Biogenesis of tail-anchored proteins: the beginning for the end?

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Summary

Tail-anchored proteins are a distinct class of integral membrane proteins located in several eukaryotic organelles, where they perform a diverse range of functions. These proteins have in common the C-terminal location of their transmembrane anchor and the resulting post-translational nature of their membrane insertion, which, unlike the co-translational membrane insertion of most other proteins, is not coupled to ongoing protein synthesis. The study of tail-anchored proteins has provided a paradigm for understanding the components and pathways that mediate post-translational biogenesis of membrane proteins at the endoplasmic reticulum. In this Commentary, we review recent studies that have converged at a consensus regarding the molecular mechanisms that underlie this process – namely, that multiple pathways underlie the biogenesis of tail-anchored proteins at the endoplasmic reticulum.

Key words: Chaperone, Endoplasmic reticulum, Membrane integration, Post-translational

Introduction

The defining feature of a tail-anchored protein is the presence of a single transmembrane (TM) segment at the extreme C-terminus of the polypeptide (Kutay et al., 1993). This TM segment provides a targeting signal for the delivery of the protein to the correct subcellular compartment, but its C-terminal location dictates that it is only freely available to cytosolic factors after the termination of protein synthesis (Fig. 1). The TM segment also acts as an anchor that retains the polypeptide in the lipid bilayer once integration has taken place (Borgese et al., 2007) (see also Fig. 1). In some cases, the role of the TM segment as a targeting signal can be modulated by the polypeptide regions that flank the hydrophobic core of the tail anchor. The amino acid composition of these elements can influence both the efficiency with which the protein is integrated into the membrane (Beilharz et al., 2003; Kim et al., 1999) and its subcellular location (Borgese et al., 2007; Henderson et al., 2007).

Irrespective of the organelle in which they reside, tail-anchored proteins are always oriented in the membrane such that the larger N-terminal region faces the cytosol (Fig. 1). This part of the polypeptide is typically important for biological function, as exemplified in the case of the SNARE proteins, in which the cytoplasmic domain plays a crucial role during vesicular transport (Jahn and Scheller, 2006). Tail-anchored proteins occur in all kingdoms of life; over 50 are predicted to be expressed in yeast and in excess of 300 in humans (Beilharz et al., 2003; Kalbfleisch et al., 2007). In eukaryotes, functionally diverse tail-anchored proteins can be found in the mitochondrial and chloroplast outer membranes, the peroxisome, and the intracellular compartments that are connected by the secretory and endocytic pathways, including the endoplasmic reticulum (ER), Golgi, plasma membrane, endosomes and lysosomes (Borgese et al., 2007). The ER is thought to act as the entry point for the many different tail-anchored proteins that occupy the compartments of the secretory pathway (Behrens et al., 1996; Kutay et al., 1995; Linstedt et al., 1995). Given its major role in producing tail-anchored proteins (see Box 1 for specific examples) and the recent advances in our understanding of this process, it is the biogenesis of tail-anchored proteins at the ER that we focus on here.

The biogenesis of tail-anchored proteins requires their post-translational targeting to a membrane followed by the integration of the TM segment into the lipid bilayer. Conceptually, this could be a simple process that requires no additional protein factors and involves the spontaneous partitioning of newly synthesised proteins into the lipid bilayer. Although there is some data to suggest that such completely unassisted biogenesis of tail-anchored proteins might occur at the mitochondrial outer membrane (Kemper et al., 2008; Meineke et al., 2008; Setoguchi et al., 2006), most studies suggest that the biogenesis of tail-anchored proteins at the ER involves one or more cytosolic factors (Abell et al., 2007; Colombo et al., 2009; Favaloro et al., 2008; Rabu et al., 2008; Yabal et al., 2003) (Fig. 1). In addition, specific membrane components also appear to facilitate the efficient integration of many tail-anchored proteins into the ER (Fig. 1) (Borgese et al., 2007; Cross et al., 2009; Rabu and High, 2007). It is worth re-iterating that the molecular mechanisms that underlie the biogenesis of tail-anchored proteins at the ER are quite distinct from the co-translational process by which most membrane proteins are synthesised at this location. During this co-translational pathway, the signal recognition particle (SRP) binds to the ER-targeting sequence of a nascent polypeptide and delivers the ribosome-bound chain to the Sec61 translocon of the ER membrane, where integration occurs (for a review, see Cross et al., 2009).

