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A Network of Cytosolic Factors Targets SRP-Independent Proteins to the Endoplasmic Reticulum

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SUMMARY

Translocation into the endoplasmic reticulum (ER) is an initial and crucial biogenesis step for all secreted and endomembrane proteins in eukaryotes. ER insertion can take place through the well-characterized signal recognition particle (SRP)-dependent pathway or the less-studied route of SRP-independent translocation. To better understand the prevalence of the SRP-independent pathway, we systematically defined the translocational dependence of the yeast secretome. By combining hydropathy-based analysis and microscopy, we uncovered that a previously unappreciated fraction of the yeast secretome translocates without the aid of the SRP. Furthermore, we validated a family of SRP-independent substrates—the glycosylphosphatidylinositol (GPI)-anchored proteins. Studying this family, we identified a determinant for ER targeting and uncovered a network of cytosolic proteins that facilitate SRP-independent targeting and translocation. These findings highlight the underappreciated complexity of SRP-independent translocation, which enables this pathway to efficiently cope with its extensive substrate flux.

INTRODUCTION

In eukaryotes, the initial biogenesis of secreted and endomembrane proteins takes place at the endoplasmic reticulum (ER), to which these proteins are directed via their hydrophobic ER-targeting sequences (Cross et al., 2009; Rapoport, 2007). To date, two types of eukaryotic ER-targeting sequences have been characterized: N′-terminal cleavable signal sequences (SS) and transmembrane (TM) domains that act as signal anchors. These determinants are recognized by cytosolic machineries that enable subsequent translocation into the ER, while preventing these substrates from folding or aggregating within the cytosol.

The first eukaryotic ER-targeting pathway identified was the signal recognition particle (SRP) and its ER-bound receptor (Rapoport, 2007). This essential pathway, which binds hydrophobic ER-targeting sequences and mediates cotranslational translocation, has since been studied in great detail and to atomic resolution (Egea et al., 2005; Saraogi and Shan, 2011).

However, some secretory pathway proteins cannot use the SRP-dependent pathway. This can be due to physical restraints that prevent the ER-targeting sequence from binding to the SRP before translation has terminated. For example, an alternate pathway is required for the ER targeting and insertion of tail-anchored (TA) proteins and short secretory proteins that cannot recruit the SRP cotranslationally (Borgese et al., 2003; Müller and Zimmermann, 1987). During the past 5 years, one alternate targeting mechanism has been characterized for these proteins and is termed the GET pathway in yeast or the TRC40 pathway in mammals (Jonikas et al., 2009; Schuldiner et al., 2008; Stefanovic and Hegde, 2007).

Additionally, some secretory pathway proteins fail to engage the SRP because they bear an insufficiently hydrophobic ER-targeting sequence (Lee and Bernstein, 2001; Ng et al., 1996). The cytosolic factors mediating this form of SRP-independent translocation have remained poorly characterized (Cross et al., 2009). However, the membranal machinery mediating the translocation of these proteins has been elucidated and includes the canonical translocon, together with auxillary factors (Rapoport, 2007). The SRP-independent translocon is a tetrameric complex, which, in yeast, comprises the essential Sec62 and Sec63 and the nonessential Sec66 and Sec72 (Feldheim and Schekman, 1994; Panzner et al., 1995; Young et al., 2001). This SRP-independent translocon enlists the lumenal chaperone BiP/Kar2 to "ratchet" its substrates into the ER (Matlack et al., 1999).

Most studies of the SRP-dependent and -independent pathways have focused on a small and well-defined group of model proteins. Due to this lack of systematic data, the fraction of the secretome that utilizes each pathway remains unknown. To date, the signals guiding SRP-independent targeting and the cytosolic protein network that recognizes SRP-independent substrates prior to their translocation into the ER lumen have only been partially elucidated (Becker et al., 1996; Caplan et al., 1992; Chirico et al., 1988; Deshaies et al., 1988). Moreover, no ER-targeting pathway has been described for these substrates.

