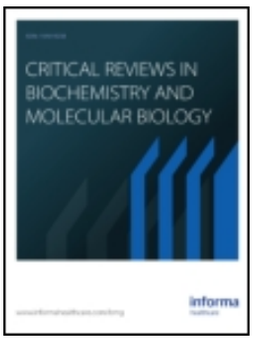

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REVIEW ARTICLE

All roads lead to Rome (but some may be harder to travel): SRP-independent translocation into the endoplasmic reticulum

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Abstract

Translocation into the endoplasmic reticulum (ER) is the first biogenesis step for hundreds of eukaryotic secretome proteins. Over the past 30 years, groundbreaking biochemical, structural and genetic studies have delineated one conserved pathway that enables ER translocation- the signal recognition particle (SRP) pathway. However, it is clear that this is not the only pathway which can mediate ER targeting and insertion. In fact, over the past decade, several SRP-independent pathways have been uncovered, which recognize proteins that cannot engage the SRP and ensure their subsequent translocation into the ER. These SRP-independent pathways face the same challenges that the SRP pathway overcomes: chaperoning the preinserted protein while in the cytosol, targeting it rapidly to the ER surface and generating vectorial movement that inserts the protein into the ER. This review strives to summarize the various mechanisms and machineries which mediate these stages of SRP-independent translocation, as well as examine why SRP-independent translocation is utilized by the cell. This emerging understanding of the various pathways utilized by secretory proteins to insert into the ER draws light to the complexity of the translocational task, and underlines that insertion into the ER might be more varied and tailored than previously appreciated.

Keywords

ASNA1, calmodulin, endoplasmic reticulum, GET, Hsp40/70, Sec62–Sec63, signal recognition particle, TRC40

History

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Introduction

All cells are characterized by an encapsulating membrane, allowing them to define and regulate their internal environment. The concept of functional isolation is further expanded upon in eukaryotic cells, which are also divided into discreet intracellular organelles, thus generating unique biochemical compartments tailored for specific biological reactions. Such lipid barriers both between and within cells are bridged by a vast array of proteins, which transverse the membrane, partially of fully, thereby allowing the cell to selectively pass information and solutes between different compartments.

The first and crucial step in the biogenesis of soluble-secreted and membrane proteins is their translocation through the membrane. While spontaneous membrane insertion has been demonstrated (Brambillasca et al., 2006; Colombo et al., 2009; Tissier et al., 2002), and might have even been a primordial translocation mechanism, it is only effective for short and highly hydrophobic peptides. In fact, the bulk of protein translocation takes place through dedicated and regulated pathways, which require energy expenditure and the action of both cytosolic and membrane factors. Although various mechanisms and routes of translocation exist, they are all characterized by a similar series of steps (Figure 1):

(1) Cytosolic translation of the precursor protein that bears a targeting sequence for its translocational membrane.
(2) Recruitment of cytosolic factors that maintain the protein in a translocational competent state.
(3) Targeting of the protein to its correct site of translocation.
(4) Insertion of the protein, either partially or fully, through the membrane.

Translocation can occur into many membranes, including the bacterial cell envelope, mitochondria, peroxisomes and chloroplasts. However, in this review, we will focus on the pathways that insert proteins into the endoplasmic reticulum (ER), which functions as the entry portal of the eukaryotic endomembrane system. It should be noted that studies of prokaryotic translocation have revealed strikingly similar themes, underlying the fact that all translocational pathways must overcome similar biological hurdles (Cross et al., 2009; Driessen & Nouwen, 2008; Park & Rapoport, 2012; Rapoport, 2007).

Translocational pitfalls – threading the needle

It is estimated that 20–40% of any eukaryotic genome encodes for proteins which must translocate into the ER (Chen et al., 2005; Choi et al., 2010; Wallin & von Heijne, 1998), as they are either secreted, membrane bound or reside within one of the endomembrane compartments. Apart from one subclass

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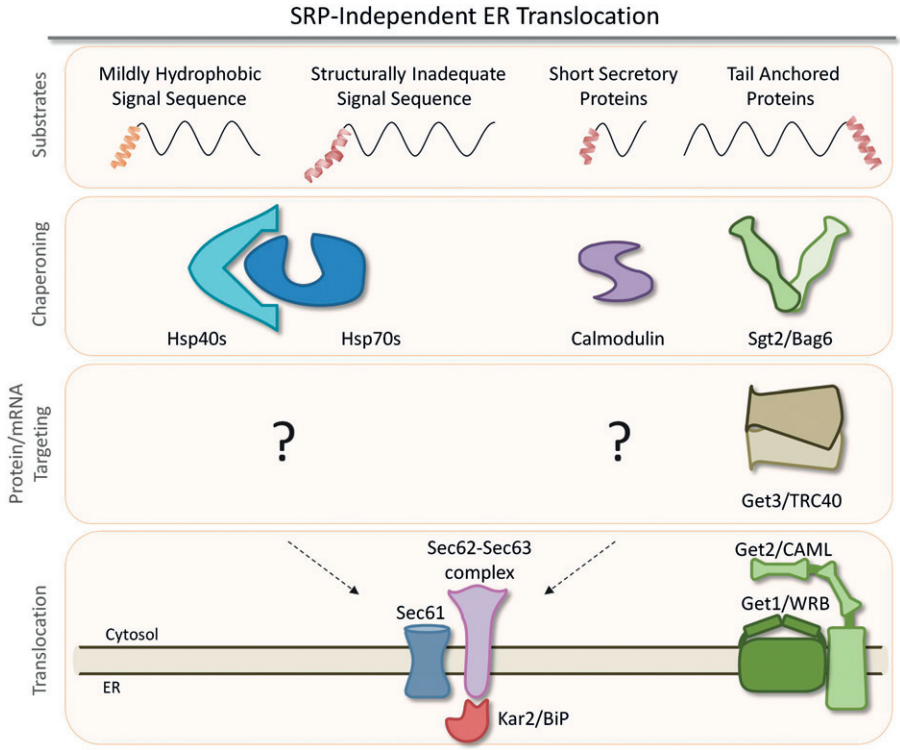

Figure 1. SRP independent pathways employ various cytosolic proteins to chaperone, target and insert their substrates into the ER. Many diverse proteins utilize SRP-independent pathways, including proteins that bear ER targeting sequences that cannot engage the SRP (due to hydrophobicity or structure) or cannot engage the SRP co-translationally (due to their length or location of ER targeting sequence). Upon their translation, these proteins must be chaperoned, so that they do not aggregate and become translocationally incompetent while in the cytosol. For some SRP-independent proteins, an ER targeting pathway has been described that rapidly relays these substrates to the ER, and other pathways most likely exist. Finally, membrane bound machinery mediates the controlled and unidirectional insertion of these substrates into or through the lipid bilayer. It should be noted that SRP-independent substrates can utilize several pathways to mediate their journey from the cytosol to the ER. (see colour version of this figure at www.informahealthcare.com/bmg).

of proteins (see TA proteins below), this protein flux is inserted into the ER through the translocational pore, named the translocon (Gorlich & Rapoport, 1993; Stirling et al., 1992). The translocon is made up of three subunits in eukaryotes – Sec61α, β and γ (Sec61, Sbh1, and Sss1 in yeast) – which together make a protein conducting channel (Park & Rapoport, 2012). The characteristics of this channel dictate the various hurdles that must be overcome in order to maintain efficient translocation (Figure 2).