A key breakthrough in our understanding has been the realisation that the biogenesis of tail-anchored proteins can occur via distinct pathways (Borgese et al., 2007; Favaloro et al., 2008; High and Abell, 2004; Kim et al., 1997; Rabu and High, 2007; Stefanovic and Hegde, 2007). One early indication that such multiple pathways exist was the clear difference in nucleoside triphosphate (NTP) requirements for the biogenesis of different tail-anchored proteins (see Box 2). Combining this information with the recent outcomes

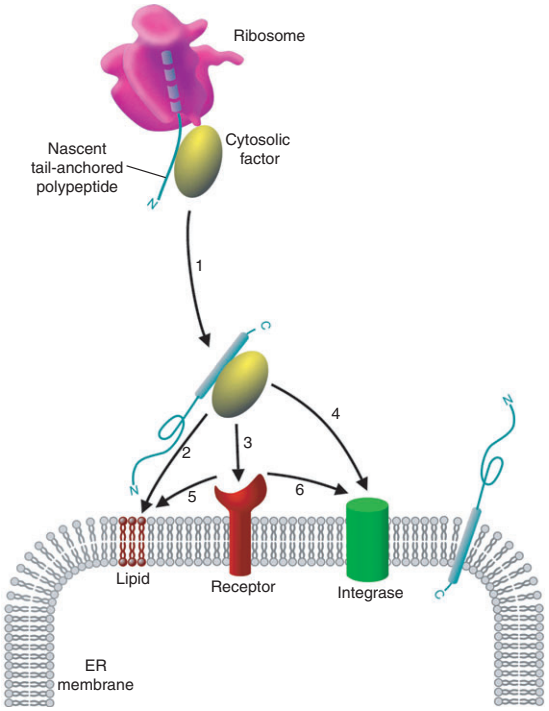

Fig. 1. Theoretical general scheme for the post-translational targeting and insertion of tail-anchored membrane proteins at the ER. The protein precursor is recognised by cytosolic factors as the TM segment folds inside the ribosome, or just after it emerges from the exit tunnel, thus forming a TM-segment recognition complex (1). At the ER membrane, this complex either directly supports unassisted partitioning into the membrane (2), docks with a receptor (3) or hands the substrate to a dedicated integrase (4). Alternatively, after docking with the receptor (3), the tail-anchored protein might then either undergo unassisted partitioning (5) or be passed on to the integrase (6).

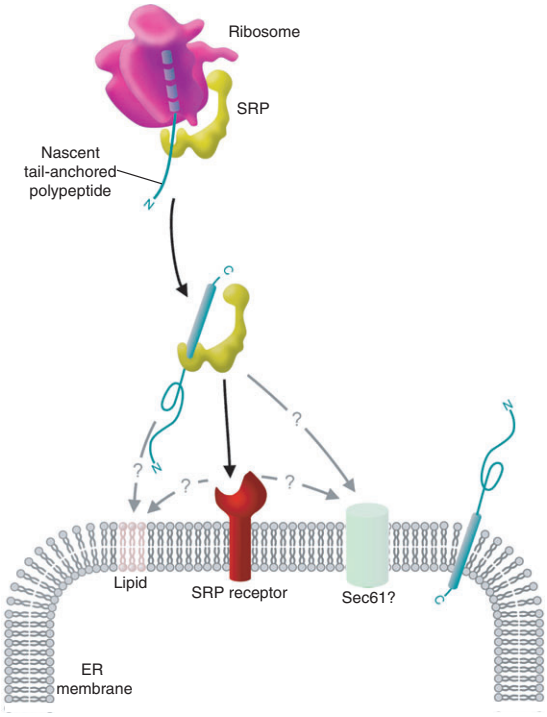

Fig. 2. SRP-dependent pathway for biogenesis of tail-anchored proteins at the ER. The interaction of the SRP-tail-anchored protein complex with the ER-localised SRP receptor most probably depends on both components being in the GTP-bound form. The SRP receptor is normally associated with the Sec61 complex, raising the possibility that tail-anchored proteins that utilise the SRP pathway might be integrated via this well-characterised ER translocon (Cross et al., 2009). Alternatively, the nascent chain could simply freely partition into the lipid bilayer after docking at the SRP receptor. In this model, the role of GTP hydrolysis is to facilitate the disassembly of the SRP–SRP-receptor complex to enable further rounds of SRP-dependent targeting to the ER (Cross et al., 2009).

of novel genetic approaches such as epistatic miniarray profiling (EMAP) (Schuldiner et al., 2005) in *Saccharomyces cerevisiae*, puts us in a position to describe three pathways for which a specific facilitator has been identified. In this Commentary, we discuss the SRP pathway, the heat shock protein 40 (Hsp40)-heat shock cognate 70 (Hsc70) pathway and the arsenical pump-driving ATPase protein (Asna1) pathway in the chronological order that they have been implicated in the biogenesis of tail-anchored proteins. We also speculate on how these pathways might be orchestrated in a cellular context. It should be noted that a very recent study by Colombo and colleagues suggests that additional, and as-yet-unknown, cytosolic factors might contribute to the biogenesis of certain tail-anchored proteins such as cytochrome b5 via a pathway that remains to be fully delineated (Colombo et al., 2009) (Fig. 1).