To address these questions, we focused on one of the most extensively researched eukaryotic secretion systems—that of the baker's yeast, *Saccharomyces cerevisiae*. We systematically categorized the translocation mechanisms of the *S. cerevisiae* secretome and further characterized the cytoplasmic effectors

of SRP-independent translocation. Through hydropathy-based analysis to identify proteins that would not engage the SRP, we could show that a substantial number of proteins are predicted to use this pathway for ER entry in yeast. Further analysis of one family of SRP-independent substrates, glycosylphopatidylinostol (GPI)-anchored proteins, demonstrated that their C′-terminal anchor sequence plays a previously uncharacterized role in ER targeting. Finally, we identified the cytoplasmic protein network that recognizes, chaperones, and targets this SRP-independent protein family to the ER. This analysis sets the foundations for more a detailed understanding of this central cellular pathway.

RESULTS

A Systematic Analysis of the Yeast Secretome Uncovers the Prevalence of SRP-Independent Translocation

Recent studies have suggested that SRP-independent translocation is more prominent than previously appreciated (Johnson et al., 2012; Lakkaraju et al., 2012; Shao and Hegde, 2011). However, even in the well-studied secretory pathway of yeast, the scope of SRP-independent translocation remains unknown. It has been demonstrated that, in yeast, the less hydrophobic the N′-terminal ER-targeting sequence is, both in terms of overall length and hydrophobicity, the more likely it is to be overlooked by the SRP (Ng et al., 1996). We therefore decided to implement these findings to systematically identify the repertoire of SRP-independent proteins in the yeast secretome.

First, we compiled a list of all yeast proteins that are predicted to be translocated into the ER (Figure 1A, left, and Table S1 available online). We then analyzed the first 60 amino acids (aa) of these 1,145 proteins for hydrophobic features that have been shown to modulate SRP binding (Figure 1A, middle) (Hatsuzawa et al., 1997). Briefly, each protein's maximum hydropathy was plotted over a window of either 9, 11, or 19 aa using the Kyte-Doolittle scale, and the length of the hydrophobic stretch was analyzed by the Phobius algorithm (Käll et al., 2004). By combining both of these parameters, we generated a compound hydropathy score for the majority of the yeast secretome (Figure 1A, right, and Table S1), excluding 32.8% of the secretome that does not contain significant hydropathy within its N′-terminal 60 aa. To ascertain that the compound hydropathy score correlates with the ability of an ER-targeting sequence to bind to the SRP, we analyzed the hydropathy score of the handful of empirically validated translocation substrates (Ng et al., 1996). Reassuringly, substrates that are known to be SRP independent generated low compound hydropathy scores, whereas those that are either partially or completely reliant on the SRP bear median or high compound scores, respectively (Table S1). Because the compound scores generated for all maximum hydropathy windows show significant correlation (Figure S1A), we chose to continue our analysis on the compound score with a window size of 9 aa, as recent structural work on an archeal SRP in complex with an ER-targeting sequence exhibited a binding interface of this length (Janda et al., 2010).

Interestingly, the compound hydropathy scores that we obtained were not distributed unimodaly (p value 0.0196), as might be expected from targeting sequences that have evolved to mediate two different translocational pathways (Figures 1A, right, and S1B). Two subpopulations were evident, representing targeting sequences with either a low score (median = 30.6, SD = 7.6) or a high score (median = 58.9, SD = 12.2). In light of this distribution, we predicted that the population bearing a compound hydropathy score below 40 would be SRP-independent substrates (Figure 1A, right). Following this criteria, we found that 333 proteins, or 43.3% of the analyzed yeast secretome, would most likely be SRP-independent substrates, whereas 436 proteins, making up 56.7% of the analyzed secretome, would engage the SRP either occasionally or consistently during their translation (Figure 1B and Table S1). It should be emphasized that SRP independence is predicted solely on the hydropathy of the first 60 aa for each protein and that highly hydrophobic segments that occur more C′-terminally could engage the SRP as translation continues. Thus, we further identified a high-confidence SRP-independent group of 173 proteins that have a compound hydropathy score below 40 and are not predicted to contain other highly hydrophobic domains, which could override their N′-terminal translocation preference (Table S2).

