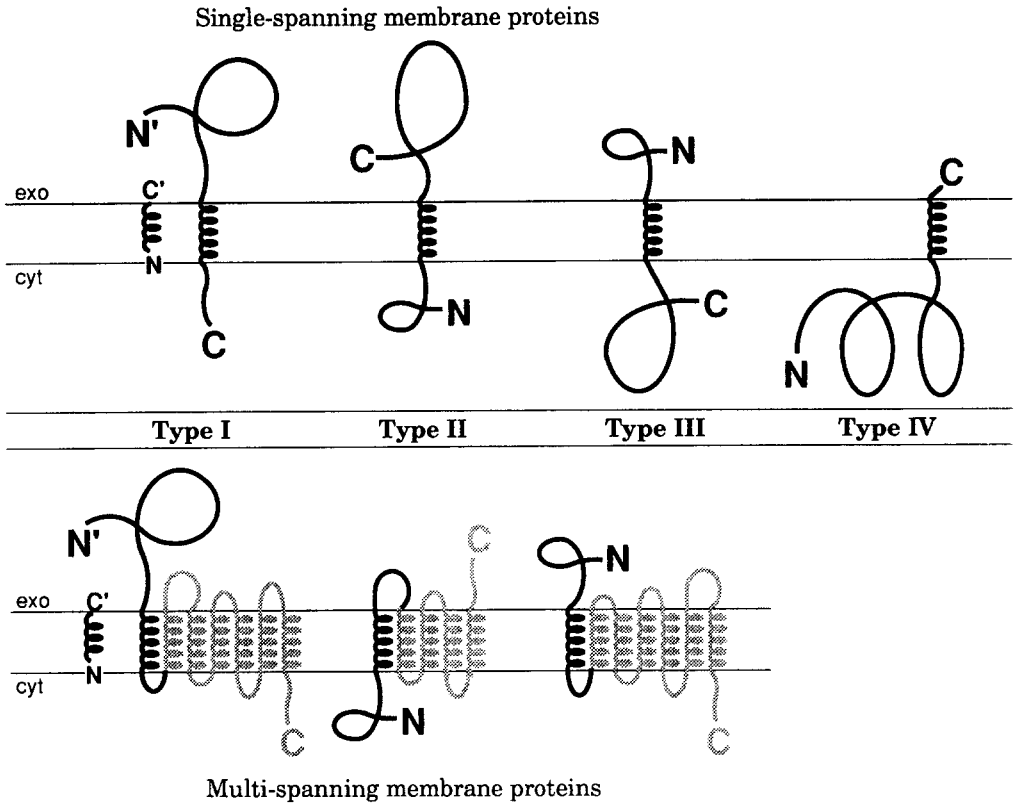

Fig. 1. Classification of membrane proteins. Different types of single-and multi-spanning membrane proteins are schematically illustrated. The classification is an extension of that by von Heijne and Gavel [27]. In the literature, type III proteins have also been defined as 'type I without cleaved signal' or type Ib. Examples of single spanning membrane proteins are the LDL receptor (type I), the transferrin receptor (II), cytochrome *P*450 (III), and synaptobrevin (IV). Examples of multi-spanning proteins are the thrombin receptor (type I), band 3 anion transporter (II), and the β-adrenergic receptor (III). Cytoplasmic and exoplasmic sides of the membrane are indicated by **cyt** and **exo**, respectively.

3. Folding of the N-terminal domain affects protein orientation

An obvious difference in translocation of the C- and the N-terminus is that the former occurs cotranslationally and is thus unaffected by protein folding. The N-terminal domain, however, is translocated essentially in a posttranslational manner, i.e. after it has been synthesized and has had an opportunity to fold in the cytoplasm (see Fig. 2). Folding of this segment and its size are thus potential obstacles for transfer across the membrane. This was tested experimentally using a model type II protein that due to mutation of the charged residues flanking the signal-anchor inserted into the membrane to equal extent with either orientation. A small zinc finger domain or the full coding sequence of dihydrofolate reductase were fused to the N-terminus (Denzer et al., submitted). These stably folding domains hindered or even prevented their translocation and yielded almost exclusive type II insertion. Disruption of their structure by destabilizing point mutations largely recovered N-terminal translocation. The efficiency of type III insertion, however, did not depend on the size of the N-terminal domain within the range of 40 to 237 amino acids.