Creating specificity

A translocon in its ''resting state'', i.e. not participating in protein insertion, forms a plugged pore that maintains the solute barrier between the ER and the cytosol. This plug might be formed by several mechanisms, including the luminal binding of Kar2/BiP chaperone, an internal α-helical domain which acts as a cork, or a gasket-like seal of the residues lining the translocon pore (Hamman et al., 1998; Park & Rapoport, 2011; van den Berg et al., 2004). Upon its cytosolic binding to a hydrophobic segment, characteristic of all ER targeting sequences, the translocon plug is released, thus enabling the translocated protein to transverse the pore freely (Plath et al., 1998). However, the secretory pathway is only one of the many membranes in the eukaryotic cell and organelles such as mitochondria, chloroplasts and peroxisomes also contain proteins bearing highly hydrophobic segments. The ER translocon will support the translocation of a wide range of proteins bearing hydrophobic patches, as attested to by the fact that 20% of random sequences will restore the secretion of a secretory protein lacking its ER targeting sequence (Kaiser et al., 1987). Thus, another layer of cytosolic ''proofreading'' is required to specifically target endomembrane proteins to the ER for translocation.

Preventing protein folding while in the cytosol

Functional studies of the large Sec61α subunit have demonstrated that it forms a dynamic pore through the membrane, whose diameter adjusts between a constricted or translocationally-active open state (Gumbart & Schulten, 2006; Haider et al., 2006; Saparov et al., 2007; Tian & Andricioaei, 2006). However, structural and biophysical analysis of Sec61α has revealed that this pore's diameter does not exceed 15–60 Å, indicating that it cannot accommodate more than a secondary structure during translocation (Hamman et al., 1997; Tsukazaki et al., 2008; van den Berg et al., 2004; Zimmer et al., 2006). In other words, proteins that translocate into the ER cannot fold in the cytosol-dictating that they must either undergo translation proximal to the translocon pore, or once synthesized in the cytosol, be maintained in a loosely folded structure.

Providing a driving force for unidirectional threading

The translocon cannot dictate the directionality of translocation. Indeed, some reports have linked Sec61 to retrotranslocation from the ER lumen to the cytosol (Plemper et al., 1999; Wiertz et al., 1996). Therefore, accessory factors are required to provide the vectorial force that drives the translocation of preinserted proteins. Three possible mechanisms could provide such a driving force (Walter & Johnson, 1994). First, coupling protein synthesis to the translocation process could utilize the GTP-driven translation elongation to direct protein insertion into the ER. Alternatively, a cytosolic, energy consuming factor could engage the preinserted protein at the translocon and push it into the translocational pore. In a complementary approach, a luminal motor could engage proteins as they have translocated and prevent their retrotranslocation to the cytosol. Indeed, all three vector

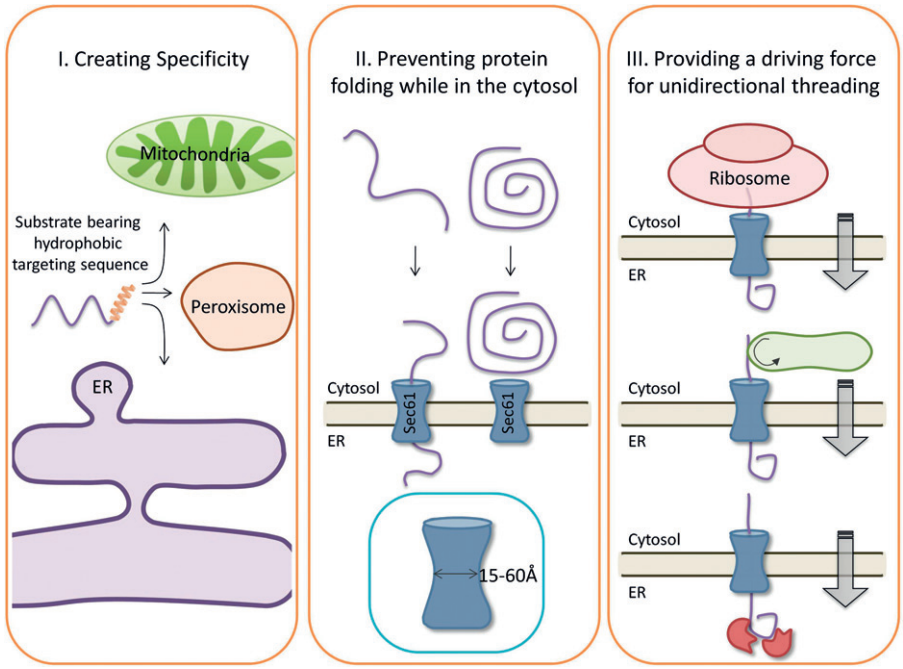

Figure 2. Several hurdles must be overcome by any translocation system to ensure that it functions in an efficient and timely manner. In order to target and translocate any protein through the ER membrane, three central challenges must be overcome. First, many organelles support the translocation of proteins bearing hydrophobic targeting sequences besides the ER, including the mitochondria, peroxisome and chloroplast (not illustrated here). Thus, endomembrane and secreted proteins must be recognized in the cytoplasm and preferentially targeted to the ER. Second, the Sec61 translocon pore has an hourglass shape, which constricts proteins that must undergo translocation to a diameter of 15–60 Å. Therefore, preinserted translocational substrates must retain a loosely folded conformation in the cytosol to efficiently transverse this pore. Finally, the translocon itself does not confer vectorial movement, and therefore, translocation must be coupled to a unidirectional moving force. This force can either be the GTP-dependent elongation of the ribosome, a cytoplasmic factor which drives proteins into the pore, or a luminal factor which traps proteins as they emerged from the translocon. (see colour version of this figure at www.informahealthcare.com/bmg).

generating models can be found in various translocational pathways.

Signal recognition particle – an elegant solution to the translocation riddle

Groundbreaking work in the 1980s established the signal recognition particle (SRP) cycle as a major pathway of protein targeting to the secretory pathway. The SRP cycle was shown to be composed of the ribonucleoparticle SRP and its ER bound SRP receptor (SR) (Gilmore et al., 1982; Walter & Blobel, 1980, 1982). Extensive *in-vivo* and *in-vitro* analysis has delineated the ordered series of events through which the SRP cycle recognizes preinserted endomembrane proteins and conducts their translocation into the ER (Grudnik et al., 2009; Keenan et al., 2001). The SRP cycle is initiated during the translation of substrates, when an ER-specific hydrophobic targeting sequence emerges from the ribosome exit tunnel, and is recognized by the SRP co-translationally (Walter & Blobel, 1981a). Consequently, the SRP dictates a transient elongational pause in the translating ribosome (Walter & Blobel, 1981b), and the ribosome-nascent chain-SRP complex is targeted to a free translocon by virtue of the SRP–SR interaction. Upon binding the SR, the SRP undergoes substantial structural rearrangements, enabling the transfer of the ribosome-nascent chain complex to the Sec61 translocon and the dissolvement of the SRP–SR interaction (Connolly & Gilmore, 1989; Connolly et al., 1991). Thus, the cycle ends as the ribosome resumes translocation directly into the translocon, feeding the protein into the translocation pore aided by the pushing force of GTP-mediated elongation (Crowley et al., 1993), while SRP is free for another round of ER targeting. Interestingly, contradictory genetic data in bacteria (de Gier et al., 1996; Yosef et al., 2010) has generated an alternative interpretation of the SRP cycle, whereby ribosomes are targeted to the ER as they synthesize the SR and later remain on the ER surface translating the local mRNA pool which is enriched for translocational substrates (Bibi, 2012).

While the SRP cycle was initially identified and characterized in mammalian cells, consequent studies revealed that this pathway is conserved in yeast and bacteria (Hann et al., 1989; Poritz et al., 1990). In fact, all sequenced genomes analyzed to date contain genes encoding for SRP and SR homologues (Bohnsack & Schleiff, 2010). This ubiquitous conservation of the SRP cycle has generated the hypothesis that this is a primordial translocation system, perhaps even preceding the requirement for a translocation pore (Ng et al., 2000). However, it should be noted that the SRP has markedly evolved between different organisms (Althoff et al., 1994). Comparative analysis of various SRP homologs has revealed that the common components in all of these ribonucleoproteins are the eukaryotic SRP54 subunit (also called Ffh in prokaryotes), bound to the SRP RNA. The SRP54 subunit has been shown to recognizes the hydrophobic core of ER targeting sequences, through its hydrophobic signal binding cleft (High & Dobberstein, 1991; Janda et al., 2010; Zopf et al., 1990). While the domain generating this cleft is structurally similar in all organisms, it is not identical, suggesting that different SRPs might have altered recognition requirements (Althoff et al., 1994).