SRP-mediated pathway of ER integration

The first cytosolic factor to be identified as playing some role in the biogenesis of tail-anchored proteins was SRP, a component that is considered a hallmark of the classical co-translational pathway for membrane protein biogenesis (Abell et al., 2004; Cross et al., 2009). An in vitro crosslinking approach showed that the tail-anchor region of synaptobrevin 2 (Syb2; also known as VAMP2, vesicle-associated membrane protein 2; see Box 1) is transiently associated with the signal sequence-binding domain of SRP (Fig. 2). Furthermore, the efficient membrane insertion of Syb2 requires an intact SRP receptor, which is consistent with the idea that SRP has a post-translational role in the binding and delivery of tail-anchored proteins to the ER membrane (Fig. 2). This SRP-dependent mechanism seems to favour a small subset of tail-anchored-protein precursors with more hydrophobic TM segments (Box 1). The role of SRP is probably to shield the hydrophobic tail-anchor region of the precursor protein and to maintain the polypeptide in a membrane-integration-competent state prior to its delivery to the target membrane. One issue with this model is that the amount of cellular SRP is low when compared with the number of ribosomes [there is approximately 1 SRP per 40 ribosomes (see Raue et al., 2007)]. Therefore, if SRP is only recruited when the hydrophobic tail anchor emerges from the ribosomal exit tunnel (Abell et al., 2004), many potential substrates might be missed and the pathway would be inefficient (Fig. 2). However, it has recently been discovered that a hydrophobic TM segment can stimulate the binding of SRP to ribosomes while this segment is still inside the ribosomal exit tunnel (Berndt et al., 2009). This evidence for such ribosome priming provides a paradigm whereby SRP might be specifically recruited to ribosomes that are synthesising tail-anchored proteins before the hydrophobic TM segment emerges from the exit tunnel. This is also consistent with the suggestion that, after the release of newly synthesised Syb2 from the ribosome, SRP has a short window of opportunity during which it can promote its biogenesis at the ER (Abell et al., 2004).

Box 1. Model tail-anchored proteins

Protein	Core TM sequence	Calculated hydrophobicity*	Predicted $\Delta G^{\ddagger}$
Synaptobrevin 2	MMIILGVICAIILIIIIVYF	64	–3.80
RAMP4	GPWLLALFIFVVCGSAIFQII	41	–1.27
Cytochrome b5	WTNWVIPAISAVAVALMYRLYM	25	+0.20

*(Kalbfleisch et al., 2007).
‡The predicted ΔG provides an alternative indication of biological hydrophobicity (Hessa et al., 2007). The more negative the value, the more hydrophobic the TM segment shown.

Synaptobrevin 2
Synaptobrevin 2 (Syb2; also known as VAMP2, vesicle-associated membrane protein 2) is a SNARE protein that is involved in vesicular transport (Jahn and Scheller, 2006) and that has been widely studied as a model tail-anchored protein (Kim et al., 1997; Kutay et al., 1995). Syb2 has a particularly hydrophobic TM segment, and it might be delivered to the ER membrane via both the Asna1-mediated (Stefanovic and Hegde, 2007) and SRP-mediated (Abell et al., 2004) routes, but does not use the Hsp40-Hsc70-mediated pathway (Rabu et al., 2008).

RAMP4
RAMP4 (ribosome-associated membrane protein 4; also known as SERP1, stress-associated endoplasmic reticulum protein 1) is a Sec61 translocon-associated protein that is upregulated following ER stress (Hori et al., 2006). The protein has a moderately hydrophobic TM segment and is delivered to the ER via an Asna1-mediated route (Favaloro et al., 2008).

Cytochrome b5
Cytochrome b5 was the first tail-anchored protein to be studied (Anderson et al., 1983), well before the term was used to describe a specific class of integral membrane proteins (Kutay et al., 1993). The ER isoform of cytochrome b5 has a TM segment with relatively low hydrophobicity (Brambillasca et al., 2006) and does not use the Asna1-mediated pathway for delivery to the ER (Favaloro et al., 2008; Stefanovic and Hegde, 2007). Its biogenesis might require a cytosolic component(s) (Colombo et al., 2009; Yabal et al., 2003), including the Hsp40-Hsc70 chaperone combination (Rabu et al., 2008) and/or another as-yet-unidentified factor (Colombo et al., 2009). There is currently no evidence that any membrane proteins are required for cytochrome b5 to be efficiently integrated into the lipid bilayer (Brambillasca et al., 2005; Yabal et al., 2003) and cytochrome b5 thus provides a paradigm for the unassisted integration of tail-anchored proteins at the ER (Brambillasca et al., 2006).

Following the SRP-receptor-dependent release of Syb2 from SRP at the ER membrane, the basis for subsequent membrane integration is unclear and controversial. Reconstitution experiments show that phospholipids alone are not sufficient to mediate the membrane insertion of the Syb2 TM segment (Brambillasca et al., 2006; Kutay et al., 1995), implying a role for one or more membrane-localised proteins in this process. Furthermore, if ER-derived microsomes are treated with protease before analysing the biogenesis of Syb2 in vitro (Abell et al., 2004; Kim et al., 1997; Kutay et al., 1995), integration is prevented. Whether the protease-sensitive membrane component affected in these experiments is the SRP receptor (Abell et al., 2004), or whether additional membrane proteins play a role in this pathway, remains to be determined (Fig. 2).

