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REVIEW

Helix insertion into bilayers and the evolution of membrane proteins

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Abstract Polytopic α-helical membrane proteins cannot spontaneously insert into lipid bilayers without assistance from polytopic α-helical membrane proteins that already reside in the membrane. This raises the question of how these proteins evolved. Our current knowledge of the insertion of α-helices into natural and model membranes is reviewed with the goal of gaining insight into the evolution of membrane proteins. Topics include: translocon-dependent membrane protein insertion, antibiotic peptides and proteins, in vitro insertion of membrane proteins, chaperone-mediated insertion of transmembrane helices, and C-terminal tail-anchored (TA) proteins. Analysis of the *E. coli* genome reveals several predicted C-terminal TA proteins that may be descendents of proteins involved in pre-cellular membrane protein insertion. Mechanisms of pre-translocon polytopic α-helical membrane protein insertion are discussed.

Keywords α-Helical membrane proteins · Translocons · Bacteriocins · Chaperones · Tail-anchored proteins

Introduction

Polytopic α-helical membrane proteins enter lipid bilayers through translocon channels. These channels are themselves polytopic α-helical transmembrane proteins, such as the proteins in the YidC/Oxa/Alb3 family, or the SecYEG/Sec61 family [1–4]. How did this system evolve? If translocon channels are essential for translocation, then they would first have to be embedded in a membrane for them to become embedded in a membrane (Fig. 1). This raises a profound evolutionary question [5]. The early stage of biochemical evolution poses several analogous problems involving interconnected origins. For example, DNA replication requires DNA polymerase, which is itself specified by a DNA sequence. Most of these issues of origins can be resolved by postulating a precursor RNA world in which genetic information and enzyme activity reside in the same type of molecule. There has been some speculation (see below) about the co-evolution of membranes and the genetic code [6], or the co-evolution of membranes and RNA polymerase [7]. Also, the presence of RNA in the signal recognition particle, which targets proteins to membranes, suggests an important role for RNA in the evolution of the targeting mechanism [8]. However, it is difficult to imagine highly charged RNA molecules as progenitors of integral membrane proteins.

In order to derive a plausible theory of membrane protein evolution, we should consider three questions. What properties can we infer for the earliest integral membrane proteins? How did they insert into membranes? And with what sort of membranes did they interact? If proteins can spontaneously insert multiple helix-crossing segments into membranes, the formulation of a theory of the evolution of membrane proteins would be relatively simple. In this scenario, the evolution of translocon channels could have occurred at an early prebiotic stage, followed by rapid selection of two separate pools of proteins: one group of water-soluble proteins and another group of integral membrane proteins. Specific targeting of membrane proteins to particular membranes via the ancestors of the

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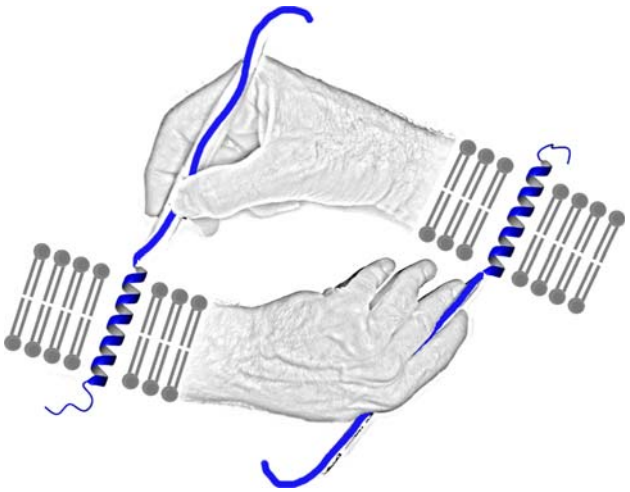

Fig. 1 Polytopic α-helical membrane proteins require polytopic α-helical membrane proteins for insertion into membranes. This raises the question of how these proteins evolved. Illustration after M.C. Escher's "Drawing Hands"

present-day translocon families would have developed at a later time. Evaluation of whether this idea is reasonable requires an examination of what we currently know about insertion of proteins into membranes.

The problem of spontaneous insertion of membrane proteins into lipid bilayers arises because of the hydrophobic nature of the lipid acyl chains. The part of an integral membrane protein that is embedded in the bilayer presents a surface of non-polar amino acid side chains to interact with the lipid hydrocarbon chains. This protein surface cannot be spontaneously inserted into the bilayer from the aqueous phase outside the membrane, because non-polar groups have an unfavorable free energy of interaction with water (the hydrophobic effect). As a consequence, integral membrane proteins form insoluble aggregates in water. Therefore, membrane proteins that are synthesized in the cytosol typically are accompanied to the membrane by chaperones that protect hydrophobic surfaces from interaction with water. At the interface between the lipid bilayer and the surrounding aqueous phase, there is also a thermodynamic barrier to insertion of non-polar amino acids, due to the polar head groups of the lipids. The entry of a membrane protein into the bilayer is usually facilitated by a translocon channel. Table 1 lists the proteins which will be discussed in this paper.