By engaging the SRP, proteins that are destined to translocate into the ER gracefully overcome the various translocational hurdles. In coupling translation with translocation, additional energy expenditure for translocation is averted, specificity is ensured and proteins are synthesized directly into their natural milieu, where they can promptly proceed to mature and fold.

SRP-independent translocation – falling off the SRP train?

While the SRP appears to be an ancient and universal pathway, not all ER-destined proteins engage the SRP. The first indications that SRP-independent pathways exist came from yeast and bacterial *in-vitro* translocation systems, which demonstrated that fully-synthesized ribosome-free proteins can also transverse the membrane (Randall, 1983; Waters & Blobel, 1986). Furthermore, some yeast species are viable in the absence of the SRP pathway (Hann & Walter, 1991). While SRP-independent translocation was initially identified and characterized in prokaryotes and lower eukaryotes, studies in higher eukaryotes have also demonstrated that the SRP does not take part in the translocation of all secreted and endomembrane proteins (Johnson et al., 2012; Kutay et al., 1995; Muller & Zimmermann, 1987; Schlenstedt et al., 1990; Shao & Hegde, 2011; Yabal et al., 2003). The SRP cycle hinges on the binding of the SRP to an ER targeting signal during translation. Therefore, SRP-dependent translocation will not take place if an ER-targeting sequence cannot engage the SRP or fails to do so because of physical restrictions of its protein. There are several classes of proteins in which this occurs:

ER-targeting sequences which are not bound by the SRP cleft

ER-targeting sequences encompass cleavable N-terminal signal sequences (SS) and transmembrane (TM) domains termed signal anchors (SA), both of which are defined by a hydrophobic core. In the case of SSs, which are typically 20–30 amino acids (aa) in length, the hydrophobic core is composed of 7–13 aa (Hegde & Bernstein, 2006). SAs are composed of 20–27 hydrophobic amino acids, which span the different membrane widths within the cell (Sharpe et al., 2010). These hydrophobic stretches might preclude SRP binding for different reasons.

Mild hydrophobicity

ER targeting sequences will only engage the SRP binding cleft if they reach some basal interaction threshold, so that both hydrophobicity and the length of the hydrophobic segment affect binding (Hikita & Mizushima, 1992; Yamamoto et al., 1987). In line with these findings, proteins with mildly hydrophobic SS have been shown to utilize the SRP-independent translocational routes in *Saccharomyces cerevisiae* and *Escherichia coli* (de Gier et al., 1998; Lee & Bernstein, 2001; Ng et al., 1996). The application of this hydrophobicity threshold has recently revealed that at least 20% of the yeast secretome is predicted to be SRP-independent (Ast et al., 2013). Paradoxically, synthetic SS with extensive hydrophobic cores are not translocated efficiently *in-vitro* (Hikita & Mizushima, 1992), indicating that there is also an upper boundary of hydrophobicity for the SS.

Structural restrictions of the ER targeting sequences

Studies which have attempted to map the binding requirements of the SRP have uncovered that while ER targeting sequence hydrophobicity is the most effective predictor of SRP engagement, some hydrophobic ER targeting sequences also fail to engage the SRP (Huber et al., 2005; Matoba & Ogrydziak, 1998). A secondary feature which might affect SRP binding is the structure of the ER targeting sequence, as mutations that obstruct α-helix structures have been shown to attenuate SRP engagement (Rothe & Lehle, 1998). Another structural factor which has been linked to the ability of an ER targeting sequence to engage the SRP is the domain's radius of gyration, as a hydrophobic but linear sequence did not engage the SRP (Matoba & Ogrydziak, 1998).

ER targeting sequences which cannot be bound during translation

While there have been some reports that SRP can target proteins to the ER following their release from the ribosome (Abell et al., 2004), the *in-vivo* function of SRP appears to be largely co-translational, as is attested to by the findings that the SRP disassociates from secretory proteins once they have been released from the ribosome (Plath & Rapoport, 2000; Wiedmann et al., 1994). Therefore, proteins whose ER targeting sequence is not exposed to the cytosol during translational elongation will not be targeted by the SRP to the ER.

Short secretory pathway proteins

Structural studies of the ribosome have revealed that the exit tunnel is 80 Å long (Voss et al., 2006), and thus can accommodate a polypeptide chain of 40–50 aa, depending on the chain's secondary structure. Therefore, before the ER targeting sequence of any protein has emerged from the exit tunnel, roughly 70–80 aa of the protein must be synthesized. Proteins shorter than this will have terminated before the SRP can direct the ER targeting of the nascent chain, explaining why short secretory proteins have been shown to utilize SRP-independent translocation pathways (Schlenstedt et al., 1992; Schlenstedt & Zimmermann, 1987; Zimmermann et al., 1990). Studies which have artificially extended the C-terminus of such short secretory pathway proteins have shown that once these physical interaction barriers are removed, the ER targeting sequence of these proteins can indeed engage the SRP (Muller & Zimmermann, 1987).

While short secretory pathway proteins function in key biological processes, such as immunity, toxicity and chemokinesis, they have until recently been considered rare and unusual. This conceptual oversight might be the result of genomic sequencing projects, which set the lower boundary for computational annotation of open reading frames (ORFs) at 100 aa. However, ribosome profiling, studies on evolutionary conservation and other bioinformatic approaches have revealed that between 5% and 10% of any genome is made up of such small proteins (Ingolia et al., 2009; Kastenmayer et al., 2006). It is estimated that in the mouse secretome ∼90 small proteins bear a signal sequence (Frith et al., 2006). Thus, small secretory pathway proteins appear to be more prevalent than previously appreciated and uncovering their mode of translocation is becoming more essential.

Tail anchored proteins

Tail anchored (TA) proteins are characterized by a single TM domain, located at their most C-terminus, so that the

translocated protein is anchored in the membrane with its N-terminus functioning in the cytosol. These proteins partake in various cytosolic processes, including targeting of vesicles within the cell, protein folding and degradation (Borgese et al., 2007; Wattenberg & Lithgow, 2001). As is the case with short secretory proteins, the ER targeting sequence of these proteins (i.e. the most C-terminal TM domain) is still buried within the ribosome exit tunnel once translation has ended, dictating SRP-independent translocational routes (Yabal et al., 2003). It is of interest to note that the TA proteins are distinct from other TM-bearing proteins, in that their translocation into the ER membrane does not involve the Sec61 translocon pore (Lang et al., 2012; Yabal et al., 2003).

SRP-independent translocation – the dark side of the moon

All of the SRP-independent substrates outlined above must consequently rely on alternate pathways to achieve efficient translocation, while dealing with the same difficulties outlined above:

Chaperoning of preinserted substrates

SRP-independent substrates that are destined for insertion are maintained in a translocationally-competent state prior to their arrival at the translocon. While there have been studies showing that some SRP-independent substrates can generate a globularly folded structure in the cytosol prior to translocation (Bush & Meyer, 1996; Paunola et al., 1998), artificially generated SRP-independent substrates which fold rapidly in the cytosol display attenuated translocation kinetics (Hikita & Mizushima, 1992; Huber et al., 2005). Thus, following their release from the ribosome, SRP-independent substrates are rapidly bound by specialized protein factors that shield the various domains of the polypeptide chain from damage and premature folding (Plath & Rapoport, 2000).

