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Membrane protein integration into the endoplasmic reticulum

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Most integral membrane proteins are targeted, inserted and assembled in the endoplasmic reticulum membrane. The sequential and potentially overlapping events necessary for membrane protein integration take place at sites termed translocons, which comprise a specific set of membrane proteins acting in concert with ribosomes and, probably, molecular chaperones to ensure the success of the whole process. In this minireview, we summarize our current understanding of helical membrane protein integration at the endoplasmic reticulum, and highlight specific characteristics that affect the biogenesis of multispanning membrane proteins.

Introduction

Helical integral membrane proteins have essential roles in the cell, and account for almost one-fourth of all proteins in most organisms [1]. However, our understanding of their biosynthesis and folding lags far behind our understanding of water-soluble proteins. The targeting and insertion of most integral membrane proteins in eukaryotic cells occur cotranslationally, whereby protein synthesis and integration into the endoplasmic reticulum (ER) membrane are coupled. In this case, the targeting of the ribosome–mRNA–nascent chain complex to the membrane depends on the signal recognition particle (SRP) and its interaction with the membrane-bound SRP receptor [2], which is located in close proximity to the translocon. The translocon, a multiprotein complex, facilitates the insertion of integral membrane proteins into the lipid bilayer [3] and the translocation of soluble proteins into the ER lumen [4]. During insertion, nascent membrane proteins have to adopt the correct orientation in the lipid bilayer, undergo covalent modifications (e.g. signal sequence cleavage and N-linked glycosylation), fold properly, and interact with ER-resident proteins (e.g.

Abbreviations
cryo-EM, cryo-electron microscopy; ER, endoplasmic reticulum; RNC, ribosome–nascent chain; SR, signal recognition particle receptor; SRP, signal recognition particle; SS, signal sequence; TA, tail-anchored; TM, transmembrane; TRAM, translocating chain-associated membrane protein; TRAP, translocon-associated protein.

chaperones), to eventually adopt their native state. All of these sequential (and potentially) overlapping events take place in a very peculiar environment, the membrane, where the physics significantly differ from those in the aqueous environment. Therefore, characterization of how membrane proteins integrate into the ER membrane requires detailed knowledge of the constraints imposed by the hydrophobic lipid bilayer, as well as its response to accommodate the transmembrane (TM) segments of integral proteins. In this review, we focus on recent advances in our understanding of the targeting, insertion and folding of mammalian integral membrane proteins.

Targeting to the ER – cotranslational versus post-translational insertion

Protein targeting to the ER membrane can occur cotranslationally or post-translationally, depending on the hydrophobicity and location of the signal sequence (SS), which consist of a short span of hydrophobic residues flanked by a positively charged N-terminal region and a polar but uncharged C-terminal region [5,6]. In the cotranslational process, targeting of secretory and membrane proteins is mediated by the conserved SRP. The eukaryotic SRP, of which the mammalian particle is the best characterized, is composed of a 300-nucleotide 7S RNA and six protein subunits with molecular masses of 9, 14, 19, 54, 68 and 72 kDa [2,7]. Among SRP proteins, only SRP54 is highly conserved in all kingdoms of life, being essential for SRP function [7]. SRP54 is composed of two domains, the M-domain and the NG-domain. The M-domain (methionine-rich domain) associates with SRP RNA and provides the SS-binding site, and the NG-domain is responsible for GTP binding (G-domain) and the interaction with the ribosome (N-domain). The SRP complex binds to a hydrophobic domain (either an N-terminal SS or a TM segment) in the nascent polypeptide as it emerges from the ribosome [8]. SRP transiently arrests protein synthesis [9] and docks the ribosome–nascent chain (RNC)–SRP complex to the ER membrane via the SRP receptor (SR) [10]. SR is a heterodimer formed by the GTPases SRα and SRβ. SRα is structurally and functionally related to SRP54, also containing an NG-domain [11]. Interaction between the SRP and the SR requires GTP binding to both complexes. Subsequently, the RNC is transferred from the SRP to the Sec61 translocon, and GTP hydrolysis triggers SRP–SR dissociation [12]. Structural studies of the RNC–SRP–SR complex reveal that SR interacts with both the ribosome and SRP, leading to conformational changes in SRP that favor RNC transfer to the translocon [13]. Recent studies with prokaryotic homologs have shown an active role of the SRP RNA in coordinating SRP–SR interactions and GTP hydrolysis [14,15]. SRP disassembly leads to the resumption of translation, and membrane proteins are laterally released by the Sec translocon into the membrane bilayer, while secretory proteins are threaded through the Sec61 machinery. Despite the increasing mechanistic and structural insights into cotranslational targeting, we have limited knowledge on how SRP regulates its binding to a diverse set of signal sequences, and on the conformational changes induced by SR binding that result in transfer of the nascent chain to the translocon [16].