Assembly of membrane proteins

Structural studies have provided insight into how translocon channels insert proteins into lipid bilayers. The archaeal translocon SecYEβ consists of 12 transmembrane helices, 10 from SecY and 1 each from the SecE and Secβ subunits [9]. A central translocation pore was identified as the channel through which a nascent polypeptide enters from the ribosome. Segments of the protein sequence destined for placement in the lipid bilayer as membrane-spanning helices are ejected through a lateral gate between helices 2 and 7. A similar structure was observed for *Thermus thermophilus* SecYE [4] (Fig. 2a, b). The polarity of the amino acid sequence of the nascent chain inside the pore determines whether the sequence is translocated across the membrane or ejected laterally into the bilayer [10]. YidC contains six transmembrane helices, and Oxa1 contains five [11], forming dimers that may have a similar structures to one molecule of SecY. A low-resolution cryo-EM structure for YidC complexed with SecYEG [12] suggests that the lateral gate of SecYEG aligns laterally with the transmembrane pore of the YidC dimer. This proximity permits YidC to carry out its bilayer chaperone function, as SecYEG transfers to YidC the newly folded transmembrane helices of polytopic proteins, thus preventing them from aggregating in the bilayer during co-translational insertion.

Insertion of nuclear-encoded polytopic integral membrane proteins into the inner membrane of the mitochondria and chloroplasts is facilitated by proteins in the TIM17/TIM22/TIM23 family [13]. Low resolution single-particle electron microscopy of the TIM22 complex shows the presence of two pores, each approximately 16 Å in diameter [14]. This protein family appears to have evolved after endosymbiosis, since there are no homologs found in prokaryotes [15]. The twin-arginine translocation (TAT) pathway of chloroplasts and bacteria is mostly involved in export of whole proteins, although it also integrates some C-terminal tail-anchored (TA) proteins into membranes [16]. The transport pore, varying in size from about 30–60 Å, is made from TatA subunits, each of which contains a single transmembrane segment [16]. However, a functional translocase requires TatC, a polytopic integral membrane protein [17].

Translocons do not spontaneously insert into membranes without the presence of other translocons to assist their assembly. For example, SecYEG and YidC are inserted via SecYEG [1, 18], and Oxa1 insertion requires already-integrated Oxa1 in the mitochondrial membrane [19]. TIM22 insertion requires already-integrated TIM22 in the mitochondrial membrane [20]. TatC does not spontaneously insert into isolated thylokoid membranes, but the insertion mechanism is unclear: it does not involve any of SecYE, Alb3, or Tat itself [21]. Pre-existing translocons occur in membranes because cells arise by cell division: bacteria, mitochondria, and chloroplasts divide, thus sorting pre-existing translocons into their progeny; and pre-existing ER sorts into progeny cells during eukaryotic cell division. Although peroxisomes can divide, they are not

Table 1 Proteins discussed in this review

Protein	Insertion[a]	Function[b]	References
Sec YEG/SecYEβ	T	Translocon; protein secretion, TM protein insertion	[3, 4, 9]
YidC/Oxa1/Alb3	T	Translocon; TM protein folding, insertion	[1–3]
Tat	?	Translocon; protein secretion, TM protein insertion	[16, 17]
TOB/Omp85	?	β-Barrel insertion, outer membrane	[27, 28]
TIM22	T	Translocon; TM protein insertion, inner membrane	[13, 14]
Pex3p, Pex16p	R	Receptor; peroxisome assembly	[22–24]
Alamethicin	S	Antibiotic	[30]
Magainin	S	Antibiotic	[31]
Melittin	S	Antibiotic	[31]
Class IIa bacteriocins	S	Antibiotic	[32]
Pore-forming colicins	S	Antibiotic	[33]
Bacteriocin AS-48	C	Antibiotic	[34–36]
Cytolysin A	S	Pore-forming toxin	[37]
Bacteriorhodopsin	C, T	Proton pump	[38, 39, 41]
GDP-glucuronidyltransferase	S	TA, enzyme	[38]
HSP40/HSc70	–	Chaperone	[46]
Asna-1/TRC40	–	Chaperone	[44, 47, 71]
Viroporin 2B	CP,T?	Viral protein	[48]
Diacylglycerol kinase	C, T?	Enzyme	[53–55]
pHLIP	C	Synthetic peptide	[56–58]
TMX-1	A	Synthetic peptide	[59]
Anti-α_{IIb}	A	Synthetic peptide	[60]
Monoamine oxidase	?	TA, enzyme	[61]
Cytochrome b_5	A, CP	TA, enzyme	[63–68]
Fis1	CP	TA, fission protein	[62, 69]
Syntaxin	CP	TA, fusion protein	[70]
Bcl-2	CP	TA, anti-apoptotic protein	[72–74]
Bax	CP	TA, pro-apoptotic protein	[75, 76]

[a] Bilayer insertion mechanisms: *T* translocon, *R* receptor, *S* spontaneous, *C* chemically mediated, *CP* chaperone mediated, *A* self-associating aggregate

[b] *TM* Transmembrane, *TA* tail-anchored

autonomous, and the seed translocator proteins, Pex3p and Pex16p, are thought to derive from peroxisome precursor vesicles that bud from the ER [22–24]. It has been argued that YidC/Oxa/Alb3 probably preceded SecYEG and Tat in the evolution of translocons [25]. Molecular phylogenetics indicates that YidC preceded the evolution of Oxa1 and Alb3, but the latter did not develop directly from YidC in bacterial endosymbionts of early eukaryotes [26]. There is no evidence linking the evolution of YidC to SecY, and there are no proteins known with simpler structures from which they might have developed. It seems likely that integral membrane protein progenitors arose from some other source.