According to the model shown in Fig. 2, a signal sequence may interact with the translocation machinery in either of two orientations. The flanking charges of the signal-anchor sequence and perhaps other features determine which is the preferred one. Stable N-terminal structures may slow or block translocation of the N-terminus, since the polypeptide needs to be unfolded for transfer through the membrane (as is the case in essentially all translocation-competent organelles [16]). It appears that, if N-terminal translocation is inefficient, the signal can reorient itself in the machinery and thus allow cotranslational transfer of the C-terminus. The result is a kinetic competition between the two pathways that may yield insertion with mixed orientations. Natural type III proteins may have evolved to be devoid of rapidly and stably folding N-terminal sequences (which indeed are often quite short), whereas some type II proteins may assure correct integration by a bulky N-terminus.

4. Short signals are treated differently than long ones

According to this model, cleaved signals (which take the left pathway in Fig. 2) are at risk to be inserted in the wrong orientation, since the hydrophilic N-terminal segment is usually very short and thus easily translocated. Yet, there appears to be a mechanism to prevent type III insertion of cleavable signals, even if the N-terminus carries little or no positive charge. Of a series of artificial N-terminal signal sequences with apolar segments consisting of 7 to 15 consecutive leucines, the short ones with up to 10 leucines inserted correctly and were cleaved [17]. The longer ones inserted at least partially with type III topology, unless the N-terminus was strongly positive. Similarly, shortening the hydrophobic segments of two forms of

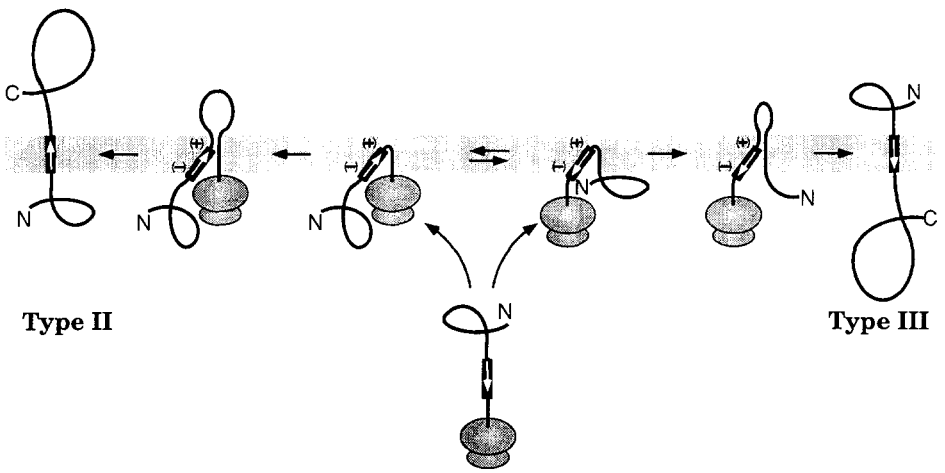

Fig. 2. Model for the membrane insertion of signal-anchor proteins. For simplicity, SRP, SRP receptor, and the components of the translocation machinery have not been drawn. (+) and (−) symbolize the putative charged regions in the translocation complex that are expected to interact with the flanking charges of signal sequences. The N-to-C direction in the signal-anchor sequence is indicated by an arrow.

cytochrome *P*450 type III signal-anchors promoted type II insertion [18]. These results correlate with the observation that internal signal sequences with short apolar segments of up to 14 leucine residues are differently positioned in the translocation machinery than those with longer apolar segments [19]. This was determined by measuring the minimal distance between the apolar segment of the signal and a potential glycosylation site to reach the oligosaccharyl transferase active site.

A candidate component of the translocation machinery that might function in retaining short N-termini of type I signals in an orientation facing the cytosol is TRAM (translocating chain-associated membrane protein [20]). Photocrosslinking experiments suggested that TRAM contacts N-terminal regions of nascent polypeptides at an early stage in the transport reaction [20]. In in vitro reconstitution experiments, TRAM was found to be required for translocation of several secretory and type I proteins with short hydrophilic N-terminal sequences, whereas it is only stimulatory for other secretory proteins, such as preprolactin, and for the type II ASGP receptor H1 [21]. This may suggest that TRAM requirement depends on the length and polarity of the region preceding the apolar segment of the signal sequence [22].

5. Conclusion

Translocation of N-terminal sequences across the bacterial plasma membrane resembles that across ER membranes with respect to the dependence on the distribution of charged residues (the positive-inside rule) [23]. In bacteria, it is also generally limited to short sequences of less than 50–60 residues [24], although there is an example of a 100-residue aminoterminal periplasmic domain in ProW [25]. It is likely that unfolding of the polypeptide is required as well and may be limiting for the process. In contrast to the eukaryotic system, however, there is clear evidence that different mechanisms are involved in transfer of N- and C-terminal sequences, the former being independent of functional SecA and SecY [24,26].

Further analysis of topological determinants and of the translocation mechanism(s) should soon make it possible to predict the orientation of membrane proteins more successfully than by flipping a coin.

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