In the post-translational route, proteins are targeted and inserted (or translocated) after translation by cytosolic ribosomes. In yeast, where this pathway is especially prominent, a dedicated complex, termed the Sec62–Sec63 complex (also present in mammalian cells), cooperates with the Sec61 translocon in post-translational translocation of soluble (secretory) proteins [17]. In this pathway, cytosolic Hsp40/Hsp70-type chaperones maintain polypeptides in a translocation-competent state [18], and several luminal chaperones are required to pull the precursor across the membrane [19]. Another subset of proteins is targeted post-translationally to the ER membrane by the TRC40–GET pathway. This subset of proteins comprises membrane proteins with a C-terminal TM segment, also known as tail-anchored (TA) proteins [20]. Although remarkable progress has been made in the identification of targeting factors, the molecular basis underlying TA membrane protein integration remains to be fully clarified. The two post-translational targeting mechanisms appear to be more complex than cotranslational biogenesis of membrane proteins. Hence, up to three distinct targeting pathways have been described so far: the SRP-mediated pathway, the ATP-dependent Hsp40/Hsc70-mediated pathway, and the TRC40–GET pathway, which is also dependent on ATP hydrolysis [21].

Translocon structure

The translocon complex is responsible for the insertion of most integral membrane proteins into the lipid bilayer, as well as for the translocation of secretory proteins across the ER membrane [4]. The gating capability of this complex in two directions (i.e. across the membrane and laterally into the lipid bilayer) differentiates it from the rest of the cellular channels. In mammalian cells, this proteinaceous complex is composed of the Sec61 α-subunit, β-subunit and γ-subunit plus the

translocating chain-associating membrane protein (TRAM) [22]. As translocon activity can be reproduced by *ab initio* reconstitution of these four membrane proteins in pure lipids [23], these proteins constitute the core components of the mammalian translocon [3].

Sec61 complex

The eukaryotic Sec61 complex is a heterotrimeric membrane protein complex (Sec61α, Sec61β and Sec61γ), called SecYEG in bacteria and archaeons. On the one hand, the α-subunit and γ-subunit are highly conserved in all kingdoms, and are required for survival in both *Escherichia coli* and *Saccharomyces cerevisiae*. The β-subunit, on the other hand, is not required, and does not have significant sequence homology between eukaryotes and eubacteria. The high-resolution structure of mammalian Sec61 is not yet available. However, we have the homologous structures from *Methanococcus jannaschii* [24], *Thermus thermophilus* [25], *Thermotoga maritima* [26] and *Pyrococcus furiosus* [27], the last two lacking the non-essential β-subunit. The fitting of the crystal structure of SecYEβ from *M. jannaschii* into the cryo-electron microscopy (cryo-EM) density map of an active mammalian Sec61 [28], and of the cryo-EM structure of SecYEG from *E. coli* with the mammalian Sec61 in a resting state [29], indicate a high degree of structural similarity between all Sec complexes.