The first translocon for helical membrane proteins could not have been a β-sheet protein. Integral membrane proteins containing β-sheets form β-barrel pores that are known only in bacterial, mitochondrial, and chloroplast outer membranes. They are inserted into the membrane through channel proteins that belong to the TOB/Omp85 family [27, 28]. These proteins are themselves integral β-barrel proteins, and they are inserted through the TOB/Omp85 pathway. Thus, like α-helical integral membrane proteins, β-barrel proteins require pre-existing proteins integrated into the membrane for their own biogenesis [29]. Although the mechanism of insertion is not clear, it probably does not involve a lateral gate analogous to that proposed for SecY. Unlike the lateral separation of transmembrane helices, β-strand separation in the membrane is thought to require substantial energy to break backbone hydrogen bonds [28]. In any case, the insertion mechanism must be specific for β-barrel proteins.

Antibiotic peptides and proteins

A variety of antibiotic peptides and proteins are known to spontaneously insert into lipid bilayers. Among the best characterized is alamethicin [30], a 20-amino acid α-helical peptide that adsorbs on membrane surfaces at low concentrations and forms transmembrane ion-conducting channels at high concentrations (Fig. 2c, d). Alamethicin is of fungal origin, and other antibiotic peptides produced by eukaryotes include magainins (frogs) and melittin (bees) [31]. Peptides with similar properties are secreted by a variety of bacteria. For example, class IIa bacteriocins are unstructured peptides in water but form transmembrane helices after interaction with lipid bilayers [32, 33]. In the membrane, the helices associate to form ion-conducting pores that are the cause of their antibiotic properties. The monomeric peptides have simple structures in the membrane, consisting of essentially a single transmembrane segment.

Other pore-forming toxins are known that have more complex insertion mechanisms. Bacteriocin AS-48, from *Enterococcus faecalis*, binds to bacterial cytoplasmic membranes and causes pore formation. The 3D structure shows a compact 70-amino acid α-helical peptide with a highly asymmetric distribution of positive charges, which suggested a non-insertion electroporation mechanism of membrane disruption [34]. Subsequently, a pH-dependent conformational change in AS-48 was discovered that exposes hydrophobic helices which could penetrate the outer leaflet of lipid bilayers [35]. This might catalyze the formation of toroidal pores. Colicins are toxic proteins produced by *E. coli* and various other bacteria and targeted against specific related bacterial strains. Pore-forming colicins contain a C-terminal globular water-soluble domain comprising about 200 amino acids folded into ten α-helices. Upon binding to the inner membrane of the target bacteria, a rearrangement occurs in which two helical segments insert into the lipid bilayer and form an ion-conducting channel [36]. Cytolysin A is a hemolytic pore-forming toxin produced by *E. coli* and related enterobacteria that is directed against eukaryotic cells. CytA monomers, containing about 300 amino acids, are elongated water-soluble bundles of parallel α-helices. Upon binding to target membranes, a large conformational change occurs [37] in which the N-terminal helical segment rotates 180° and inserts into the lipid bilayer. Also, a *β*-strand loop rearranges into an α-helix and inserts into the bilayer. The pore is formed of 12 protomers.

The currently known antibiotic peptides thus provide a great deal of information about how spontaneous insertion of single transmembrane domains occur. However, as discussed below, it is unlikely that any of these antibiotic peptides are descendents of a translocon progenitor.

In vitro insertion of translocon-dependent polytopic helical membrane proteins

Although polytopic helical transmembrane proteins are thought to require a translocon for membrane insertion, there are various reports of spontaneous insertion of polytopic membrane proteins in vitro. For example, the archaeal proton pump bacteriorhodopsin can be inserted into unilamellar dimyristoyl phosphatidylcholine vesicles in conditions where membrane packing defects were introduced [38]. However, this was actually a membrane fusion process, rather than a transmembrane protein insertion, because bacteriorhodopsin was incorporated as small fragments of archaeal membranes containing bacteriorhodopsin already embedded in a bilayer prior to insertion. This same study [38] also reported that pig liver GDP-glucuronidyltransferase could be inserted in vesicles containing packing defects. However, this protein contains only a single C-terminal transmembrane-sequence. Insertion of single C-terminal helices is a different process from integrating polytopic membrane proteins (see below). There are reports of spontaneous insertion of polytopic membrane proteins, such as bacteriorhodopsin, from cell free protein synthesis systems. Integral membrane proteins were found to be co-translationally inserted into small unilamellar vesicles (SUVs) [39], large unilamellar vesicles (LUVs) [40], or nanolipoprotein particles [41], all in the apparent absence of the translocon apparatus. However, the composition of the cell-free protein translation extract used in these experiments is not fully known. The extracts may contain chaperones or other proteins that promote co-translational membrane protein insertion.