It has previously been suggested that the various ER insertion pathways might have adapted to better translocate specific subsets of the secretome. Therefore, we examined whether the predicted translocational groups shared unique structural characteristics (Figure 1B). We found that the predicted SRP-independent protein group, not constrained to the high-confidence SRP-independent substrates, is enriched for proteins that contain cleavable signal sequences (p value 3.16×10^{-71}). Many of these substrates also harbor a C′-terminal GPI-anchoring site (2.47×10^{-16}), which undergoes cleavage and anchoring within the ER. Conversely, the SRP-dependent substrate group is comprised mostly of TM-bearing proteins (3.3×10^{-49}). Indeed, a cleavable SS is less hydrophobic than a TM domain, explaining its lack of dependence on the SRP machinery.

These striking correlations suggest that a protein's translocation route is not random but rather that proteins with similar attributes utilize a specific insertional pathway. Furthermore, these findings put forth a model (Figure 1B) whereby the yeast SRP binds highly hydrophobic sequences such as signal anchor TM domains, as does the bacterial SRP (Huber et al., 2005). In contrast, although some SSs were predicted to engage the SRP, most were not and might rather bind to a different set of chaperoning and ER-targeting proteins.

These hydropathy-based predictions uncovered an entire repertoire of SRP-independent substrates that have never been studied before. We therefore decided to verify the predictions by looking at the in vivo translocation dependence of predicted SRP-independent substrates. To this end, we selected high-confidence SRP-independent substrates that could be visualized with a C′-terminal green fluorescent protein (GFP) fusion and picked the 83 relevant strains from the GFP library (Huh et al., 2003) (Table S2). These strains were then deleted for the nonessential *SEC72* gene, which encodes for a component of the SRP-independent translocon (Rapoport, 2007). It should be noted that the deletion of *SEC72* does not completely halt SRP-independent translocation (Feldheim and Schekman,