The α-subunit

Sec61α constitutes the protein-conducting channel of the translocon complex, crossing the membrane 10 times, with both its N-terminus and C-terminus facing the cytosol. Viewed from the top, the protein adopts a square shape that can be divided into two pseudosymmetric halves, the N-terminal half containing TM segments 1–5 and the C-terminal half comprising TM segments 6–10 (red and blue TM segments in Fig. 1, respectively). These two parts form an indentation in the centre through which the nascent chain passes, and is aligned with the ribosomal exit tunnel [28]. From a lateral view, Sec61α has a rectangular contour and the channel within an hourglass shape [30]. When it is in an inactive state, the cytoplasmic entry to the channel has a diameter of ~ 20–25 Å [24]. Close to the middle of the membrane, the translocation pore reaches its narrowest point (5–8 Å), composed of a ring of bulky hydrophobic residues followed by a short helix (TM segment 2a) that blocks the channel pore (Fig. 1). After this 'plug', the channel widens again towards the ER lumen. Nevertheless, it has been reported that there is a significant increase in the pore diameter [31], which is probably needed to accommodate the multiple TM segments of multispanning nascent chains that may leave the translocon in pairs or groups (see below).

The β-subunit

The β-subunit is the smallest component of the Sec61 complex. It contains a single TM domain located next to TM segments 1 and 4 of Sec61α (Fig. 1A). Although this subunit is not essential either for translocation across the ER membrane or for insertion of TM segments into the lipid bilayer, it has been reported to kinetically facilitate cotranslational translocation [32],

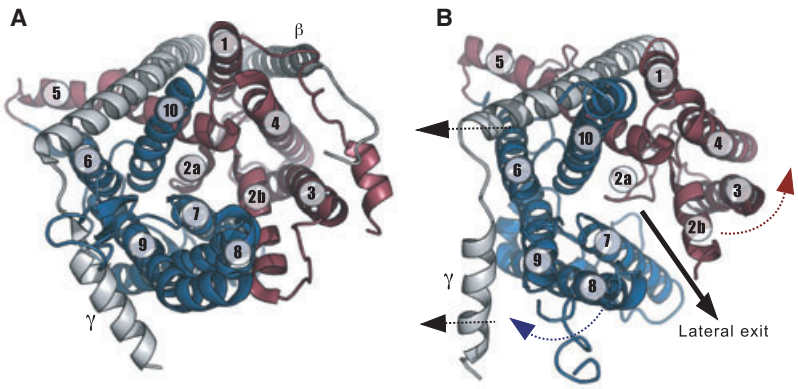

Fig. 1. Translocon structure. Top view of the translocon structure. (A) Closed structure of the translocon from *M. jannaschii* (Protein Data Bank ID code: 1RHZ) [20]. (B) Partially open structure of the translocon from *P. furiosus* (Protein Data Bank ID code: 3MP7) [23]. In both panels, all TM segments of Sec61α are colored (red and blue for each half; see text) except for the β-subunits and γ-subunits, which are shown in gray. All TM segments are numbered for easy comparison between the open and closed structures. The dotted arrows in (B) indicate the helix displacements required for the widening of the channel and opening of the lateral gate. A solid arrow shows the lateral gate exit pathway of a TM segment from the interior of the channel into the membrane.

and to interact with the SR heterodimer, probably facilitating recognition of unoccupied translocons by the RNC–SRP–SR complex [33]. The participation of Sec61β in the translocation process is also supported by its direct interaction with the nascent chain and the ribosome [34].

The γ-subunit

Sec61γ has two helices connected by an extended loop (Fig. 1). The first helical region, an amphipathic helix, sits parallel to the cytosolic side of the membrane and contacts the cytoplasmic side of the Sec61α C-terminal half. The second helix crosses the membrane diagonally, interacting with both N-terminal and C-terminal parts of Sec61α, and acts as a clamp that brings both halves of Sec61α together [24].