Hsp70 chaperones and their Hsp40 co-chaperones

The 70 kDa heat shock proteins (Hsp70s) take part in a wide array of co- and post-translational folding processes, through their ability to engage hydrophobic patches in an ATP dependent manner (Kastenmayer et al., 2006). By repetitive substrate binding and release, the Hsp70 secludes the hydrophobic domains of its substrates, thus preventing protein aggregation and possibly also driving local unfolding (Kastenmayer et al., 2006). However, Hsp70s are inherently weak ATPases, and the Hsp70 by itself cannot maintain continuous cycles of substrate engagement (Blond-Elguindi et al., 1993, Palleros et al., 1993). *In-vivo*, the ATPase activity of Hsp70s is stimulated by their co-chaperones, the Hsp40s, fostering a productive chaperone-substrate cycle (Liberek et al., 1991, Szabo et al., 1994). Thus, the various Hsp40s serve to fine-tune the chaperoning cycle and recruite the Hsp70 to specific folding tasks by virtue of their localization or structure (Sahi & Craig, 2007). In yeast there are 4 Hsp70s and 22 Hsp40s that cover a broad range of cellular roles (Walsh et al., 2004; Werner-Washburne & Craig, 1989,). In humans, this number has predicted to expand to over 40 Hsp70s and hundreds of potential Hsp40s (Brocchieri et al., 2008, Hennessy et al., 2000). Therefore understanding which of these Hsp70/40 pairs plays an important role in translocation is a major challenge.

The first indication that Hsp70s might take part in the preinsertional chaperoning of SRP-independent substrates came from parallel fractionation and genetic experiments in yeast (Chirico et al., 1988, Deshaies et al., 1988). These studies demonstrated that, in extract, Hsp70s are recruited to SRP-independent proteins with mildly hydrophobic SSs and are required for their efficient translocation. This *in-vitro* dependence on Hsp70s could be bypassed by preincubating the preinserted proteins in urea (Chirico et al., 1988), further stressing the role of these chaperones in maintaining the loosely folded preinsertional conformation. It should be noted that Hsp70s cannot restore the translocation potential of aggregated preinserted proteins, but rather seem to work as ''holdases'' until these proteins have reached the translocon (Ngosuwan et al., 2003). Subsequent genetic work in yeast identified one cytosolic Hsp40 which recruits Hsp70s to this pretranslocational task – the ER bound Ydj1 (Becker et al., 1996; Caplan et al., 1992). The systematic deletion of all nonessential cytosolic Hsp40s in yeast has recently revealed that two other Hsp40s, namely Apj1 and Jjj3, might also mediate the preinsetional chaperoning of SRP-independent proteins (Ast et al., 2013). The role of the Hsp70/40 cycle is not restricted to yeast, as it has also been linked to SRP independent substrates in *E. coli* as well as TA and short secretory protein biogenesis in higher eukaryotes (Hendrick et al., 1993; Rabu et al., 2008; Zimmermann et al., 1988), highlighting this chaperoning route as a central link in the SRP-independent translocational pathway.

Calmodulin

Recent fractionation assays on short secretory proteins in mammalian cells have identified calmodulin as a preinsertional-binding factor which promotes the translocation of these SRP-independent substrates (Shao & Hegde, 2011). Calmodulin is an intracellular calcium sensor found in all eukaryotic cells, which undergoes calcium-induced conformational changes regulating processes such as growth, movement and proliferation (Chin & Means, 2000). In cells depleted of calmodulin, small secretory proteins were rerouted from translocation to off-target pathways, such as degradation, indicating that calmodulin might be the major preinsertional chaperone for this protein family in mammals. Calmodulin was shown to be recruited to these small proteins by binding their SS, consistent with previous findings that calmodulin can interact with the SS of pre-prolactin and HIV-1 p-gp160 (Martoglio et al., 1997). Furthermore, calmodulin also appears to bind mammalian TA proteins in a calcium dependent manner, although this interaction inhibits TA protein translocation (Colombo et al., 2009).

Calmodulin has been previously shown to engage amphiphatic α-helixes that are 16–35 aa long, with little sequence specificity (O'Neil & DeGrado, 1990). Structural work has shown that the substrate binding pocket on calmodulin is characterized by large hydrophobic patches, flanked by regions of highly negative electrostatic potential explaining its substrate preference (Babu et al., 1988; Kretsinger et al., 1986). This structure demonstrates that calmodulin can

effectively shield the hydrophobic SS in the unnatural cytosolic environment, thus precluding degradation or aggregation. While based on this structure it is tempting to speculate that other secretory pathway proteins might also engage this pathway for preinsertional chaperoning, the generality of this pathway remains to be verified. Furthermore, the interplay between calmodulin's calcium sensing role and its translocational responsibilities are unclear as of yet. Interestingly, large-scale immuneprecipitation studies of calmodulin in yeast uncovered that it interacts with the SR (Zhu et al., 2001), hinting that this protein might also take part in a targeting mechanism.

Sgt2/Bag6

Sgt2 is a small glutamine-rich tetratrico-peptide repeat (TPR) containing protein found in yeast and highly conserved across eukaryotes (Angeletti et al., 2002; Kordes et al., 1998). Recent genetic and biochemichal work has identified Sgt2 as both a chaperone and scaffold protein, which can capture TA proteins as they are released from the ribosome. Sgt2 seems to be the first partner in the TA protein translocation pathway, also termed the GET pathway, handing these substrates off to subsequent partners (Wang et al., 2010). The C-terminus of Sgt2 is rich in methionine residues, a feature which is prevalent in hydrophobic interaction domains, while its N-terminus binds the Get4–Get5 complex (Liou & Wang, 2005; Simon et al., 2013; Wang et al., 2010). In turn, the Get4–Get5 complex serves as a mediator, recruiting the Get3 targeting factor that relays secretory pathway TA proteins to the ER surface (Jonikas et al., 2009; Schuldiner et al., 2008).

Once an endomembrane TA protein is released from the ribosome, it is rapidly bound by Sgt2, thereby preventing its aggregation in the cytosol or mistargeting to mitochondria (Wang et al., 2010). The Get4–Get5 complex raises the local concentration of Get3 found around Sgt2, while at the same time priming the Get3 for receiving TA proteins (Chartron et al., 2010, 2011). Thus, the handoff of the TA protein from Sgt2 to Get3 is achieved, gracefully linking the chaperoning and targeting stages of this pathway.

In mammals a similar relaying of the TA protein takes place, whereby nascent TA proteins are captured by an analog of Sgt2–Bag6. Bag6 has been shown to bind translating ribosomes, and hence is effectively positioned to capture the TM domain post-translationally (Leznicki et al., 2010; Mariappan et al., 2010). TA proteins bound by Bag6 are then fed to the mammalian homologue of Get3-TRC40. This handoff is also bridged by a mediator complex, TRC35 and Ubl4A, homologous to the Get4–Get5 components (Mariappan et al., 2010; Wang et al., 2011). Interestingly, Bag6 does not appear to have a yeast homologue. The mammalian homologue of Sgt2–SGTA also recognizes TM domains and binds Bag6 (Leznicki et al., 2010; Winnefeld et al., 2006), however it appears to have evolved to regulate off-target pathways of preinserted TA proteins (Leznicki & High, 2012).

While recent years have exponentially expanded our knowledge regarding the roles of Sgt2 and Bag6 in TA protein translocation, these proteins might have broader cellular functions. The TPR domain in Sgt2 has been shown to recruit Hsp70s and Hsp90s (Angeletti et al., 2002), although this interaction is not required for efficient TA protein translocation (Wang et al., 2010). This interaction between Sgt2 and Hsp70s serves to recruit chaperones to sites of non-TA prion protein aggregation (Kiktev et al., 2012). Furthermore, the Bag6-SGTA complex appears to function in the targeting of both nascent preinserted secretory pathway and ER retrotranslocated proteins to degradation (Hessa et al., 2011; Leznicki & High, 2012; Xu et al., 2012; Wang et al., 2011). These results hint at a more global role for this conserved chaperone in triaging cytosolic secretory pathway proteins to their various intracellular fates.