Chaperone-mediated and chemically mediated insertion of transmembrane helices

Many membrane proteins are integrated into bilayers post-translationally, rather than co-translationally. Examples of post-translational insertion include the nuclear-encoded membrane proteins of mitochondria and chloroplasts, the outer membrane proteins of bacteria, and the membrane proteins of peroxisomes. These proteins require chaperones to escort them to membranes so that they will not aggregate [42–44]. At the membrane surface, the chaperones transfer the membrane proteins to receptors and insertion channels for membrane assembly [20, 23, 28, 45]. TA proteins, containing single transmembrane helices that are inserted from the cytosolic side of the membrane, have a unique insertion mechanism (see below). TA proteins are chaperoned to the membrane by Hsp40/Hsc70 or Asna-1/TRC40 in an ATP-dependent manner [46]. The type of chaperone appears to be correlated with the hydrophobicity

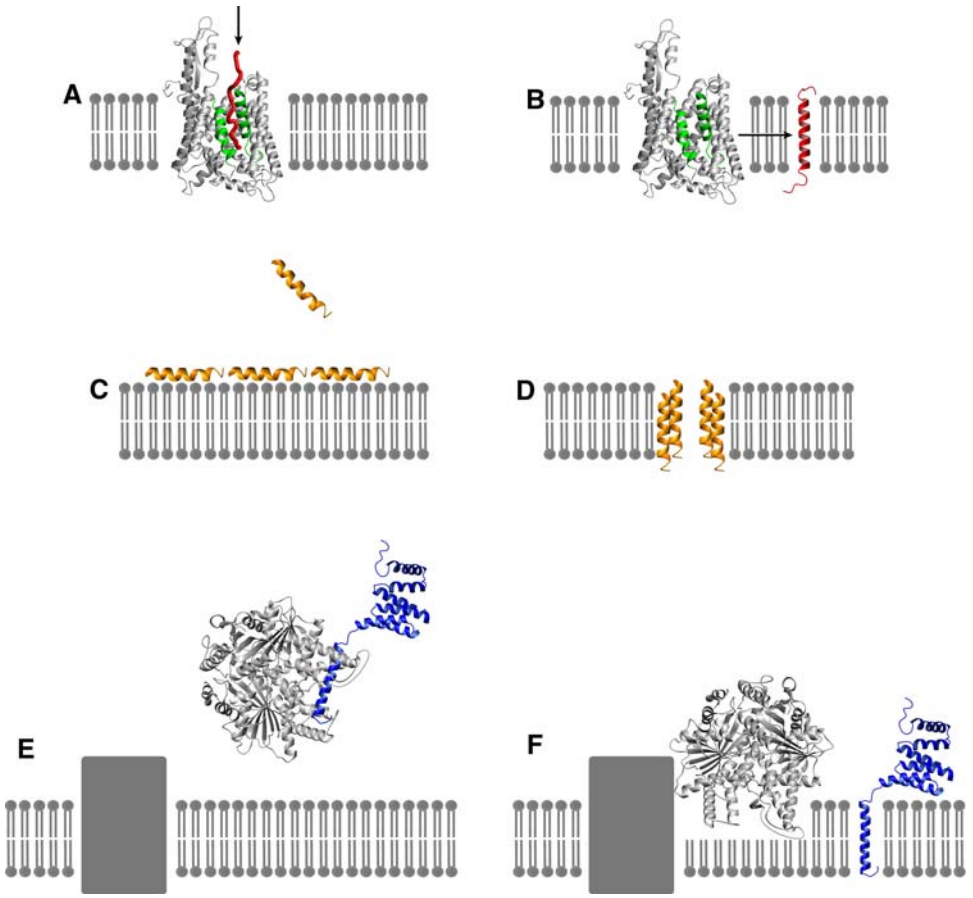

Fig. 2 Insertion of helical transmembrane (TM) proteins. **a** SecYE translocon from *Thermus thermophilus* [4] (Protein Data Bank 2ZJS). Nascent polypeptide chain (*red*) shown entering translocon channel from ribosome (*arrow* indicates direction of movement). Lateral gate, helices 2 and 7 (*green*). **b** TM helix (*red*) shown after insertion. *Arrow* indicates direction of motion through lateral gate. Translocon channel can accommodate two TM segments, thereby having the capability of inserting proteins with multiple TM domains and with connecting loops on both sides of membrane. **c** Peptide antibiotic alamethicin (*yellow*) (Protein Data Bank 1AMT) binds to membrane surface. **d** Alamethicin peptides associate to form TM channel. **e** Get3, a chaperone in the Asna1/TRC40 family [47] binds C-terminal tail-anchored membrane proteins and docks with receptor (*gray rectangle*) on target membrane (Protein Data Bank 2WOJ). TA protein (*blue*) modeled from water-soluble domain of Fis1 (Protein Data Bank 1PC2) and TM domain of monoamine oxidase (Protein Data Bank 2Z5Y). **f** After binding to receptor, chaperone inserts TM domain of TA protein into bilayer, possibly by entering bilayer outer leaflet. Protein structures drawn using MOLMOL software [90]

of the TA transmembrane sequence. The crystal structure of a yeast TRC40 homolog, Get3 [47], suggests that the chaperone may partition into the outer leaflet of the bilayer to release the C-terminal tail after docking with a membrane receptor protein (Fig. 2e, f).