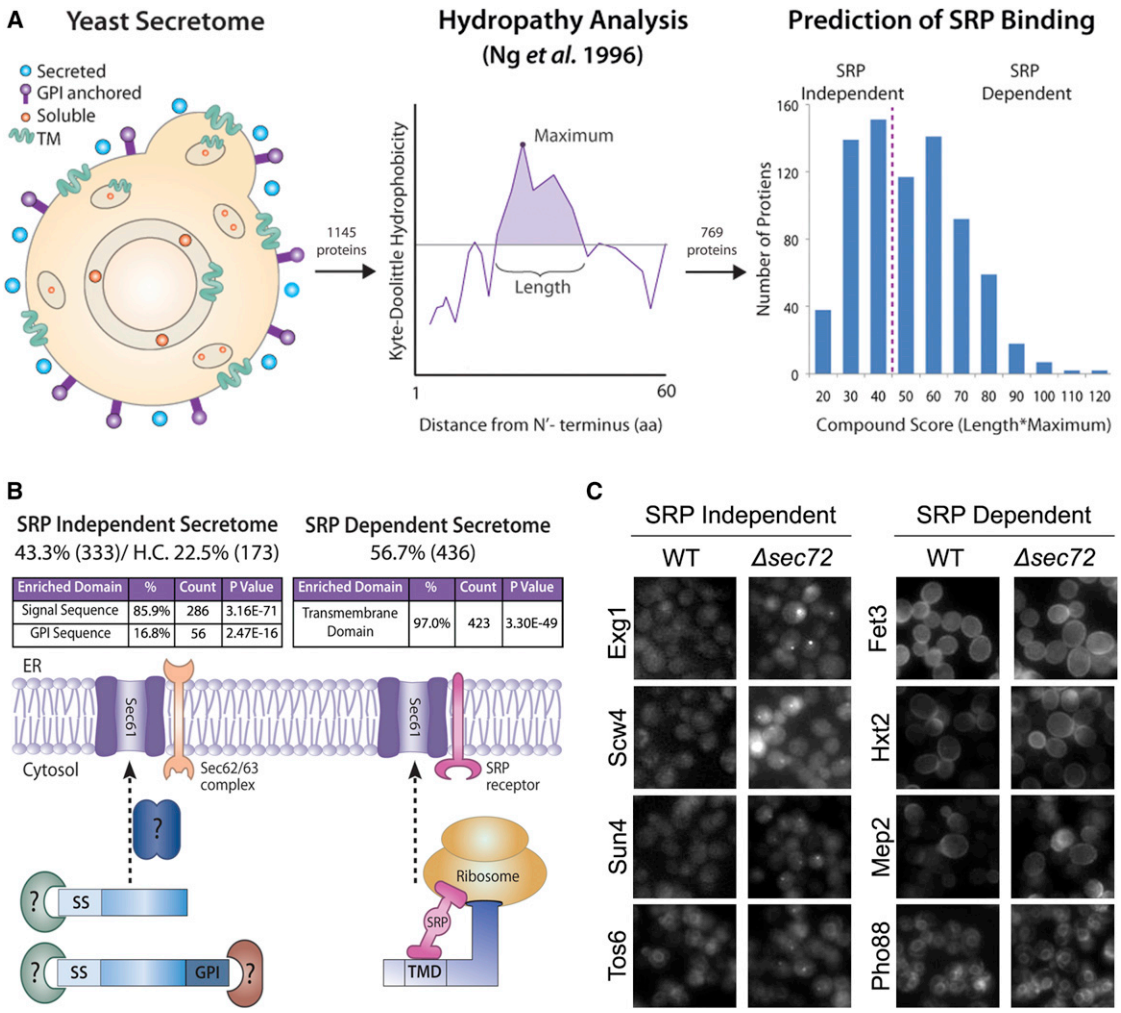

Figure 1. Hydropathy-Based Analysis Predicts that a Substantial Fraction of the Yeast Secretome Will Not Bind the SRP

(A) A comprehensive list of the yeast secretome, which constitutes 1,145 proteins, was generated (left). The first 60 aa of each protein were analyzed to characterize its ER-targeting sequence (Ng et al., 1996), scoring for both the length of the hydrophobic segment and its maximum hydrophobicity (middle). A potential hydrophobic segment could be identified for 769 proteins. For each of these proteins, a compound hydropathy score was generated using both of these parameters. This score exhibited a bimodal distribution within the population. Proteins scoring below a threshold of 40 were ranked as SRP-independent substrates (left). See also Figure S1 and Tables S1 and S5.

(B) SRP-independent proteins are predicted to make up 43.3% of the yeast secretome (HC, high confidence). Domain analysis uncovered that predicted SRP-independent proteins are enriched for SS- and GPI-anchoring sequences. In contrast, SRP-dependent proteins (56.7% of the secretome) utilize their first TM domain as an SRP-recruiting signal anchor. In parentheses is the number of proteins in each category.

(C) To validate the hydropathy-based predictions, the localization of predicted SRP-independent and -dependent proteins was analyzed when the SRP-independent translocon is impaired (*Δsec72*). Representative strains are shown here. Indeed, predicted SRP-independent substrates were mislocalized in *Δsec72* cells, accumulating in cytosolic aggregates, whereas proteins predicted to be SRP dependent exhibited a WT phenotype in the *Δsec72* mutant (lens 60×). See also Tables S2 and S3.

1994). By imaging the wild-type (WT) and *Δsec72* strains, we could verify 38 substrates that are mislocalized in the absence of Sec72, representing 46% of the tested strains that most likely undergo the severest cases of translocational attenuation (representative strains in Figure 1C; Table S2). In contrast, the localization of more than 100 GFP-tagged predicted SRP-dependent proteins was not altered in the absence of Sec72 (representative strains in Figure 1C; Table S3). These results suggest that our hydropathy-based analysis can indeed reveal whether a protein utilizes the SRP-independent pathway for ER targeting.