Targeting preinserted substrates to the ER

Targeting at the mRNA level

Until recently, the ER localization of mRNAs encoding for secretory pathway proteins was presumed to be a secondary effect of the SRP cycle: as the SRP docks the ribosome-nascent chain complex onto the translocon, the mRNA is a passive passenger of this targeting step. However, recent systematic studies looking into mRNA localization within the cell have overturned this assumption. While the cytosolic face of the ER is enriched for mRNAs encoding for endomembrane proteins, these were not the only proteins enriched on the ER surface, which also included transcripts of cytosolic and nuclear proteins (Diehn et al., 2000, 2006; Reid & Nicchitta, 2012). Strikingly, knocking down SRP subunits did not globally alter this localization pattern, although translocation was impaired (Pyhtila et al., 2008; Ren et al., 2004). Furthermore, halting translation does not affect the membrane targeting of RNAs encoding for several endomembrane proteins (Nevo-Dinur et al., 2011). This translation-independent membranal localization of mRNA is abolished when the membranes are pretreated in basic pH, indicating the involvement of a membrane bound factor in this phenomenon (Pyhtila et al., 2008). These findings shed light on an alternate pathway that might target endomembrane proteins to the ER.

It is still unclear which sequence factors possibly target mRNAs to the ER. The study of specific secretory pathway proteins such as Pmp1 and Hac1 has revealed a role for sequences in the 3'UTR in directing ER localization (Aragon et al., 2009; Loya et al., 2008). More systematic analyses have uncovered that the transcript encoding for membrane proteins are significantly U-rich, while signal sequence encoding segments are A-poor (Palazzo et al., 2007; Prilusky & Bibi, 2009). This would suggest that specific protein factors exist that bind such motifs. As for ER localized mRNA-binding proteins, to date several have been identified (Cui et al., 2012, Frey et al., 2001; Furic et al., 2008; Gelin-Licht et al., 2012; Schmid et al., 2006), principally in yeast, and their recognition motifs have been characterized. High throughput bioinformatic identification of all mRNAs that can bind to a RNA-binding protein has remained problematic, as the recognition sequences tend to be promiscuous and often a structure is recognized and not a sequence (Kraut-Cohen & Gerst, 2010). Thus, only a handful of mRNA targets are known for each RNA-binding protein. However, recent biochemical work in metazoans has uncovered a potential

role for the ER-bound protein, p180, in mediating general mRNA binding (Cui et al., 2012). p180 was originally identified as a potential ribosome receptor at the ER (Savitz & Meyer, 1990), and its overexpression promotes the ER-mRNA association. Thus it appears that there is a wide variety of mRNA targeting routes to the ER, encompassing both general and specific mRNA transcripts, possibly ensuring that the protein is synthesized proximally to its site of translocation.

Get3/TRC40

In the past 5 years, a powerful mixture of yeast genetics and *in-vitro* translocation assays has unraveled a long-standing scientific riddle as to the ER targeting and insertion mechanism of TA proteins. These complementary approaches have placed the yeast Get3 and its mammalian homologue TRC40 at the heart of this pathway. Several independent lines of investigation identified this cytosolic 40 kDa ATPase to bind to nascent TA proteins, and mediate their targeting to the ER (Favaloro et al., 2008; Schuldiner et al., 2008; Stefanovic & Hegde, 2007). In the absence of this factor, TA proteins accumulate in cytosolic aggregates in yeast (Schuldiner et al., 2008). Structural work has demonstrated that Get3 functions as a dimer which transitions between an ''open'' and ''closed'' state driven by ATP hydrolysis. In the open state the Get3 dimer creates a large hydrophobic groove that most likely envelopes the TM domain of the TA protein (Hu et al., 2009; Kubota et al., 2012; Mateja et al., 2009; Suloway et al., 2009). Following its binding to a nascent TA protein through its indirect interaction with Sgt2, Get3 is recruited to the ER membrane by the transmembranal Get1–Get2 complex (WRB and CAML in mammals), where it releases its protein load onto the ER membrane (Schuldiner et al., 2008; Vilardi et al., 2011b; Yamamoto & Sakisaka, 2012). It appears that the binding of Get3 to the Get4–Get5 relaying factors as well as its subsequent interactions with Get1–Get2 might take place through a similar motif on both protein complexes, hinting at parallel mechanisms inducing the uptake and release of TA proteins by Get3 (Chang et al., 2012; Stefer et al., 2011).

Recently, the possible cellular role of Get3 has been expanded, when mammalian short secretory proteins were shown to bind TRC40 as an ER targeting factor, via their SS (Johnson et al., 2012). While it appears that these TRC40-bound short secretory proteins also rely on the WRB receptor, it remains to be clarified whether upstream factors such as Bag6 are also required for these substrates. It should be noted that the immunodepletion or the inhibition of TRC40 did not halt the translocation of small secretory proteins, demonstrating that there are several overlapping ER translocation pathways for these substrates. Furthermore, Get3 has also been shown to bind and target the SRP-independent family of glycophosphatidylinositol-anchored proteins to the ER in yeast (Ast et al., 2013). Thus, it appears that Get3 has a much broader binding range than previously appreciated, enabling it to partake in the ER targeting of varied SRP-independent substrates.

Translocation across the ER membrane

Following the relaying of endomembrane proteins to the cytosolic leaflet of the ER, novel translocation components must take over to free the substrate and allow it to insert into the lipid bilayer. Although spontaneous insertion is possible for hydrophobic polypeptides (Colombo et al., 2009; Brambillasca et al., 2006; Tissier et al., 2002), this process is unregulated and inefficient. Therefore, the channel forming translocons together with accessory factors as well as the Get1–Get2 complex are essential for this process.

Sec61 with the Sec62–Sec63 complex

The molecular details of the SRP-independent membranal machinery were first elucidated in yeast, where conditional lethality screens assaying for protein translocation identified components of this essential pathway (Deshaies & Schekman, 1987, 1989; Rothblatt et al., 1989, Sadler et al., 1989). SRP-independent substrates were shown to translocate through the Sec61 translocon, when it was in association with an auxiliary tetrameric complex (Panzner et al., 1995). This auxiliary complex was then shown to be made up of the essential Sec62 and Sec63 and the nonessential Sec66 (also termed Sec71) and Sec72 proteins (Deshaies et al., 1991; Feldheim et al., 1993; Green et al., 1992; Panzner et al., 1995). Although the majority of the functional domains of the tetrameric complex reside in the cytosol (Deshaies & Schekman, 1990, Feldheim et al., 1992, 1993, Green et al., 1992), it can also recruit the luminal Hsp70 – Kar2 (the yeast BiP homologue) – through a J-domain which is found on the luminal loop of Sec63 (Brodsky & Schekman, 1993; Sadler et al., 1989).

While the precise role of each protein in SRP-independent translocation remains obscure, the general scheme assuring that these substrates are threaded through the Sec61 translocon has been well outlined. SRP-independent translocation can be roughly divided into two stages – protein recruitment to the translocon followed by a ratchet-like insertion process. First, upon its arrival at the ER membrane, the SS of SRP-independent substrates is recognized by the Sec61 translocon, as well as Sec62 and Sec66 (Plath et al., 1998). This interaction data is supported by recent structural work on the SRP-independent translocon, which shows that Sec62 and Sec66 are found adjacent to Sec61 (Harada et al., 2011). Therefore, Sec62 and Sec66 might play a role in directing or reinforcing the Sec61-substrate interaction on the cytosolic leaflet. Upon its binding to the translocon, the SRP-independent substrate has been shown to shed all cytosolic chaperones (Plath & Rapoport, 2000), freeing it for linear threading through the pore. Sec72 contains a tetratricopeptide repeat (Schlegel et al., 2007), a motif which binds Hsp70s/90s and might mediate this shedding process.