Translocation and insertion of a nascent chain

During cotranslational insertion/translocation, the nascent polypeptide is extruded into the translocon from the ribosome exit tunnel. The precise stoichiometry and structure of the actively engaged translocon–ribosome complex has been a subject of great controversy over the years. Initial cryo-EM studies indicated that three or four copies of the Sec61 complex could interact with the ribosome at the same time [35]. However, biochemical studies and the structures that have recently become available strongly suggest that only one copy of the Sec61–SecY complex is required for translocation [24,27–29,36,37]. Biochemical analysis of Sec61 point mutants [38], and the cryo-EM reconstructions of the ribosome–translocon pair, indicate that the loops between TM segments 6/7 and 8/9 of the translocon are involved in this association [28,39]. In fact, point mutations within those loops of *E. coli* SecY are known to affect the ribosome–SecY interaction [39]. However, similar changes in loop L6 of the yeast translocon did not affect binding to the ribosome [28]. All of this indicates that, despite small differences, the ribosome–Sec junction is well conserved among species.

Although many details remain unknown, significant insights into the mechanism of membrane insertion have come from structural studies. The process starts with the engagement between the translocon complex and its cytosolic partner (i.e. the ribosome in the cotranslational pathway). Either this contact or the presence of the SS triggers the widening of the cytosolic side of the channel [25], including the hydrophobic ring, which increases from ~ 5 to ~ 14 Å [27]. In this pre-open state, displacement of TM segments 6, 8 and 9 from their position in the closed configuration would create a lateral 'crack' between the two halves of Sec61α (i.e. at the interface between TM segments 2b and 7/8), which would occur only in the cytosolic side of the channel. However, segment 2a retains its location, keeping intact the permeability barrier. Once the SS enters into the channel as a loop, its first amino acids interact with the cytosolic residues of TM segment 8. At the same time, the hydrophobic core of the SS contacts TM segments 7 and 2b on both sides of the channel and the phospholipids through the already open lateral crack [40]. As the elongation of the nascent chain continues, two rearrangements occur in Sec61α. First, the plug is displaced to leave room for the nascent polypeptide, which can now completely expand the channel. Second, the pairs formed by TM segments 2/3 on one side and 7/8 on the other half move apart from each other (Fig. 1B), creating a lateral gate across the entire channel, which exposes the nascent polypeptide to the core of the membrane [27,41]. The sequence within the translocon can then partition into the lipids if it is hydrophobic enough, as the SS would do, or continue through the translocon into the ER lumen. The structural changes in the α-subunit are accompanied by a dramatic shift (Fig. 1B) in the location of the N-terminal helix of Sec61γ/SecE [27], which releases the clamp over Sec61α. Nevertheless, the opening of the lateral gate is not required to accommodate a translocating peptide within the channel [28]. Therefore, it is possible that the opening of the lateral gate is triggered by the presence of a TM segment inside the translocon, which would adjust its dynamic structure according to the nature of the polypeptide within the channel. During this process, the permeability barrier is kept by the coordinated in and out movement of the 'plug' and the widening/narrowing of the hydrophobic ring, while the opening/closing of the lateral gate exposes hydrophobic segments to the lipid bilayer, allowing their partition into the membrane.

TRAM

TRAM was identified by crosslinking methods in reconstituted proteoliposomes [22]. Although it is recognized as an essential component for the translocation or insertion into the membrane of several secreted and membrane proteins, its precise function remains unknown. TRAM is an integral membrane protein with eight TMs and both the N-terminus and C-terminus facing the cytosol [42]. The role of TRAM in the translocation of secretory proteins is restricted to the insertion of the SS into the membrane [43], where

TRAM has been found to be required for the insertion of SSs with either short hydrophobic sequences or with low overall hydrophobicity. Regarding the insertion of TM segments, TRAM has also been reported to crosslink with a wide variety of TM segments [44–48], some of them containing charged residues [49–51]. These observations, together with the fact that TRAM itself contains an unusually high number of charged residues within its TM segments, led to the idea that TRAM could act as a chaperone for the integration of nonoptimal TM segments by providing a more favorable context [42].

Translocon-associated proteins

Some other membrane proteins [i.e. translocon-associated protein (TRAP), PAT-10, RAMP4 and BAP31] have been reported to interact with the translocon and modulate its function at some stage. However, their presence is not required for either insertion or translocation, and thus they are not considered to form part of the translocon core complex.