The GPI-Anchored Protein Family Depends on SRP-Independent Translocation for ER Insertion in Yeast

The predicted SRP-independent protein group was enriched for the GPI-anchored protein family. The GPI-anchored proteins undergo a posttranslational glycan-lipid modification in the ER and are estimated to make up 10%–20% of eukaryotic cell-surface proteins (Orlean and Menon, 2007). In our analysis, all but one of the 57 putative family members displayed low compound hydropathy scores (Table S1 and, by additional methods, Figure S2). We could verify this prediction for all

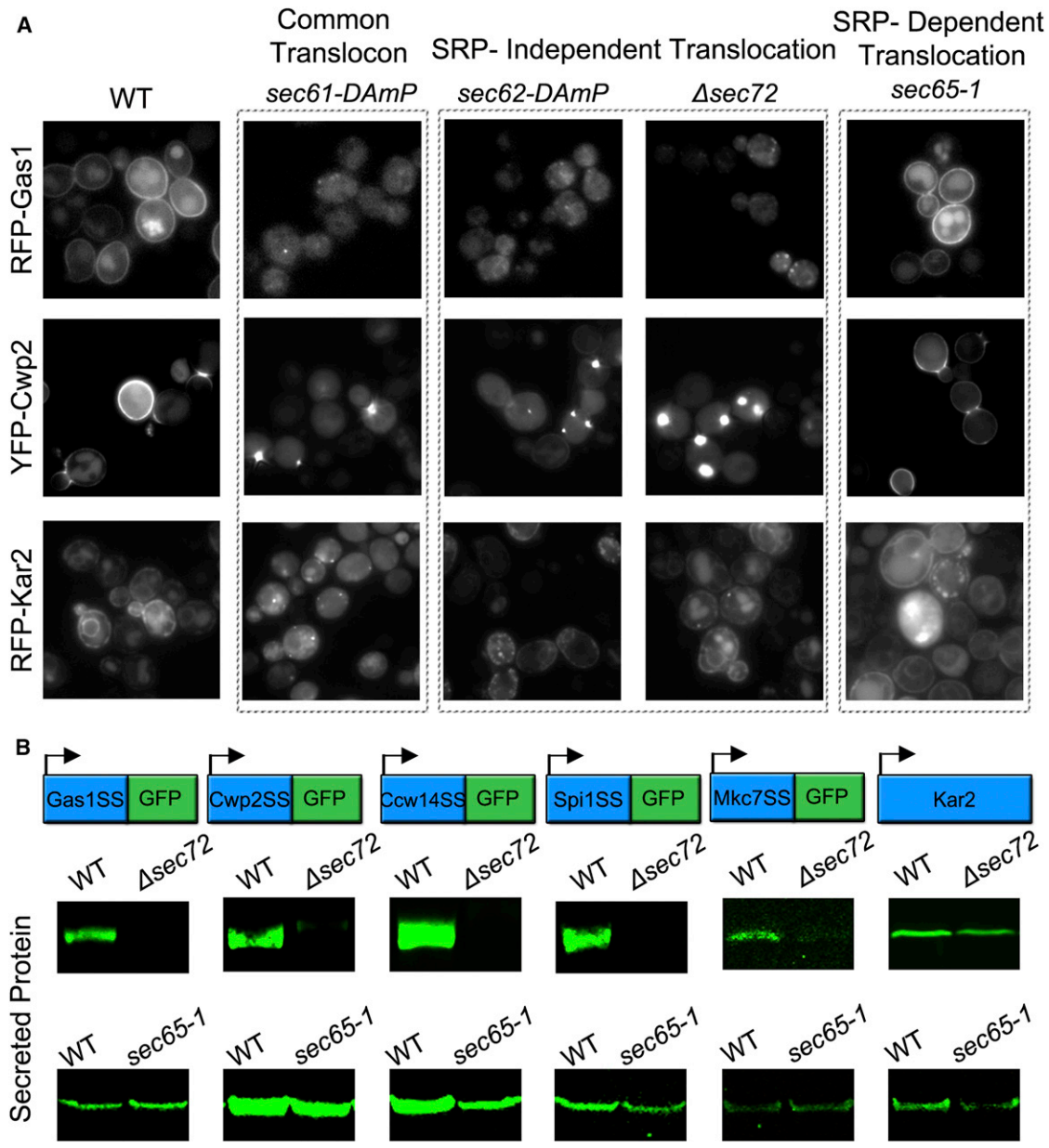

Figure 2. GPI-Anchored Proteins Depend on the SRP-Independent Pathway for ER Insertion