While the SS seems to be held in the ER for the extent of translocation (Plath et al., 1998; Shaw et al., 1988), the rest of the SRP-independent substrate is inserted into the ER by Brownian movement, which confers small-scale motility. However, Brownian motion is not directional, and can result in backsliding of the substrate into the cytosol. To overcome this, whatever segment of the substrate has been inserted into the ER lumen is rapidly bound by Kar2 (Matlack et al., 1999), which is too large to undergo retrotranslocation through the pore. Kar2 is ideal for binding the wide range of translocating proteins as it has low sequence specify when activated (Misselwitz et al., 1998). Sec63 is essential in this Kar2

ratcheting phenomenon, as it both ensures a high local concentration of Kar2 and induces the closure of the peptide-binding pocket of Kar2 around the translocating substrate, through its J domain (Corsi & Schekman, 1997; Matlack et al., 1999). Thus, it seems that a key task of this tetrameric complex is to rapidly engage substrates on the cytosolic surface and ratchet them into the ER lumen.

While the Sec62–Sec63 complex has been primarily studied in the context of SRP-independent translocation, it also partakes in other cellular processes that require the translocon. Sec63-dependent Kar2 ratcheting also functions in enabling the initial substrate insertion steps of SRP-dependent translocation in yeast and higher eukaryotes, although this function is independent of Sec62 (Brodsky et al., 1995; Tyedmers et al., 2003; Willer et al., 2003a; Young et al., 2001). Furthermore, several putative Sec62–Sec63 accessory proteins have been identified through protein–protein interaction studies in yeast, such as Hph1, Hph2, Yet1, Yet3 and Ylr301w (Pina et al., 2011; Willer et al., 2003b; Wilson & Barlowe, 2010). However, none of these accessory factors affect the kinetics of SRP-independent translocation for model proteins. Therefore, it appears that these novel factors mediate the translocation of a specific subset of proteins or act in a regulatory capacity, possibly fine-tuning the translocational apparatus to the substrate requirements.

Comparative genomics have revealed that the Sec62 and Sec63 genes are conserved in higher eukaryotes, and these proteins were found to complex with mammalian Sec61 (Meyer et al., 2000; Tyedmers et al., 2000). Initial indications that these homologues have retained their cellular function came from cancer linkage studies, which showed that mutations in Sec63 or Sec62 that cause overexpression are associated with tumors in the gastric, colorectal or prostate systems (Davila et al., 2004, Eschrich et al., 2005, Jung et al., 2006, Mori et al., 2002, Schulmann et al., 2005). Recent studies of cell-lines lacking either Sec63 or Sec62 have substantiated a role for these proteins in mammalian translocation. These studies have uncovered that human Sec62 is required for the efficient translocation of small secretory proteins(Lakkaraju et al., 2012; Lang et al., 2012), while Sec63 is linked to a subset of SS bearing proteins, among them the prion protein PrP (Lang et al., 2012). Furthermore, human Sec63 appears to be a negative regulator of multi-spanning membrane proteins, in a Sec62 independent manner (Mades et al., 2012). Thus, the yeast and mammalian Sec62 and Sec63 are similar in many ways, indicating that they have maintained some of their translocational functions and substrates. However, these proteins have evolved between yeast and man, as is underscored by the fact that the N-terminal domain of mammalian Sec62 was shown to interact with the ribosome exit tunnel (Muller et al., 2010), a function not shared by its yeast counterpart. Therefore, more work is required to understand how the Sec62–Sec63 complex helps maintain the translocational flux at the functional and regulatory levels in health and disease.

Alternate translocons

In addition to Sec61, yeast contain a second translocon which can function as an insertion channel into the ER (Finke et al., 1996). In contrast, higher eukaryotes, such as flies, mice and humans, contain two highly similar copies of the Sec61 translocon (termed Sec61A1 and Sec62A). The main subunit of this alternate translocon is the Ssh1 protein, which bears ~30% identity with the yeast Sec61 and canine Sec61α sequences. Similar to Sec61, Ssh1 is found in a complex with two other subunits – Sbh2 (which is ~50% identical to Sbh1) and Sss1 (that is common to both translocons). While structural studies have shown that Ssh1 is indeed akin to Sec61 (Becker et al., 2009), Ssh1 is not essential for viability (Finke et al., 1996). However, strains that bear both a deletion of the *SSH1* gene as well as a temperature sensitive allele of the Sec61 translocon were much more susceptible to temperature fluctuations than strains bearing either of these mutations alone. Furthermore, some SS bearing reporter proteins interact with both the Sec61 and Ssh1 translocon, although others were unique to Sec61 (Wittke et al., 2002). Therefore, it was suggested that the Ssh1 translocon functions as a backup mechanism, possibly mediating translocation when Sec61 is overloaded or compromised. However, recent crosslinking work has revealed that there is at least one substrate which is preferentially translocated through the Ssh1 translocon, namely Sec66 (Spiller & Stirling, 2011). Only in the absence of Ssh1 will Sec66 engage the Sec61 translocon, indicating that while there might be functional overlap between the two translocons, some substrates might be primarily sorted and translocated through one or the other.

Currently, it is unclear whether the Ssh1 translocon can associate with previously characterized translocation accessory factors, such as the Sec62–Sec63 complex. While pull downs of the Ssh1 components did not reveal any binding to these factors (Finke et al., 1996), the bona-fide substrate of the Ssh1 complex, i.e. Sec66, is dependent on Sec63 for translocation (Spiller & Stirling, 2011). In light of the dynamic interchange uncovered between these two translocons, it appears that more delicate tools will be required to understand the relative translocational contribution of Sec61 and Ssh1.

Get1/WRB and Get2/CAML

Get3, loaded with a TA protein, is targeted to the ER by virtue of its interaction with an ER receptor complex, made up of Get1 and Get2 (Schuldiner et al., 2005, 2008). Get1 and Get2 are membrane bound proteins, which mediate the docking and substrate release phases of TA-bound Get3. It is not known if the Get1–Get2 complex functions as a heterodimer or a hetrotetramer, although the symmetrical structure of the Get3 dimer might favor the latter. Several recent structural studies have elegantly uncovered the mechanism through which the Get1–Get2 complex interacts with Get3 (Kubota et al., 2012; Mariappan et al., 2011; Stefer et al., 2011). It appears that Get2 initially recruits Get3 from the cytosol, through a long and flexible cytosolic arm, thus docking Get3 at the complex and elevating the local concentration of Get3 (Mariappan et al., 2011; Stefer et al., 2011). Interestingly, Get2 binds to the closed conformation of Get3, explaining how futile cycles of free Get3 recruitment are avoided.

After the initial docking of Get3 at the complex, Get1 binds to Get3 through two rigid coiled coils that are proximal to the membrane (Mariappan et al., 2011; Stefer et al., 2011). The Get1–Get3 interaction serves to both align the TA protein in close proximity to the membrane and forces Get3 into an open conformation (Kubota et al., 2012), releasing the substrate from the protein-binding groove. The conformational switch between the Get3 open and closed state is mediated by ATP hydrolysis, which Get1 might catalyze as its coiled coil domain interacts with Get3s nucleotide-binding pocket. Finally, the release of Get3 from the membrane appears to be mediated by the recruitment of a new ATP molecule by Get3, which competes for Get1 binding. Following this step, the GET pathway is free to relay another TA protein to the ER membrane.

In comparison with the model recently achieved for Get1–Get2 function, our understanding of the mammalian system is lagging behind. WRB, the ER localized mammalian homologue of Get1, has been shown to bind to TRC40 through a coiled coil motif (Vilardi et al., 2011a). Identifying the counterpart of Get2 proved to be more problematic, possibly because of the intrinsically unstructured nature of its cytosolic binding arm. However, recent work has identified CAML as the missing link that both binds WRB and mediates the insertion of mammalian TA proteins from TRC40 (Yamamoto & Sakisaka, 2012). Thus, it appears that the major steps in TA protein targeting to the ER are conserved from yeast to higher eukaryotes.