In vitro insertion of integral membrane proteins can be accomplished using imitation chaperones. For example, viroporin, which normally requires the ER translocon for membrane insertion, spontaneously inserts into vesicle membranes when fused with a water-soluble domain [48]. Viroporins are small proteins produced by a variety of viruses, including poliovirus, HIV, and hepatitis C [49]. Viroporins contain one or two α-helical transmembrane domains that can form ion channels [50]. N-terminal fusion of maltose-binding protein to enterovirus viroporin 2B produced a soluble chimera that was found to spontaneously insert into LUVs, provided that the lipids were negatively charged [48]. The inserted proteins formed pores that permeabilized the vesicles to small solutes. The two transmembrane segments of the viroporin were shown to cooperate in pore formation [51].

Chemical chaperones have been used to facilitate the membrane insertion of helical proteins. Bacteriorhodopsin can be transferred from detergent micelles to detergent-saturated LUVs [52]. Diacylglycerol kinase (DAGK), a bacterial outer membrane protein, contains three transmembrane helices that interdigitate into a trimeric structure containing nine transmembrane helices [53]. DAGK can be solubilized in urea and formic acid, which acts as a chemical chaperone. Solubilized DAGK was rapidly

diluted to neutral pH and low urea concentration in the presence of lipid vesicles, and it spontaneously inserted in the membrane. The inserted DAGK folded correctly and gained catalytic activity [54, 55]. However, the yields of refolded protein were low (about 10%), suggesting that aggregation and misfolding constitute a major barrier to spontaneous insertion and folding of membrane proteins. Hydrogen ion promotes membrane insertion of a transmembrane peptide called pHLIP, derived from the C helix of bacteriorhodopsin [56, 57]. Above pH 6, pHLIP aggregates into a water-soluble tetramer. Below pH 6, pHLIP spontaneously inserts into vesicles. The pH dependence results from the protonation of two Asp residues in the transmembrane sequence that cannot be integrated into the hydrocarbon region of the bilayer in the ionized form. The pH-dependent insertion of pHLIP has been used for targeting imaging agents to tumors that are acidic [58]. An aggregated 31-residue synthetic peptide, TMX-1, was shown to be a useful chaperone for transfer of itself to bilayers [59], although the authors seemed disappointed by the peptide's self-association. Aggregated TMX-1 forms a stable non-precipitated solution which can spontaneously insert peptides in lipid bilayers in a transmembrane conformation. With palmitoyl–oleoyl phosphatidylcholine vesicles at lipid concentrations greater than 0.5 mM, more than 90% of the peptide was transferred from solution to the vesicles. The rate of insertion of a designed peptide, anti-α_{IIb}, into vesicles from an aggregated state in aqueous solution was reported [60]. Stopped-flow kinetics showed three separate kinetic phases, attributed to membrane binding, insertion, and peptide dimerization. Binding occurred on a time scale of tens of milliseconds, and insertion occurred on a time scale of hundreds of milliseconds.

Although the mechanisms of these chaperone- and chemically assisted insertion processes are not fully understood, it seems clear that assisted insertion of single transmembrane helices is a relatively common event, whereas assisted insertion of polytopic membrane proteins is inefficient and uncommon.

Membrane insertion of proteins with C-terminal transmembrane helices (TA proteins)

Many proteins are anchored to membranes by a single transmembrane segment that is near the C-terminus of the protein, oriented with the N-terminus on the cytosolic side of the membrane. The tail anchors are likely to be transmembrane α-helices, as shown by the crystal structure of monoamine oxidase in a lipid-like detergent environment [61], although the tails may be unstructured in water [62]. A protein with a single transmembrane helix oriented with a cytosolic N-terminus cannot be directly inserted into the membrane through the Sec61 translocon (although this topology could be produced post-translationally by proteolysis). Instead, these C-terminal TA proteins are inserted by special mechanisms. Examples of TA proteins are cytochrome b_5, Fis1, syntaxin, and Bcl-2.

Cytochrome b_5 can be purified from liver microsomes in a form that is stable in aqueous solution as an aggregate (approximately an octomer) [63]. Soluble cytochrome b_5 spontaneously inserts into SUVs and LUVs made of purified lipids [64] in the absence of any added chaperones. This insertion behavior is similar to some of the membrane-inserting synthetic peptides discussed above. In vitro, the conformation of the membrane anchor is variable. Some of the inserted cytochrome b_5 tail forms a loop, so that the C-terminus is exposed on the vesicle surface [65]. By contrast, in the ER, the membrane anchor crosses the membrane so that the C-terminus is on the luminal rather than the cytoplasmic side of the membrane [66, 67]. In a cell-free assay, added Hsp40/Hsc70 chaperone increases the amount of cytochrome b_5 inserted into added HeLa cell membranes [46]. However, cytochrome b_5 free of ATP could not only insert into lipid vesicles, but it could also translocate up to 85 amino acids fused at the C-terminus, indicating that chaperones are not needed [68].