(A) Two fluorescently tagged GPI-anchored proteins, Gas1 and Cwp2, were imaged following attenuation of general (*sec61-DAmP*), SRP-independent (*sec62-DAmP* and *Δsec72*), or SRP-dependent translocation (*sec65-1* at restrictive temperature). Both of these GPI-anchored proteins accumulate in cytosolic aggregates in the absence of either the translocon or the SRP-independent pathway (lens 100×), while remaining unaffected by the mutation in the SRP. As a control, Kar2, which is known to utilize both translocation mechanisms, is affected in all mutants. See also Figures S2 and S3.

(B) The SSs of GPI-anchored proteins were N′-terminally fused to GFP, and the efficiency of translocation of these fusion proteins was measured by analyzing their secretion and probing for GFP. Whereas, in WT and SRP-depleted (*sec65-1*) cells, secretion of these GFP constructs takes place, GFP is no longer found in the media when the SRP-independent translocon is impaired (*Δsec72*). In contrast, Kar2, which enters the ER through both pathways, displays a diminished rate of secretion in both mutants.

13 C′-terminally GFP-tagged GPI-anchored proteins that were tested (Table S2).

As C′-terminal GFP tagging masks the C′-terminal GPI anchor sequence, it might alter the translocation dependency of GPI-anchored proteins. Therefore, we analyzed the translocational preference of two fluorescently tagged GPI-anchored proteins (Figure 2A), Gas1 and Cwp2, which maintain their correct SS and GPI anchor sequence organization (Castillon et al., 2009; Fujita et al., 2006). Indeed, the localization of these GPI fusion proteins was only affected by mutations in the translocon (*sec61-DAmP*) or SRP-independent complex (*sec62-DAmP* and *Δsec72*). Kar2, which is known to utilize both pathways (Ng et al.,

1996), was also mislocalized in an SRP mutant (*sec65-1*). The foci imaged in the absence of the SRP-independent translocation do indeed represent a preinserted aggregated form of these proteins as indicated by their running size on an SDS-PAGE gel and colocalization with inclusion markers (Figures S3A and S3B). We also ensured that dependence on the SRP-independent machinery was not an artifact of the fluorescent tagging, as endogenous GPI-anchored proteins demonstrate the same translocational dependence (Figure S3C and S3D).

To further ascertain that the dependence of GPI-anchored proteins on the SRP-independent pathway is a family-wide phenomenon in vivo, we fused the SS of five verified GPI-anchored proteins to GFP. We assayed the ER insertion of these fusion proteins by following GFP secretion into the medium (Figure 2B) in either WT cells or cells that are defective in the SRP-dependent or independent insertion pathways (*sec65-1* and *Δsec72*, respectively). Whereas WT and *sec65-1* (at the restrictive temperature) cells secrete the various SS-GFP fusion proteins into the media, the secretion of all five was attenuated in *Δsec72*. In contrast, Kar2 secretion into the media is weakened, but not abolished, in both the *sec65-1*and *Δsec72* mutants (Figure 2B, right). These results imply that the SS-GFP constructs—and ergo their representative GPI-anchored proteins—fail to enter the secretory pathway in the absence of SRP-independent translocation.

Every GPI-anchored protein that we have studied has shown the same reliance on SRP-independent translocation. Moreover, all GPI-anchored proteins have a similar ER-targeting domain. These two facts strongly support the notion that, in yeast, this entire protein family requires SRP-independent translocation for ER entry. More generally, these findings indicate that the SS is usually linked to SRP-independent translocation in yeast.