TRAP is a tetrameric complex (α, β, γ and δ) of integral membrane proteins [52]. It is associated with ribosome–Sec61 complexes with a 1 : 1 stoichiometry [29]. It has been proposed that TRAP facilitates the initiation of protein translocation [53], although the details of the mechanism remain unknown. PAT-10 was discovered as a translocon-associated protein during a search for Sec61 partners during opsin nascent chain insertion [50]. It is a membrane protein that crosslinks with some of the opsin TM segments [54]. This interaction is independent of the presence of N-glycosylation sites, the amino acid sequence, or the topology of its first TM segment. Apparently, PAT-10 binding is triggered by the relative location of this TM segment within the opsin nascent chain. RAMP4 was also found to be tightly associated with the translocon [23]. RAMP4 is a small (66-residue) TA membrane protein implicated in promoting correct integration/folding of integral membrane proteins by facilitating subsequent glycosylation [55]. In a translating ribosome–translocon complex, RAMP4 is recruited to the Sec61 complex before the TM segment emerges from the ribosome exit tunnel; hence, it has been postulated that it is the presence of a TM sequence within the ribosome that triggers this recruitment [56]. Another protein that has been reported to interact with the translocon complex is BAP31. This multispanning integral membrane protein participates in the identification of misfolded proteins at the ER and their retrotranslocation to the cytoplasm. The finding that BAP31 interacts with both Sec61β and TRAM [57] suggests a role of the translocon in membrane protein quality control. The increasing number of interacting partners of the translocon also indicates that different functions of the channel may be performed in association with different cellular components. Indeed, the Sec61 complex might be merely the common player in a wide variety of transient complexes, each one performing different but related functions.

TM domain requirements

Hydrophobicity

Individual TM helices follow an ordered insertion pathway, in which they pass from the tunnel in the large ribosomal subunit into the Sec61 translocon channel, and then exit the channel laterally into the surrounding lipids [30,58]. Generally, the hydrophobicity of the TM sequence drives integration into the membrane. However, the efficiency of insertion of TM segments by the translocon depends on amino acid composition, the positions of residues within the segment, TM segment orientation, and helix length [59–62], suggesting that membrane insertion is fundamentally a fine-tuned thermodynamic partitioning process. Several TM segments from multispanning membrane proteins contain charged amino acids that are nevertheless tolerated in the membrane [63,64]. Computational modeling suggests that integration of TM sequences with a central ionized residue might be assisted by helix–helix interactions within the membrane more than the stabilization of this ionized group by the translocon [65]. *In vivo* and *in vitro* studies suggest that the translocon may act as a facilitator in the insertion/selection process [59,60,66], whereby protein–lipid interactions 'decide' the successful integration of the TM segment into the membrane through favorable acyl chain solvation [67], which is also affected by lipid composition [68]. Indeed, recent work in yeast has shown that mutations in the hydrophobic constriction ring of Sec61p influence translocation efficiency, modifying the hydrophobicity threshold for membrane insertion [69]. Such a mechanism based on lipid-mediated partitioning would accommodate the diversity of sequences that pass through the translocon on their way to the membrane. Nevertheless, it has previously been suggested that the translocon complex can act as a chaperone during the integration of nonoptimal TM segments. Indeed, a recent observation that ATP depletion can halt TM segment release from the translocon into the bilayer strongly supports this chaperone function [70], which supplement the thermodynamic partitioning process.

Amino acid preferences

A recent annotation on the amino acid composition of α-helical TM segments showed that there is considerable information in sequences that relates to the intricate contacts between TM segments [71]. Indeed, there is a biased amino acid preference, depending on whether the residue is exposed to the lipid bilayer or to a soluble environment (Fig. 2). Using all annotations in the MPTopo database [72], we selected amino acids from TM segments and compared their occurrence with that of amino acids in non-TM segments. In total, there were 206 proteins with known three-dimensional structure and topology, which had 1244 TM segments. The total number of amino acids in TM segments was 25 281, as compared with a total of 63 107 amino acids in non-TM regions. As previously reported [73], the hydrophobic residues Leu and Ala make up the bulk of the amino acids in the TM segments, accounting for one-fourth (24.5%) of all amino acids that are inserted through the translocon, but these two residues are also common in the non-TM regions (16.2%). This effect is even more evident for Gly, as its prevalence is almost equal in TM and non-TM regions (Fig. 2). Interestingly, charged residues, together with Pro, are underrepresented in TM domains relative to non-TM regions. This feature is probably meaningful in terms of both hydrophobicity and helicity.