Insertion of the mitochondrial fission protein Fis1 into the mitochondrial outer membrane was shown to be independent of the TOM-dependent mitochondrial protein import pathways, and targeting appeared to depend on the sterol composition of the membrane [69]. The membrane fusion SNARE protein syntaxin is inserted into ER membranes post-translationally and sorted to its target membrane via the secretory pathway [70]. There is some evidence that syntaxin is inserted via the Asna-1/TRC40 chaperone [46, 71]. The Bcl-2 protein family consists of TA proteins involved in the intrinsic apoptosis pathway [72]. Bcl-2 inserts into ER membranes in vitro [46, 73], and the insertion is strongly enhanced by Hsp40/Hsc70 plus ATP [46]. Bcl-2 also inserts via the C-terminal helix into mitochondrial outer membranes, and it can be stimulated by a binding partner to insert two internal helices into bilayers [74]. Bax is a Bcl-2 family protein that inserts into mitochondrial outer membranes after activation by interaction with cytosolic factors, also via the C-terminal helix plus two internal helices [75]. The insertion of internal helices by Bcl-2 and Bax is similar to the pore-forming colicins. Bax monomers associate in the membrane to form a large pore [76], triggering downstream events in apoptosis. Like the Bcl-2 family, both Fis1 and syntaxin are also involved in processes that actively rearrange lipid bilayers: Fis1 in mitochondrial and peroxisomal fission [77], and syntaxin in formation of exocytotic fusion pores [78].

TA proteins thus form a large family of proteins that can spontaneously insert single transmembrane helices into

Table 2 Single C-terminal transmembrane helix proteins predicted from *E. coli* genome

ASAP identifier[a]	Length[b]	Predicted TM helix[c]	Predicted function[d]
ABE-0007487	101	80–99	Unknown; Duf883 domain
ABE-0008799	113	91–110	Unknown; Duf883 domain
ABE-0010192	101	81–98	Unknown; Duf883 domain
ABE-0011041	117	47–69	Unknown
ABE-0002210	475	451–473	DnaJ
ABE-0002951	476	438–460	NAD-dependent epimerase/dehydratase
ABE-0004919	294	260–279	Formate dehydrogenase-N, Fe–S subunit
ABE-0014416	215	146–168	Phosphoglyceromutase 2
ABE-0007667	331	313–330	Flagellar assembly protein
ABE-0009223	268	235–257	E1-like
ABE-0009834	372	332–354	Hydrogenase 2, small subunit
ABE-0010028	206	173–195	SH3-containing protein
ABE-0010728	73	50–72	Outer membrane protein
ABE-0011858	338	315–332	UDP-D-glucosyltransferase

These proteins lack signal sequences and have the predicted transmembrane (*TM*) helix near the C-terminus. The first four entries have properties expected of hypothetical descendents of translocon progenitors

[a] From the University of Wisconsin *E. coli* genome project [80]

[b] Number of amino acids in the open reading frame

[c] Amino acid sequence positions of the predicted TM helix from TMHMM [81]

[d] From National Center for Biotechnology Information

membranes. Chaperone-mediated insertion may function primarily to target the TA proteins to specific subcellular membranes and also to increase the efficiency of the insertion process.

Evolution of helical integral membrane proteins

We can now return to the three questions posed at the beginning of this article.

What properties can we infer for the earliest integral membrane proteins?

It is clear from the above discussion of membrane protein insertion that polytopic integral membrane proteins do not spontaneously insert into lipid bilayers, but there are several types of proteins known to insert without translocons or receptors. The main groups are peptide antibiotics and TA proteins. Most of the TA proteins use chaperone systems for delivery to membrane targets, and often membrane receptors are involved in targeting and integration. However, neither chaperones nor receptors are absolute requirements, as demonstrated by cases of membrane insertion in heterologous systems or insertion into liposomes. Co-evolution of separate chaperones and TA proteins could have occurred, but it would not have been necessary. In many cases, small protein aggregates can act as self-chaperones. Proteins such as Bax or pore-forming colicins undergo conformational changes that expose membrane-embedded helices prior to membrane insertion. Many peptide antibiotics and TA proteins can spontaneously form pores in membranes, which is a property that must necessarily precede the evolution of translocon activity.

Are there existing proteins that are descendents of the postulated progenitors of translocons? Everything we know about TA protein insertion, including the pore-forming Bcl-2 family and SNAREs, comes from eukaryotic cells. A component of Sec61 is actually a TA protein: Sec61β, which is inserted into the ER membrane via Asna-1/TRC40 [71]. However, Sec61β is not homologous with prokaryotic SecG, unlike its Sec partners that are the eukaryotic–prokaryotic homologs, Sec61α-SecY and Sec61γ-SecE. Many of the antibiotic peptides, such as magainins and melittin, are exclusively eukaryotic. The bacterial pore-forming toxins, such as colicins or bacillus toxins, are limited to narrow clades. If they had been derived from a progenitor in a common bacterial ancestor, the sequences should have wider distribution across deeply branched species. Bacteriocins are a heterogeneous group of proteins including both pore-forming peptides and other proteins, such as the linocin family, that do not interact with membranes [79]. In order to sample the extent of prokaryotic TA proteins, I examined the 4,510 identified coding sequences of *E. coli* K12 MG1655 [80], using the TMHMM algorithm [81] for