One question which has yet to be answered is how TA proteins, once at the ER membrane, insert into the lipid bilayer, as this step is known to be independent of Sec61 (Yabal et al., 2003). One suggestion is that TA insertion is spontaneous, and the GET pathway simply targets the secretory pathway proteins to the correct intracellular membrane. This hypothesis is supported by the fact that some TA proteins insert efficiently into liposomes, which lack any protein component (Brambillasca et al., 2006). Furthermore, it is known that the insertion of TA proteins is amenable to changes in membrane lipid composition (Brambillasca et al., 2005; Krumpe et al., 2012), and unassisted insertion agrees with thermodynamic constraints (Leznicki et al., 2011). Another option put forward is that the TM domains of Get1 and Get2 play a role in this insertion process, either by forming a channel or by wedging the TM domain into the membrane through local membrane distortion. This option is supported by the fact that spontaneous insertion might have undesirable results, such as recognition for degradation or aggregation. As tools for studying membrane insertion have already been developed for Sec61-dependent proteins, this last stage of the puzzle is ripe for analysis.

SRP-independent translocation – why has it stuck around?

The SRP pathway presents a straightforward solution to the translocation conundrum – by coupling the translation of a secretory pathway protein to its translocation, the cell ensures that the protein is always found in its natural environment. In contrast, SRP-independent translocation appears to require the presence of several cytosolic partners that divert a significant amount of attention and energy to the tasks of chaperoning and targeting these precursor proteins. Moreover, the SRP-dependent pathway appears to mediate translocation at a faster rate, as translation is estimated to proceed at 5–10 aa/s (Waldron et al., 1977), while models of the SRP-independent Sec62–Sec63 pathway suggest that it translocates ~1 aa/s (Liebermeister et al., 2001). Finally, the translocation of SRP-independent proteins into the ER lumen appears to be more energetically costly for the cell, as it entails an ATP-driven ratcheting mechanism, and does not harness the elongation force. As the SRP pathway is ubiquitous across the tree of life, one immediate question is presented: Why has SRP independent translocation remained?

One could argue that for proteins whose structure prevents binding to the SRP, such as short secretory or TA proteins, this pathway is an innate default. However, previous studies have shown that these structural ''shortcomings'' can be overcome by simple elongation – assuring that the ER targeting sequence will exit the ribosome before the termination of translation. Another possibility is that these proteins are merely in ''evolutionary limbo'', whereby they will ultimately develop to bind the SRP. At least for mildly hydrophobic SS bearing proteins, this does not appear to be the case. Although there is very little conservation between different SSs (von Heijne, 1985); SSs have been shown to evolve slowly, indicating a stabilizing selection force (Kim et al., 2002; Williams et al., 2000).

Together, these findings suggest that while the SRP is an essential and key component of translocation, alternate pathways might also confer a selective advantage for a subset of the secretome. While this theory has not been extensively studied, the potential advantages of SRP-independent translocation are highlighted in studies examining the functional and regulatory aspects of these pathways (Figure 3).

Regulatory control

Uncoupling translation and translocation and introducing additional mediating factors expands the number of potentially regulated steps between the biogenesis and ER insertion of an endomembrane protein. This might allow for a more delicate and rapid sensing and reaction mechanism between the cytosol and ER, which would, in turn, modulate the flux of proteins between these two compartments in accordance with their various requirement or the ERs changing capacities.

ER unfolded protein stress

The ER constitutes the primary site of protein folding in the secretory pathway, so that proteins targeted to subsequent organelles have reached their correct tertiary and quaternary structure most often before leaving its lumen. While this is an efficient process, the ER's capacity for protein folding can be overwhelmed, either due to an extensive inwards flux of proteins or environmental perturbations, resulting in misfolded proteins accumulating within the ER lumen (Kozutsumi et al., 1988; Ron & Walter, 2007). Under these conditions, it would be beneficial for the ER to halt the

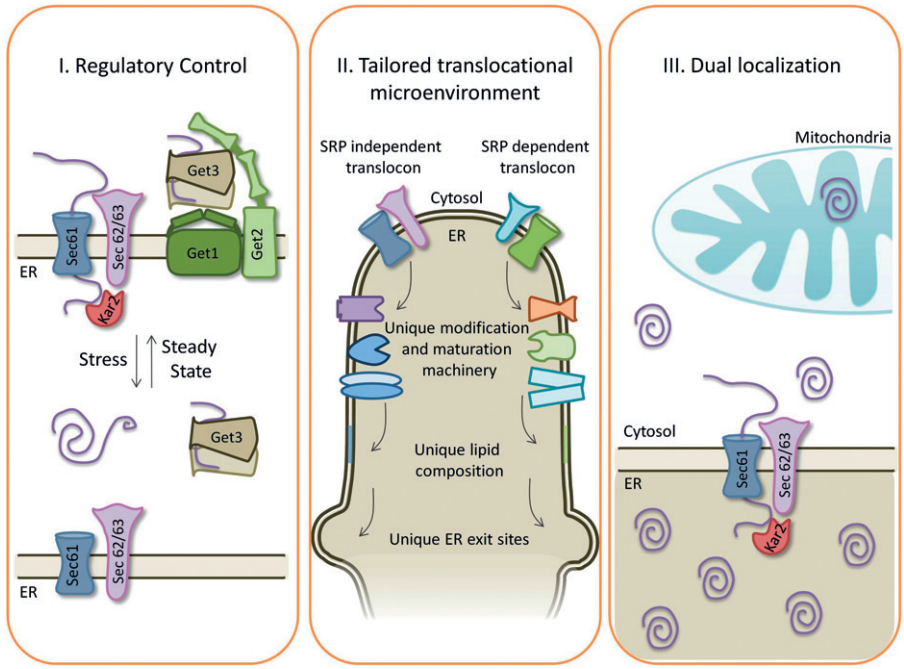

Figure 3. SRP-independent translocation might impart distinct regulatory and functional advantages. There are several potential benefits that might result from possessing and maintaining SRP-independent substrates. First, as the SRP-independent pathway is more costly, it might be more attune to stresses in the cell, thus allowing it to rapidly regulate the translocational influx. During unfolding or energy stress, targeting and translocating machineries are diverted from their steady state tasks, thereby quickly halting the translation and folding burden on the ER. Second, as there is a notable difference in the abundance of the translocational and modificational machinery within the ER, the presence of several translocational routes might generate microdomains within the ER specialized to distinct translocational clientele. Thus, protein maturation and perhaps exit from the ER might be tailored to the various translocational substrates. Third, as SRP-independent translocation allows the preinserted protein to reside within the cytosol, it might represent a preferential pool of dually targeted proteins. In this scenario, apart from their function in the endomembrane system, a small part of the SRP-independent substrates might also function in other organelles, such as the cytoplasm or mitochondria. (see colour version of this figure at www.informahealthcare.com/bmg).

insertion of novel secretory pathway proteins and deal with the misfolded proteins at hand. One potential mechanism governing this interchange could be a limited pool of luminal chaperones, such as Kar2/BiP, which would mediate both protein folding and translocation. Thus, the luminal chaperone would be titrated away from translocation should the folding burden take precedence, providing a rapid translocational halting mechanism. Indeed, such a preemptive quality control pathway has been defined for the prion protein, PrP and other proteins with ''weak'' SS, which were shown to be rerouted to cytosolic degradation just minutes after the induction of ER misfolding stress (Kang et al., 2006). In fact, artificially rerouting PrP to the ER lumen under these conditions resulted in its aggregation. Moreover, this artificially rerouted PrP caused such excessive luminal burden that the cells expressing it failed to recover from the folding offense. As SRP-independent proteins are predominantly reliant on the chaperone ratcheting mechanism to enter the ER, they are the primary candidates for this pre-emptive quality control pathway. Furthermore, studies of misfolded stress in yeast have shown that the luminal chaperone Kar2 is indeed a limiting factor, as its overexpression can alleviate translocational defects in known SRP-independent targets (Ng et al., 2000). Thus, it seems that translocation and the ER's processing capacity are tightly intertwined for SRP-independent proteins.