Helical conformation of TM segments

The formation of an α-helix is critical for membrane insertion of a TM segment. Even the most hydrophobic polypeptides could not insert into lipid bilayers without concomitant secondary structure formation [74]. One of the most intriguing challenges that membrane proteins have to face is desolvation and partitioning of the polar peptide bond from water into the membrane, which is as unfavorable as that of a charged side chain [75]. However, the formation of intramolecular hydrogen bonds (i.e. adoption of secondary structure) can compensate for the loss of hydrogen bonds between the polypeptide backbone and water molecules [76]. Where does a predestined TM segment adopt its α-helical conformation? According to the two-stage model (see below), TM segments fold during insertion into the membrane and, in the case of multispanning membrane proteins, before helix association [77]. However, some TM α-helices have been shown to be already folded in the ribosomal tunnel [78–81], even before reaching the translocon or inserting into the lipid bilayer, suggesting that the folding inside the ribosome may regulate the fate of the nascent polypeptide.

Integration mechanism in multispanning membrane proteins

During the biogenesis of multispanning membrane proteins, several TM segments in a single polypeptide need to be integrated by the Sec61α translocon. Unfortunately, our knowledge of the molecular mechanism underlying this process is still very limited. During translation, and once the SS or a TM segment has reached the translocon, this first hydrophobic segment has to be relocated to accommodate the following TM segment within the translocon pore. Whether, at this point, multiple TM segments partition into the membrane sequentially (that is, each TM segment exits the translocon individually [49]), or several TM segments can accumulate inside or in the proximity of the

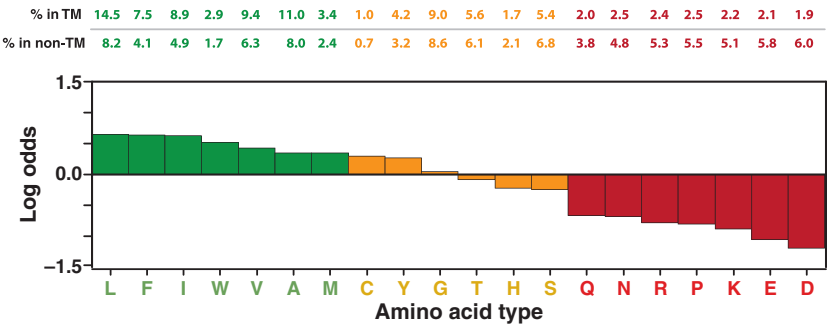

Fig. 2. Amino acid preferences in TM segments as compared with loop regions (non-TM) in membrane protein structures. The top two rows show the percentage of occurrence of all amino acid types in TM segments and non-TM segments in membrane proteins of known structure. The lower plot shows the log odds ratio of the occurrence. Briefly, a log odds ratio is the $\log_{10}$ ratio of the odds of an amino acid occurring in TM segments versus the odds of it occurring in a non-TM segment. Positive log odds indicate overoccurrence of the amino acid type in TM segments. Negative log odds indicate underrepresentation of the amino acid type in TM segments. Amino acids are colored according to an arbitrary division of their log odds (i.e. green for log odds > 0.3; orange for 0.3 ≤ log odds ≥ −0.3; and red for log odds < −0.3).