It has been established that artificially engineered versions of Syb2 can be N-glycosylated at C-terminal extensions (Abell et al., 2004; Kutay et al., 1995). Therefore, post-translationally integrated Syb2 chains can access the ER-localised oligosaccharyl-transferase complex in a similar fashion as proteins that are delivered via the co-translational pathway that relies on the Sec61 translocon for integration into the ER membrane (Cross et al., 2009). Furthermore, it has been shown that newly synthesised Syb2 chains are sufficiently close to the Sec61 translocon that they can be crosslinked to Sec61 subunits (Abell et al., 2003). However, despite such circumstantial evidence (Fig. 2), there is currently no functional data to support a role for the Sec61 translocon during the membrane integration of Syb2 (Kutay et al., 1995) or of any other tail-anchored protein (Steel et al., 2002; Yabal et al., 2003). Thus, the molecular basis for the membrane integration of tail-anchored proteins that are delivered to the ER via a post-translational SRP-mediated pathway is unknown (Fig. 2).

Hsp40-Hsc70-mediated pathway of ER integration

In vitro studies have indicated that the post-translational biogenesis of most, if not all, tail-anchored proteins is stimulated by ATP (Abell et al., 2007; Favaloro et al., 2008; Kim et al., 1999; Kim et al., 1997; Kutay et al., 1995; Steel et al., 2002; Yabal et al., 2003) and the participation of at least one ATP-dependent cytosolic factor in this process was suggested several years ago (Yabal et al., 2003). Certain secretory proteins are translocated across the ER membrane by an unusual post-translational mechanism involving cytosolic members of the Hsp70 family of molecular chaperones (Ngosuwan et al., 2003; Zimmermann, 1998); by extrapolation, it is possible to speculate that such chaperones play a similar role during biogenesis of tail-anchored proteins (Box 2). In the case of Hsc70, two lines of evidence show that this cytosolic ATPase can function with its co-chaperone, Hsp40, to promote the biogenesis of tail-anchored proteins. First, a combination of Hsp40 and Hsc70 can promote the ATP-dependent biogenesis of many tail-anchored proteins in the absence of other cytosolic factors (Abell et al., 2007; Rabu et al., 2008). These studies indicate that Hsp40 and Hsc70 are sufficient to promote biogenesis of tail-anchored proteins in vitro. The limitation of this experimental approach, however, is that it does not address the possibility that there might be competition between different cytosolic factors for binding to tail-anchored proteins. Thus, an in vitro system containing all known cytosolic factors was exploited in combination with small molecule inhibitors of Hsc70 (Fewell et al., 2004) to explore the issue of whether Hsp40 and Hsc70 are necessary for biogenesis of tail-anchored proteins (Rabu et al., 2008). Using this approach, it was concluded that the biogenesis of a specific subset of tail-anchored proteins is promoted by the Hsp40-Hsc70 chaperone combination (Fig. 3). This subset of proteins, which includes cytochrome b5,

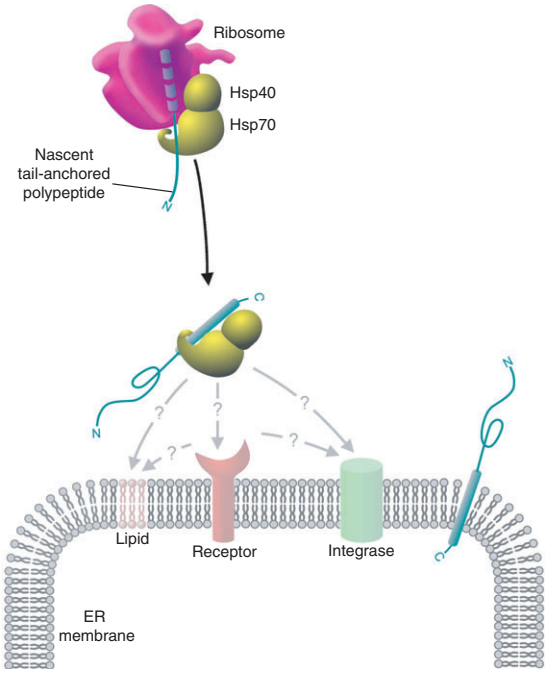

Fig. 3. Hsp40-Hsc70-dependent pathway for the biogenesis of tail-anchored proteins at the ER. General chaperones of the heat-shock family can accept and shield tail-anchored proteins with a TM segment of relatively low hydrophobicity (Rabu et al., 2008), presumably preventing their aggregation. This interaction might simply enable the subsequent unassisted integration of the TM segment by direct partitioning into the lipid bilayer, or allow a similar process for which a dedicated ER-membrane receptor is a prerequisite. No such receptor has been identified to date and, similarly, any role for an integrase during the Hsp40-Hsc70 pathway for biogenesis of tail-anchored proteins remains hypothetical.

is characterised by TM segments of comparatively low hydrophobicity (see Box 1).