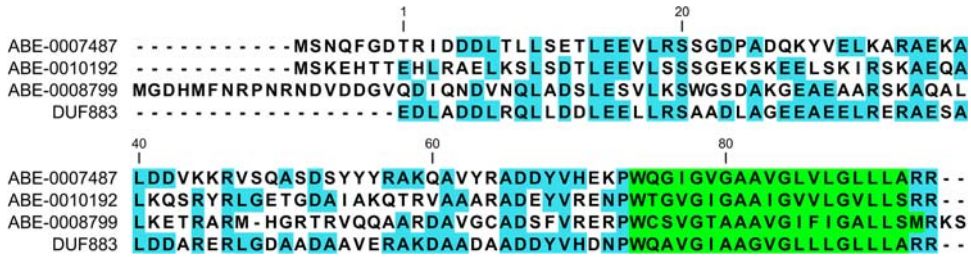

Fig. 3 Sequence alignment of three tail-anchored proteins from *E. coli*. Sequences are aligned with the consensus DUF883 domain (pfam05957). *Green* putative C-terminal transmembrane domain, *light blue* sequence identities with the consensus DUF883 domain

prediction of transmembrane sequences. I identified 242 sequences containing only one transmembrane segment, 25 of which lacked likely signal sequences. Of these, 14 had the predicted transmembrane sequence near the C-terminus (Table 2). Three proteins have Duf883 superfamily domains, of unknown function (Fig. 3). The phylogenetic distribution of proteins containing Duf883 domains is widespread, with representatives in eukaryotes as well as all major bacterial clades and at least one representative in Euryarchaeota. This indicates a common ancestor more than 4 billion years ago [82], although later evolution with extensive lateral transfer could have occurred. Another protein, with a single predicted transmembrane segment 48 amino acids from the C-terminus, is widely distributed in bacteria, with similar sequences found in Proteobacteria, Actinobacteria, and Firmicutes, indicating a possible common ancestor at least 3.4 billion years ago. One small protein with a predicted C-terminal membrane segment is identified as an outer membrane protein, and therefore it is unlikely to have appeared early in membrane evolution. The other nine putative TA proteins all have much larger N-terminal domains. Eight are known proteins, and most of their functions do not suggest ancestral involvement in biogenesis of membrane proteins. However, one has an N-terminal DnaJ-domain: a membrane-anchored member of the DnaJ chaperone family [83]. Membrane-anchored chaperones could be descendants of primitive translocons that were combined with chaperone activities.

How did the first polytopic proteins insert into membranes?

In the absence of a translocon, could polytopic integral membrane proteins form spontaneously, under the conditions existing before cells evolved? One can imagine primordial insertion mechanisms similar to techniques commonly used today. For example, consider the detergent dialysis method [84], or the related detergent dilution [85] or detergent adsorption [52] methods. Mixtures of detergent-solubilized lipids and integral membrane proteins are diluted below the detergent critical micelle concentration, resulting in the spontaneous formation of bilayer vesicles with inserted membrane proteins. Amphiphiles capable of forming both micelles and bilayers have been proposed as the main chemical constituents of early membranes [86]. The dilution requirement for the detergent–dilution technique would work against the encapsulation of a useful combination of RNA and substrates that could permit the selection necessary for early evolution. Amphiphile removal by adsorption would not decrease internal solute

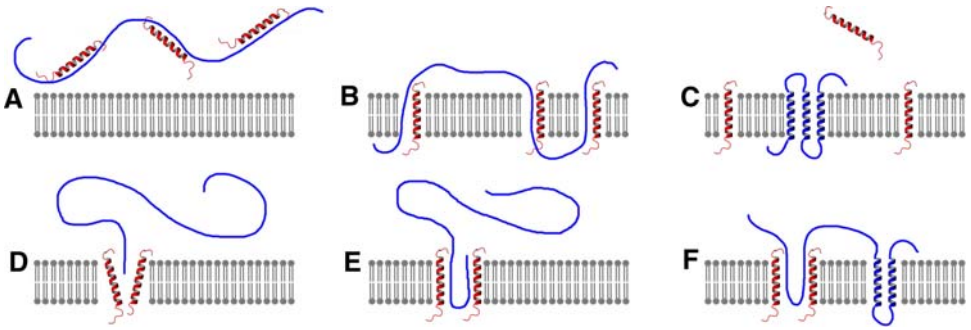

Fig. 4 Two possible mechanisms for pre-translocon insertion of polytopic helical membrane proteins. Single transmembrane (TM) helix proteins that spontaneously insert into bilayers (*red*) could facilitate polytopic membrane protein (*blue*) insertion. **a–c** Single TM helix proteins separately chaperone target transmembrane segments of larger protein, followed by dissociation of chaperones along with strong interaction of transmembrane helices to form folded structure. **d–f** Single TM helix proteins associate to form a translocon-like channel, probably involving four or more subunits (only two are shown). Binding of target protein opens lateral gate to release helical segments into bilayer. The polytopic protein in (**d**) and (**e**) is either chaperoned or cotranslationally inserted; the chaperones or ribosome are not shown

concentrations, but it would destabilize the proposed type of amphiphile membranes [86]. However, the amphiphile membranes can be controlled by changes in pH or ionic composition [86], providing a possible mechanism for vesicles to encapsulate solutes at much higher concentrations while also embedding integral membrane proteins in the bilayer. A general problem with the idea of the evolution of membrane protein insertion from an amphiphile mechanism is the reversibility of the insertion. By contrast, translocon-inserted membrane proteins become irreversibly embedded in the lipid bilayer.