The GPI-Anchoring Sequence Has an Uncharacterized Role in ER Targeting

Although GPI-anchored proteins carry an N′-terminal SS, they also contain a C′-terminal GPI anchor sequence, which tags them for a posttranslational modification with a glycolipid anchor in the ER lumen (Orlean and Menon, 2007). The secretion assay outlined above demonstrated that the SS itself is sufficient to guide SRP-independent translocation, as expected. However, it has been previously shown that the hydrophobic core of the GPI anchor can substitute for that of the SS and maintain ER translocation (Yan et al., 1998). We therefore wondered whether, at its native C′-terminal location, the GPI anchor sequence also partakes in any pretranslocation processes.

To address this question, we expressed either soluble GFP, GFP tagged N′-terminally with the SS of Gas1 (Gas1SS-GFP), or GFP tagged C′-terminally with the GPI anchor sequence of Gas1 (GFP-Gas1GPI) in WT cells (Figure 3A). Although untagged GFP was found in the cytosol, the Gas1SS-GFP construct was secreted from the cell, generating a weak cytosolic pattern (see also Figure 2B). Strikingly, the GFP-Gas1GPI fusion protein exhibited a ring-like ER pattern, indicating that this domain mediates ER targeting.

To assay whether the GPI anchor sequence takes part solely in ER targeting or whether it also undergoes translocation, we performed a proteinase protection assay on purified ER-derived microsomes from the Gas1SS-GFP- or GFP-Gas1GPI-expressing strains (Figure 3B). Microsomes extracted from both strains contained the fusion proteins, demonstrating that both the SS and GPI anchor sequence support ER association. However, only the Gas1SS-GFP construct could generate a proteinase-protected band, indicative of its insertion into the protective ER lumen. Furthermore, this band is lost when the Gas1SS-GFP microsomes are treated with both the membrane-perturbing detergent Triton and proteinase. Therefore, it appears that the

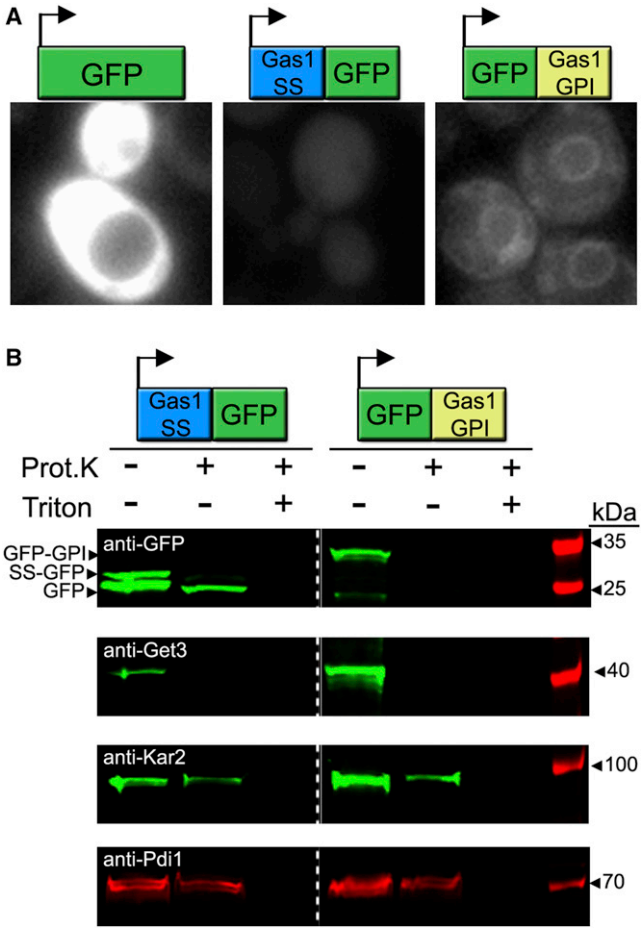

Figure 3. Both the SS and the GPI-Anchoring Sequence Can Direct ER Targeting

(A) Constructs encoding for either GFP, GFP N′-terminally fused to Gas1's SS, or GFP C′-terminally fused to Gas1's GPI-anchoring sequence were imaged in WT cells (lens 100×). Whereas GFP accumulates in the cytosol, Gas1SS-GFP is secreted from the cell (see also Figure 2B). GFP-Gas1GPI generates a ring-like ER pattern, indicating that the GPI-anchoring sequence undergoes ER targeting.