translocon and be released into the bilayer in pairs or groups [44,50,82,83], is thought to be protein-dependent. Recent structural data have shown that, in the pre-open state, the hydrophobic ring is widened to ~ 14 Å in the direction of the lateral exit site [27], which is enough for the accommodation of more than one helix, especially because these dimensions could be further increased in a fully open state [31]. It is also known that hydrophobic TM segments leave the translocon sequentially from the N-terminus to the C-terminus [82], and less hydrophobic segments interact with other TM segments at early stages of membrane integration [46,54,84,85]. More hydrophilic TM segments are forced by downstream hydrophobic sequence to adopt a TM disposition [86,87]. However, whether these hydrophilic helices are spontaneously inserted or helped by the Sec61 translocon to insert together with their partner helices is still unknown. Nevertheless, it has been suggested that interhelical interactions are required to neutralize polar groups in TM sequences [76,88]. Indeed, recent comparison of helix–helix interactions in available membrane protein structures reveal that they constitute one of the most distinctive characteristics of multispanning membrane proteins with more than four TM segments [89]. These helix–helix interactions might be coordinated *in vivo* by the translocon or its associated proteins. For example, TRAM (see above) plays a role in assisting the integration of hydrophobic sequences containing charged residues [43,51]. Therefore, unraveling the functions of translocon-associated proteins will provide new insights into the integration mechanism of noncanonical TM segments.

Topology

During integration, nascent membrane proteins have to adopt the correct topology (that is, it has to define the number of TM segments and their orientation with respect to the plane of the lipid bilayer [90]), which is probably influenced by the translocon. However, whether a TM segment adopts an N-teminal cytosolic or reverse orientation depends on several factors. First, it has been observed that the folding state of an extramembrane domain preceding a TM segment precludes its translocation, and consequently forces the TM segment towards an N-terminal cytoplasmic orientation [91]. Second, the hydrophobicity of the TM sequence influences membrane orientation. For example, highly hydrophobic sequences promote N-terminal translocation despite the fact that the presence of moderately hydrophobic TM segments favors the opposite orientation [92]. Third, and most important, it has been long known that the distribution of charged residues between the flanking regions of a TM segment is a major determinant of topology in membrane proteins [93,94]. The so-called 'positive-inside rule' was first observed for prokaryotic proteins, where bacteria maintain a net negative-inside electrical potential across the membrane, and a cytoplasmic bilayer leaflet enriched in negatively charged lipids also promotes charge bias. A similar skewed distribution was also identified later in eukaryotes [95], where the balance between positive and negative charges drives protein topology. Indeed, changing the flanking charges by site-directed mutagenesis can reverse the topology of a TM segment [96]. Moreover, it has been recently demonstrated that certain residues of the translocon also contribute to the positive-inside orientation of signal sequences [97,98]. Therefore, the amino acid sequence appears to be the primary determinant of final topology that is initially interpreted by the translocon. Nevertheless, it has also been reported that membrane lipid composition also influences the final topological orientation of membrane proteins [99]. In summary, both the amino acid sequence of a membrane protein and the collective determinants in the bilayer membrane influence protein topology.

Multispanning membrane proteins generally adopt their native orientation depending on the insertion of the SS or the first TM segment, which determines that the subsequent TM segments would insert sequentially with opposite orientations. Nevertheless, drastic changes in loop regions that favor inverted orientations have only local effects [100]. Furthermore, it has recently been shown that the topology of a full-length protein can be changed by simply adding a positively charged residue, irrespective of the region of the protein where the mutation is placed, including the C-terminal end of the protein [101]. Unfortunately, the molecular mechanisms by which downstream determinants contribute to the topology are as yet unknown [102]. Therefore, experimental evidence is now challenging the classic static view of the attainment of membrane protein topology. For example, some proteins may adopt multiple topologies, depending on the cellular localization or environment [103], whereas others, such as viral membrane proteins, have a strong preference for a specific topology [104].