Even less is known about the events that occur at the membrane for Hsp40-Hsc70-mediated integration of tail-anchored proteins than the events involved in integration mediated by SRP (Figs 2 and 3). Cytosolically oriented membrane receptors for chaperones have been identified for precursor proteins that are delivered to mitochondria and chloroplasts (Young et al., 2003; Soll and Schleiff, 2004). However, a receptor for the Hsp40-Hsc70-dependent biogenesis of tail-anchored proteins at the ER membrane has not been identified. It has been suggested (Hartl and Hayer-Hartl, 2002) that the role of the molecular chaperones during post-translational targeting is mainly to prevent the aggregation of hydrophobic regions of polypeptide (such as tail-anchor segments), thereby maintaining these substrates in a membrane-integration-competent conformation (Ngosuwan et al., 2003; Zimmermann, 1998). In this scenario, there might be no need for an ER-localised receptor. Indeed, if cytochrome b5 can be considered a representative for all tail-anchored proteins that exploit the Hsp40-Hsc70-mediated route (Rabu et al., 2008), then the membrane integration of these proteins probably occurs by simple partitioning (Fig. 3) and requires no integral membrane proteins (Brambillasca et al., 2005). The efficiency of this unassisted route for integration of tail-anchored proteins depends on the phospholipid composition of the membrane, as shown by the fact that the presence of cholesterol strongly inhibits membrane integration (Brambillasca et al., 2005).

Box 2. Nucleotide requirements for biogenesis of tail-anchored proteins

SRP-mediated pathway

It has been established that SRP-mediated protein targeting uses GTP as an energy source (Cross et al., 2009). In experiments that assayed the biogenesis of Syb2, membrane integration was substantially diminished when NTPs were depleted using apyrase. The addition of a non-hydrolysable GTP analogue (GMPPNP), but not of an ATP analogue (AMPPNP), partially restored Syb2 integration. Furthermore, the addition of GTP in combination with purified SRP stimulated the membrane integration of Syb2 by more than threefold (Abell et al., 2004).

Hsp40-Hsc70-mediated pathway

In contrast to the SRP-mediated pathway, the Hsp40-Hsc70 pathway depends on ATP. The biogenesis of an in vitro-translated tail-anchored protein (Sec61β) can be strongly stimulated by the addition of purified Hsp40, Hsc70 and ATP (Abell et al., 2007). For other intracellular processes that rely on Hsc70, it is known that the activity of the chaperone is regulated by ATP-controlled cycles of substrate binding and release (Bukau and Horwich, 1998; Hartl, 1996). Hsp40 proteins, in turn, are implicated in regulating the ATPase function of Hsc70 proteins (Young et al., 2004). The Hsp40-Hsc70-mediated pathway seems to be important for the integration of tail-anchored proteins with TM segments of a lower net hydrophobicity (Box 1), whereas the Asna1-mediated pathway is preferentially used for the integration of substrates with more hydrophobic TM segments (Rabu et al., 2008).

Asna1- and GET 3-mediated pathways

Several observations strongly suggest that tail-anchored protein biogenesis via the Asna1 (TRC40) and GET pathways is an ATP-dependent process, and that Asna1 and Get3 both display a robust ATPase activity (Kao et al., 2007; Kurdi-Haidar et al., 1998; Shen et al., 2003; Tseng et al., 2007). Interestingly, the binding of a tail-anchored protein substrate to Asna1 does not depend on ATP hydrolysis (Favaloro et al., 2008). Furthermore, biogenesis of tail-anchored proteins could be inhibited by adding a dominant-negative ATPase-deficient mutant of Asna1 (Stefanovic and Hegde, 2007). However, despite evidence that ATP hydrolysis underlies Asna1 and Get3 function, a thorough mechanistic investigation of the role of the ATPase in the putative GET cycle (see Fig. 4) is still lacking.

Most naturally occurring tail-anchored proteins have a very short hydrophilic region at the C-terminus of the hydrophobic tail anchor, meaning that there is very little polypeptide that needs to be translocated across the membrane into the ER lumen (Fig. 1). Remarkably, when the C-terminus of cytochrome b5 is extended by the addition of an artificial segment of polypeptide, the resulting protein can still be efficiently integrated into the membrane in a post-translational manner, implying that large polypeptide domains can translocate across the phospholipid bilayer via this unassisted route (Brambillasca et al., 2006). Although this unassisted pathway is exploited by cytochrome b5 and protein tyrosine phosphatase 1b (PTP1B; also known as PTPN1), it appears that there are many other tail-anchored proteins that cannot become membrane-integrated by this mechanism. Intriguingly, the tail-anchored proteins that can be membrane-integrated without assistance are those that have been suggested to utilise Hsp40-Hsc70 when exposed to cellular extracts (Box 1) (Brambillasca et al., 2006; Colombo et al., 2009; Rabu et al., 2008). However, whether such tail-anchored proteins are obligatory substrates for the Hsp40-Hsc70

pathway is disputed (Colombo et al., 2009; Rabu et al., 2008), and any role for these chaperones during biogenesis of tail-anchored proteins in vivo remains to be demonstrated.