Spontaneous insertion of single transmembrane helix proteins, perhaps like an ancestor of the existing Duf883 domain proteins discussed above, could lead to subsequent evolution of polytopic membrane proteins in at least two ways: by direct facilitation of membrane incorporation, or by formation of precursor translocons.

Direct facilitation

TA proteins are likely to insert by mechanisms similar to those shown for peptides such as alamethicin: first binding to the water-bilayer interface, and then associating and integrating into the hydrocarbon region of the bilayer. If, instead of self-associating to form a pore, the TA protein associates with a non-polar region of a potential polytopic membrane protein, the insertion of the polytopic protein could be facilitated. This would result in the integration of polytopic membrane proteins without a translocon-like mechanism (Fig. 4a–c). Insertion of pairs of helices would be facilitated by the TA protein, followed by displacement of the individual TA proteins as the transmembrane helices of the polytopic protein associate to form a compact structure. Subsequent selection on these polytopic membrane proteins would eventually lead to the evolution of translocon channels similar to YidC and SecY.

Precursor translocons

Primitive transmembrane pores formed by TA proteins would bind a variety of partners, including other protein chains from the aqueous compartment adjacent to the membrane. Some pores would undoubtedly have properties conducive to stabilization of a bound peptide chain as a transmembrane helix. A primitive lateral gate mechanism in a pore made of individual single-helix TA subunits would be analogous to the pore-forming process itself: an equilibrium between addition of subunits to and dissociation of subunits from the pore (Fig. 4d–f). This would result in the slow accretion of polytopic membrane proteins. Selection on these proteins would result in the evolution of the polytopic translocon ancestors of YidC and SecY that would eventually replace the earliest self-inserting TA pores.

What membranes were present for interaction with early membrane proteins?

It is far from clear what sort of lipid bilayers, if any, existed at the time membrane proteins evolved. The prebiotic environment may not have been capable of forming hydrocarbon chains from small molecule precursors by heat, electrical discharge, or light energy, since these products are not plentifully made in prebiotic synthesis reactions. It has been suggested that lipids and membranes could have originated from carbon carried to Earth by meteorites [86, 87]. Furthermore, it is difficult to conceive of a common ancestor for the fundamentally different eubacterial and archaeal lipids. This problem has been circumvented by the proposal that lipid bilayer membranes developed after the evolution of cells [88]. It was suggested that cells initially occupied micrometer-scale compartments in the porous structure of iron sulfide precipitates near undersea vents. Another pathway to precursor cells without lipids could have been protein-lined compartments, similar to the multi-enzyme complexes and microcompartments in extant cells [79]. If early cells consisted of semi-permeable microcompartments, with protein and/or mineral shells, the shells could have been slowly replaced by lipid–protein membranes. Compartments lined with protein shells would not have required any precursor translocon, as the insertion of polytopic membrane proteins would have initially been facilitated by the protein subunits that composed the protein shell.

Lipid bilayers have been proposed as an essential scaffold for the evolution of the genetic code and protein synthesis [6], with polynucleotides anchored to bilayer vesicles (“obcells”) by polypeptide tails. These tails are proposed to have been N-terminal, since the C-terminus must couple to the 3′ OH of the polynucleotide. Bilayers have also been proposed as catalysts for polynucleotide synthesis [7]. During this early stage of biochemical evolution, it is likely that lipoproteins existed to transport lipids to and from the surfaces of membranes (or protein/mineral shells). This suggests another possible progenitor for membrane proteins. Bacterial lipoproteins are transported between membranes by carrier proteins that bind to the lipid acyl chains [89]. The ancestors of these proteins could have been both transporters of lipoproteins and also partly embedded in lipid bilayers. A segment of a lipid-binding protein could move almost seamlessly between contact with transported acyl chains and interaction with the membrane into which the lipids insert. The processes of lipid- and helix-insertion into membranes seem to have many similarities. We recently found that the rates of

dodecyl sulfate hydrocarbon chains inserting into lipid bilayers (R. Renthal and I. Peña, unpublished data) are approximately the same as the reported rates of transmembrane helix insertion [60].

Conclusions

Two different scenarios seem most probable for the origin of polytopic integral membrane proteins: (1) if protein evolution preceded lipid bilayer evolution, membrane proteins could have evolved from lipoproteins and the protein or mineral shells of microcompartments; or (2) if lipid bilayers and proteins evolved together, the first membrane proteins could have been small, single-transmembrane helix proteins, perhaps resembling currently known TA proteins. These proteins could have chaperoned polytopic membrane proteins, which then evolved into the first translocons. Alternatively, the TA proteins themselves could have evolved into a primitive translocon, after which polytopic membrane proteins could enter membranes, resulting in development of translocons resembling those now known.

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