Hydrophobic matching

The effect of the so-called hydrophobic matching on the assembly and orientation of TM segments has been widely studied [105]. A 'mismatch' occurs when the hydrophobic thickness of the membrane does not match the length of the hydrophobic region of a TM

segment [106]. Two types of hydrophobic mismatch have been described: (a) positive, when the membrane is not thick enough for a TM segment; and (b) negative, when the length of the hydrophobic section of a TM segment is too short to span the hydrophobic core of the lipid bilayer. In both scenarios, either the membrane or the polypeptide will adapt to minimize the exposure of hydrophobic residues to the aqueous media (positive mismatch) or the extrusion of polar amino acids within the hydrophobic core of the membrane (negative mismatch) [107]. Both rearrangements are known to be important for determining the final assembly of a membrane protein, as shown by fluorescence [108–110] and chimeric overexpression of dimerizing TM segments in membrane-mimetic environments [111,112]. The ability of the Sec61 translocon to handle negative mismatch has recently been studied [62]. In this work, it has been demonstrated that polyleucine segments as short as ~ 10 residues integrate efficiently into the ER membrane. Finally, hydrophobic matching may reflect an evolutionary strategy to regulate the activity of membrane proteins by allowing the adaptation of TM segment lengths to bilayer thickness in different cellular membranes [113].

Folding and assembly of multispanning membrane proteins

Forces behind the folding of membrane proteins

Next, we briefly introduce the molecular interactions driving protein folding within membranes. For a recent complete review, see [74,75]. Although hydrophobic collapse is a major driving force in the folding of soluble proteins, its role in membrane proteins is mostly limited to the formation of secondary structures across the lipid bilayer. Similarly, salt bridges and aromatic interactions do not make a great contribution to membrane protein folding. Conversely, interhelical hydrogen bonding [114,115] and, especially, van der Waals forces have been identified as major promoters of membrane protein folding [116,117]. Therefore, the restrictions imposed by the lipid bilayer allow for effective folding of TM segments of integral membrane proteins, despite the low contribution of hydrophobic forces and the reduced effect of salt bridges and aromatic interactions [118].

Folding and assembly of membrane proteins – the two-stage model

The folding and assembly of helical membrane proteins was schematized more than two decades ago as a two-stage process [77]. First, each TM helix is formed and independently inserted into the lipid bilayer. Second, these helices interact with each other to establish the final structure of the protein. Although this simplified view has since been refined, it still constitutes a valid conceptual approach.

In vivo, the insertion into the ER membrane occurs cotranslationally via the translocon complex. In this scenario, a TM segment does not insert into the membrane spontaneously; instead, the translocon facilitates its partition from the aqueous environment within the translocon pore into the lipid bilayer. After insertion or, for some proteins, during insertion, the TM helices interact with each other to form higher-order structures. These interactions create a microenvironment that permits further changes in the protein structure, such as insertion into the membrane of re-entrant loops or short polypeptides, membrane packing of non-α-helical segments, and binding of prosthetic groups [119].

Finally, the influence of the specific lipid environment during the assembly of TM segments should also be taken into account. The lipid and protein components of biological membranes have coevolved, allowing membrane proteins to assemble and function in the heterogenic environment provided by the diverse lipid bilayers in a cell. As well as membrane thickness, membrane lateral pressure [120], charge density [121] and even unique lipid–protein interactions [122] have been identified as structural determinants of membrane proteins. Furthermore, very recent cryo-EM studies using RNC complexes bound to SecY reconstituted in nanodisks revealed an interaction of the ribosome with lipids, leading to disorder in the lipid microenvironment adjacent to the translocon, which may favor membrane insertion of TM segments [123]. All in all, the final structure of a multispanning membrane protein will not be defined solely by protein–protein and lipid–protein interactions but also by the folding of its soluble domains. Thus, the aqueous environment on both sides of the membrane imposes restrictions on the folding of the extramembrane regions, and, by extension, on the overall protein structure.

Concluding remarks

Membrane protein integration appears to be orchestrated by multiple determinants and factors that, in unlimited combinations, give rise to native protein structures. During protein targeting, TM segment insertion and assembly into the membrane, several interconnected processes occur simultaneously. Structural studies of the translocon, together with *in vitro* quantitative thermodynamic analyses and biophysical

dissection of TM interactions, have resulted in significant advances in our understanding of membrane protein integration into the lipid bilayer. Our current knowledge, coupled with bioinformatics analysis [124], is opening new opportunities for *de novo* membrane protein structure prediction and design.

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