GET pathway of ER integration

Several independent lines of investigation led to the discovery of a novel ATP-dependent pathway for biogenesis of tail-anchored proteins at the ER (Box 2) in which the cytosolic ATPase Asna1 acts as the 40-kDa component of a TM-recognition complex (hence also designated TRC40 in mammals). Mammalian Asna1 was discovered by two groups that both exploited in vitro crosslinking approaches to identify the cytosolic components that contact tail-anchored-protein precursors prior to membrane integration (Favaloro et al., 2008; Stefanovic and Hegde, 2007). Asna1 belongs to a NTPase superfamily of proteins with archeal or archeoeukaryotic origin, and homologues in various organisms have previously been associated with a response to metal stress (see Box 3). Independently of this biochemical approach, the yeast equivalent of Asna1 was identified via an EMAP approach that was designed to identify functionally related sets of genes and predict physical complexes between gene products (Jonikas et al., 2009; Schuldiner et al., 2005). By using such genetic interaction approaches in *Saccharomyces cerevisiae*, the yeast Asna1 homologue Get3 was discovered to participate in the function of the early secretory pathway and was subsequently shown to be necessary for biogenesis of tail-anchored proteins (Schuldiner et al., 2005; Schuldiner et al., 2008). Genetic and physical interaction analyses have now implicated four proteins that interact with the cytosolic Get3 component during the biogenesis of tail-anchored proteins as part of the GET pathway (Auld et al., 2006; Ito et al., 2001; Jonikas et al., 2009; Schuldiner et al., 2005). The genes were initially named Golgi-ER trafficking (GET) because of the vesicular transport defects that were observed in the corresponding deletion mutants (Schuldiner et al., 2005), but have now been renamed as guided entry of tail-anchored proteins (GET) (SGD database, http://www.yeastgenome.org; see also Fig. 4). Get1 and Get2 are membrane proteins that form a receptor at the ER membrane (Schuldiner et al., 2008), whereas Get4 and Get5 interact with Get3 in the cytosol to form the yeast TM-recognition complex (Jonikas et al., 2009). It is not yet clear to what extent the GET pathway differs in lower and higher eukaryotes, and we will therefore discuss each component individually and highlight specific insights that have been derived from studies of the different organisms.

What are the key pieces of evidence that Asna1 (TRC40) and Get3 are involved in the TM-recognition complex? In reticulocyte lysate, the crosslinking of Asna1 to several different mammalian tail-anchored-protein precursors can be detected when membrane integration of the precursors is prevented by the absence of target ER-derived microsomes (Favaloro et al., 2008; Stefanovic and Hegde, 2007). Asna1 binding occurs in the presence of ADP or ATP and requires the TM region of the tail-anchored-protein precursor (see Box 2). Additional evidence for a TM-specific interaction was obtained using yeast Get3 and various yeast tail-anchored proteins in a two-hybrid assay (Schuldiner et al., 2008). Furthermore, Stefanovic and Hegde demonstrated that an ATPase-deficient mutant form of Asna1 has a dominant-negative effect on biogenesis, supporting the theory that there is a functional interaction between tail-anchored proteins and Asna1 (Stefanovic and Hegde, 2007).

A refined EMAP screen has recently been used to identify two additional genes that closely resemble *GET3* in their genetic

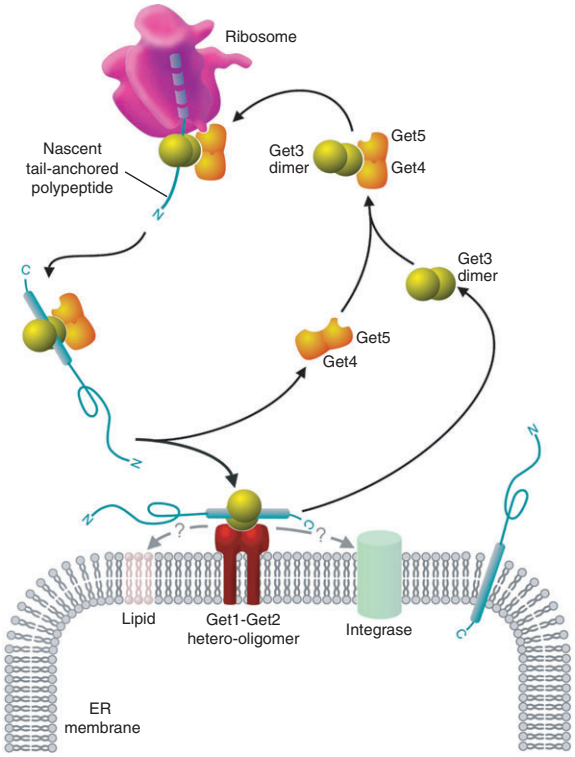

Fig. 4. GET pathway for the biogenesis of tail-anchored proteins at the yeast ER. Get3 is part of a TM segment recognition complex that contains Get4 and Get5 (Jonikas et al., 2009; Schuldiner et al., 2008) and that is most probably conserved in higher eukaryotes (Stefanovic and Hegde, 2007; Favaloro et al., 2008). The integral membrane proteins Get1 and Get2 form a hetero-oligomeric receptor at the ER membrane that binds to Get3 bound to a tail-anchored protein (Auld et al., 2006; Schuldiner et al., 2005; Schuldiner et al., 2008). Whether the subsequent integration of tail-anchored proteins into the lipid bilayer occurs by direct partitioning or via an integrase is currently unknown.