Energy depletion stress

SRP-dependent and -independent pathways involve different energy expenditures while mediating translocating proteins into the ER. The SRP pathway relies primarily on the ribosomal GTP-expenditure and is itself regulated by GTP as both the SRP and the SR are GTPases (Rapiejko & Gilmore, 1997). In contrast, the SRP-independent pathways require additional steps of ATP hydrolysis. A ready ATP energy source is required for the chaperoning action of the Hsp70s, the Sec62–Sec63/Kar2 ratcheting mechanism and TRC40/Get3 regulation. Thus, it is possible that the SRP-independent pathways are more finely tuned to the energy reserves of the cell. This correlation would explain why a fusion protein targeted to the ER with an SRP-independent SS accumulated in the yeast cytosol during stationary phase, when nutrient stores were depleted (Wang & Da Silva, 1993). This cytosolic accumulation could be overcome if a carbon source was reintroduced to the media. Moreover, recent work has uncovered that upon glucose starvation, Get3 acts as a ''holdase'' which moves with TA proteins to cytosolic aggregation sites (Powis et al., 2012). Thus, Get3 appears to segregate these substrates to defined cytosolic quality control compartments under conditions that do not support their insertion. Taken together, these results argue that the SRP-independent pathway, by virtue of its more pronounced dependence on ATP-consuming factors, might attenuate the flux of proteins into the secretory pathway under unfavorable energetic conditions.

Tailored translocational microenvironment

Upon its insertion into the ER lumen, a secretory pathway protein is rapidly assaulted by an army of ER machineries that mediate its folding and modification. These reactions often occur co-translocationally, and include achieving the correct orientation of TM domains, the removal of a SS if present, the introduction or corrections of disulfide bonds, the addition of N- or O-linked glycan trees, the addition of glycan-lipid modifications such as glycophosphatidylinositol anchors, and folding quality control. These complex tasks require a myriad

Table 1. Abundance of components of the translocation and modification machinery.

	Protein	Abundance	
Name*	Function	TAP library**	GFP library***
Sec61p	Translocon	24 800	2664.0
Sec11p	Signal peptidase	3150	204.5
Stt3p	N′-linked glycosylation	3000	364.3
Pmt1p	O′-linked glycosylation	41 500	93.5
Pmt2p	O′-linked glycosylation	6510	58.9
Pmt4p	O′-linked glycosylation	1270	51.1
Gpi8p	GPI anchoring	1560	142.5

*Each protein is the central/catalytic subunit of the process.
**Ghaemmaghami et al. (2003).
***Newman et al. (2006).

of ER-bound and luminal factors, which recognize and carry out the maturation requirements of each inserted protein. Further complicating this picture, the catalytic subunits of the various complexes carrying out these reactions are an order of magnitude less abundant than the translocon (Table 1). Thus, despite the empirically tight link between translocation and maturation, it appears impossible that every translocon is constantly being monitored by the various modification complexes.

One mechanism which might overcome this ratio barrier is the division of substrates between the translocons so that proteins with similar modificational needs are inserted through specific translocons. Indeed, there is evidence indicating that different translocon species exist. In yeast, the Sec61 translocon is equally distributed between three protein fractions – ribosome bound and unbound fractions as well as a Sec62-Sec63 bound form (Panzner et al., 1995). Furthermore, the SRP receptor does not seem to be in the proximity of the Sec62–Sec63 complex, as assayed by a spilt ubiquitin system (Wittke et al., 2002). Thus, it appears that the SRP-dependent and independent pathways are indeed spatially distinct. In addition, the Ssh1 translocon also mediates SRP dependent, and perhaps also independent, translocation. Thus, four potential translocons exist in yeast. This theme of distinct translocons also appears to hold true in higher eukaryotes, where translocons engaged with the Sec62–Sec63 complex do not interact with the SRP receptor (Meyer et al., 2000), and several forms of translocation-associated proteins are proposed to exist (Wang & Dobberstein, 1999).

One pertinent question which arises from the presence of various translocon species is whether they engage or cluster with different modificational machineries, thus tailoring the translocational microenvironment. Although this phenomenon has not been widely investigated, one testimony that this may be the case comes from the study of the oligosaccharyl transferase (OST) complex, which catalyzes the covalent addition of an N-linked glycan to its translocating substrate. It appears that two different OST complexes exist in yeast, which can either contain the Ost3 or Ost6 subunits (Schwarz et al., 2005; Yan & Lennarz, 2005). These subunits alter both the interactions and activity of the OST complex, as the Ost3 containing form interacts with the Sec61 translocon and glycosylates its substrates with high affinity, while the Ost6-bearing complex interacts with the Ssh1 translocon and has lower affinity for its substrates (Spirig et al., 2005; Yan & Lennarz, 2005). In mammalian cells, two different isoforms of the OST complex also exist, and seem to differ with regards to their activity and donor affinity (Kelleher et al., 2003; Shibatani et al., 2005). Further evidence for such spatial separation comes from the study of ER exit sites, which indicates that proteins with different structures also utilize distinct sites for this final stage of maturation (Castillon et al., 2009; Stephens & Pepperkok, 2002). Thus, it seems that the seemingly continuous ER landscape is more varied than previously appreciated, postulating connections which streamline the biogenesis of secretory pathway proteins.

Dual localization

A protein's localization is usually regarded as a constant, singular outcome of its functional requirements. However, there are few documented examples of dually localized proteins which function both in the secretory pathway and other cellular compartments, such as the cytosol or mitochondria (Regev-Rudzki & Pines, 2007). In these cases, it appears that while only a small fraction of the protein is found outside of the secretory pathway, its alternate localization allows it to take part in basic cellular processes, such as glucocorticoid receptor-mediated gene activation or intracellular calcium modulation (Gkika et al., 2004; Shaffer et al., 2005). While this dual localization can be a steady-state phenomenon (Levine et al., 2005), it might also be triggered by ER specific stresses (Anandatheerthavarada et al., 1999; Robin et al., 2002; Sun et al., 2006). SRP-independent proteins might provide a key protein pool which can be shared between the secretory pathway and other cellular compartments, as these proteins transiently reside in the cytosol.

While large strides have been made in the last decade to systematically analyze protein localization, the extent of dually localized proteins is presently unknown. This is most likely the outcome of current localization assays, which hinge on fractionation or direct imaging of the protein of interest. In these cases, should a small portion of the protein pool be located in a second cellular compartment, this will most likely either be overlooked or disregarded. This is especially true if the second compartment is the cytosol, which is much larger than all other organelles, and thus would generate a much more diluted fluorescent signal. Thus, unless a second location is actively assayed for, it is likely to be overshadowed by the more predominant cellular compartment. With the advent of new technologies for accurate localization of proteins (Ben-Menachem et al., 2011) it should now be possible to categorize proteins which are present at low levels in a given cellular compartment. The systematic application of such tools will reveal the true spectrum of protein localization, and uncover what role the SRP-independent pathway plays in this plasticity.

Summary

The past 30 years of study have placed the SRP pathway in the limelight of ER translocation. Apart from furthering our understanding of this conserved route in its own right, studying the SRP pathway has uncovered the intrinsic problems that must be overcome when uncoupling a protein's

ultimate localization from its initial site of translation. However, a growing body of literature is starting to map SRP-independent pathways that can also answer these translocational needs. This emerging understanding of the variety of translocational pathways also highlights the conceptual similarities between the different mechanisms, which must ensure that the protein is relayed from the cytosol to the ER in a translocationally competent state. Perhaps it is time to stop referring to these pathways as SRP-independent, thus defaulting to the SRP pathway, and name them in their own right.

The presence of multiple pathways brings to mind several questions: are there other pathways that have been overlooked? Have SRP-independent routes been functionally conserved between lower and higher eukaryotes? What is the extent of substrate overlap between various pathways? Is there any crosstalk between the different mechanisms? How are the different pathways regulated to maintain the optimal protein flux during steady-state and stress? Thus, it appears that these studies of ''non conventional'' translocation have shed a new, complex light on the initial and crucial stage of secretory protein biogenesis, hinting that translocation is a tailored and multifaceted event.

Declaration of interest

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