interaction signature (Jonikas et al., 2009). The corresponding proteins interact physically with cytosolic Get3, and were accordingly named Get4 and Get5. Deletion of either *GET4* or *GET5* was shown to interfere with proper targeting of the tail-anchored protein Sed5 to a similar extent as the deletion of *GET3*. Furthermore, cytosol derived from a *GET5* deletion strain was unable to support integration of another tail-anchored protein, Sec22, in vitro. The *GET4* and *GET5* genes appear to be highly conserved, raising the possibility that their counterparts are present in the mammalian TM-recognition complex (see also Stefanovic and Hegde, 2007). Although Get4 has not been functionally characterized, Get5 has been found to associate with Sgt2, a factor involved in the coordination of several more ubiquitous chaperones such as Hsc70 and Hsp90 (Angeletti et al., 2002; Liou et al., 2007). Intriguingly, Get4 and Get5 have been suggested to associate with ribosomes on the basis of a proteomic analysis of yeast ribosome-associated complexes (Fleischer et al., 2006). This raises the exciting possibility that components such as Sgt2 might participate in directing nascent tail-anchored-protein precursors to different pathways that involve either Get3 or Hsp40-Hsc70.

Biochemical studies revealed that an unknown proteinaceous component of the mammalian ER is required for the Asna1-mediated post-translational integration of tail-anchored proteins (Favaloro et al., 2008; Stefanovic and Hegde, 2007). To date, the

Box 3. Asna1 and Get3 ATPases belong to a superfamily of GTPases

Asna1 (TRC40) and Get3 are members of a large class of P-loop GTPases (named SIMIBI for signal recognition particle, MinD and BioD), some of which have acquired ATPase activity during evolution (Koonin, 1993; Leipe et al., 2002). Evolutionary analysis suggests that the subfamily of ATPases to which Asna1 and Get3 belong is of archeal or archeoeukaryotic origin and has sporadically transferred to some bacterial genomes or resistance plasmids (Leipe et al., 2002). The most well-characterised family member, the ArsA ATPase, is found on a plasmid that confers arsenite resistance in *Escherichia coli* (Rosen et al., 1999; Zhou et al., 2000). The *arsA* gene contains a tandem duplication of the NTPase domain that is present in a single copy in most other family members. Detailed biochemical and structural investigation of ArsA has revealed that it contains an oxyanion-binding site, which explains why its ATPase activity is stimulated by arsenite (Kuroda et al., 1997). The exact molecular mechanism by which ArsA confers arsenite resistance is unclear, although it is known that it requires the multispanning membrane protein ArsB, a channel protein that has been proposed to exploit the ATP-powered conformational changes of ArsA for active arsenite extrusion (Kuroda et al., 1997). However, the specific cysteine residues that are involved in metal coordination in ArsA are not conserved in the eukaryotic homologues, and major pathways of arsenite resistance involve members of the ABC transporter superfamily in yeast (Ghosh et al., 1999).

Get3 does not seem to be specifically involved in arsenite resistance (Shen et al., 2003); thus, it is possible that Get3 has undergone evolutionary changes that resulted in its adaptation to a role in tail-anchored-protein insertion. However, there is a twist to this conclusion: despite the loss of the specific oxyanion metal-binding site that is present in ArsA from Get3 and Asna1, both of these eukaryotic family members act as metal stress factors. Intriguingly, the Get3 protein binds to zinc, and both its mobility on non-reducing SDS-PAGE and its subcellular localisation are affected by copper and other metals such as cobalt (Lee and Dohlman, 2008; Metz et al., 2006; Shen et al., 2003). Furthermore, the *GET3* gene is strongly implicated in general metal resistance in yeast (Ruotolo et al., 2008; Schuldiner et al., 2008; Shen et al., 2003). This raises the possibility that Get3 is a multifunctional protein or, alternatively, that tail-anchored protein biogenesis can modulate cellular metal homeostasis. The latter theory would suggest that the metal sensitivity of eukaryotic ArsA homologues serve a regulatory function in the context of tail-anchored protein biogenesis. Intriguingly, Favaloro and collegues reported that the release of RAMP4 from Asna1 is more efficient under reducing conditions, suggesting that there is some form of redox regulation of the GET pathway (Favaloro et al., 2008).

identities of such putative membrane components have only emerged from studies of the yeast system, in which the multi-spanning membrane proteins Get1 and Get2 together form a receptor that recruits Get3 to the ER membrane, resulting in GET complex formation (Schuldiner et al., 2005; Schuldiner et al., 2008). Although the gene encoding the mammalian WRB protein is a good candidate for the orthologue of *GET1,* any functional role in the biogenesis of tail-anchored proteins remains to be established. No obvious *GET2* orthologue has been identified to date, but because both Get1 and Get2 proteins expose coiled-coil-forming domains on the cytosolic surface of the ER, their receptor function could be mediated by proteins that are related in structure but have low or no sequence similarity. Our current model is that Get3 shuttles between a GET complex consisting of Get1, Get2 and Get3, and an alternative cytosolic complex consisting of Get3, Get4 and Get5. Both complexes are required for the normal biogenesis of a spectrum of tail-anchored proteins in vitro and in vivo (Schuldiner et al., 2008). At the same time, genetic analysis of the *GET* genes provides independent evidence that there are additional pathways for the biogenesis of tail-anchored proteins, as outlined below.

Redundant or alternative pathways?

In contrast to many genes that encode tail-anchored protein substrates, the *GET* genes are not essential in yeast [SGD database, http://www.yeastgenome.org (Cherry et al., 1998)]. Given that many tail-anchored proteins are required for cell viability, this observation suggests that their biogenesis can proceed via alternative routes in the absence of Get3. Nevertheless, in vivo experiments in yeast indicate that the GET pathway is particularly important for the high specificity of tail-anchored-protein insertion; in the absence of the GET pathway, tail-anchored proteins normally destined for the ER are mis-targeted to the outer membrane of mitochondria (Schuldiner et al., 2008). Surprisingly, the deletion of *GET1* or *GET2* leads to substantially more severe phenotypes than does the deletion of *GET3* (Auld et al., 2006; Schuldiner et al., 2005; Schuldiner et al., 2008). In the absence of Get1 and Get2, Get3 forms non-productive aggregates with its tail-anchored protein substrates (Schuldiner et al., 2008) and most probably prevents the use of alternative pathways. Clearly, the SRP and Hsp40-Hsc70 pathways described above are obvious candidates for such alternative routes.

The precise relationships between these different routes for biogenesis of tail-anchored proteins are unclear, but the properties of the TM segment, and in particular its hydrophobicity (Box 1) (Ng et al., 1996), appear to promote the use of a particular pathway (Rabu et al., 2008). This effect of tail-anchor composition is presumably a result of the recruitment of different cytosolic factors to the newly made precursor around the time of protein synthesis [but see also Colombo et al. (Colombo et al., 2009)]. In addition, the ribosome might play a crucial role in mediating the selection of specific cytosolic factors by the nascent tail-anchored protein, which would permit the appropriate components to be recruited before the TM region has fully emerged from the ribosomal exit tunnel (Berndt et al., 2009; Bornemann et al., 2008). If Get4 and Get5 are indeed ribosome-associated, as suggested by mass spectrometry-based proteomic screens of yeast ribosomal complexes (Fleischer et al., 2006) (see also Fig. 4), these components are in a perfect position to recruit Get3 to the ribosomal surface. However, any preference by individual tail-anchored proteins for specific cytosolic factor(s) seems to be far from absolute and, for many tail-anchored precursors, there appears to be some degree of redundancy in the mechanisms that mediate their biogenesis.

Notwithstanding the apparent redundancy of the GET pathway in yeast, the fact that mice that lack Asna1 die during embryogenesis implies that there are circumstances in which the Asna1 pathway is essential (Mukhopadhyay et al., 2006). Why might the Asna1 pathway be essential in higher eukaryotes? Multicellular organisms might have evolved tail-anchored protein substrates that are strictly Asna1 dependent, and these particular proteins might play key roles in specialised tissues and/or during development. Alternatively, specialised cells, such as the professional secretory β-cells of the pancreas, might be unable to tolerate even modest perturbations to the biogenesis of key components such as the tail-anchored SNARE proteins. Given the prominent and fundamental roles of SRP in co-translational targeting of proteins to the ER via the Sec61 translocon, and of Hsc70 in protein folding, Asna1-deficient multicellular model

organisms will provide an important platform for tackling the relative physiological contributions of these different pathways for biogenesis of tail-anchored proteins. Therefore, understanding the basis for the essential role(s) of the Asna1-dependent pathway is a prerequisite for understanding the contribution of all of the different pathways operating in vivo.

Conclusions and perspectives

Taken together, the recent studies on the biogenesis of tail-anchored proteins at the ER outlined above have revealed an unexpected level of complexity that is quite distinct from the archetypal co-translational pathway responsible for 'classical' membrane protein biogenesis, where only a single targeting mechanism is apparent (Cross et al., 2009). However, despite significant advances in our understanding of the biogenesis of tail-anchored proteins at the ER, many unanswered questions remain. The reason why such complexity in this process is needed is currently unclear, although one possible advantage is that these multiple pathways could provide a flexible biosynthetic platform that can be selectively regulated through different mechanisms under different physiological conditions. Furthermore, the relative physiological importance of the different pathways is hard to judge, primarily because our analytical approaches exploit in vitro systems or in vivo perturbations. We have little idea of the interaction or overlap between the different pathways, and lack detail regarding the mechanisms by which different cytosolic factors actually promote membrane integration. With the notable exception of the Get1 and Get2 components of the GET complex, we also do not know whether there are ER-localised receptors, and the basis by which a hydrophobic tail anchor is translocated into the phospholipid bilayer during the integration process continues to elude us.

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Note added in proof

Two recent structural studies provide the first insights into the binding of hydrophobic tail-anchor segments by Get3 family members (Mateja et al., 2009; Suloway et al. 2009).

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