(B) To assay whether the GPI-anchoring sequence directs ER targeting or entry, a proteinase protection assay was carried out on microsomes prepared from cells expressing Gas1SS-GFP or GFP-Gas1GPI, followed by an immunoblot for various proteins. After proteinase digestion, free GFP remained in the Gas1SS-GFP microsomes, whereas complete digestion took place in the GFP-Gas1GPI microsomes, indicating that only the SS can direct entry into the lumen of the ER. As controls, lumenal Kar2 is shielded during proteinase treatment, whereas cytosolic Get3 is digested. The lumenal Pdi1 serves as a loading control. All proteins are digested in the presence of the membrane-perturbing agent, Triton.

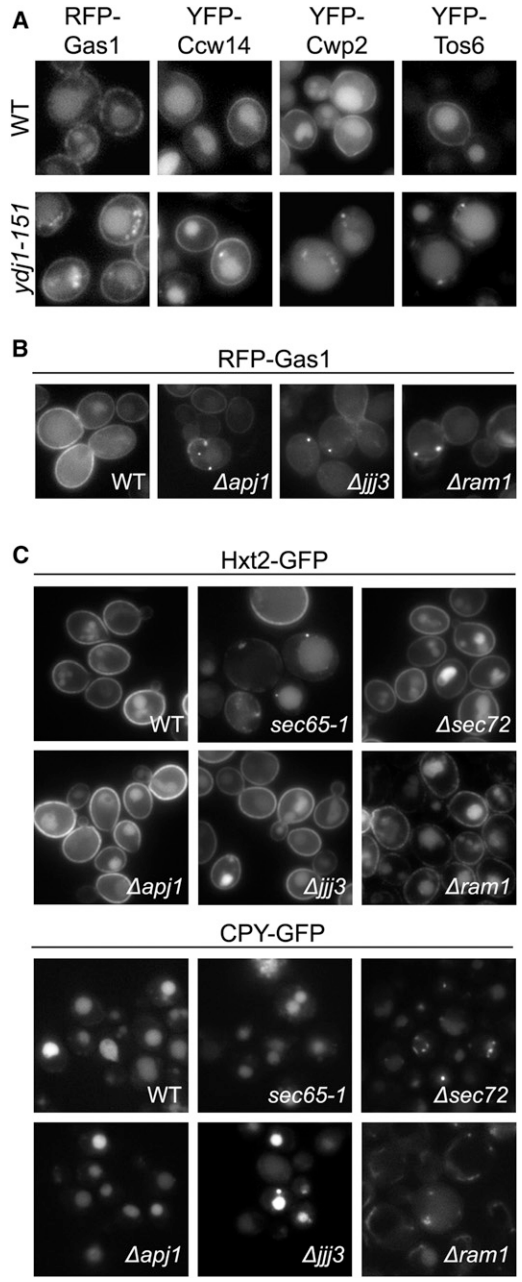

Figure 4. Three Cytosolic Hsp40s Take Part in the Molecular Chaperoning of Preinserted GPI-Anchored Proteins

(A) Four fluorescently tagged GPI-anchored proteins—Gas1, Ccw14, Cwp2, and Tos6—were visualized in WT and a temperature-sensitive mutant of the Hsp40 Ydj1 (*ydj1-151*), which cannot activate the cytosolic Hsp70 Ssa1. Although GPI-anchored proteins are normally found on the cell surface, in the *ydj1-151* mutant, at the restrictive temperature, they also accumulate in cytosolic aggregates (lens 100×), implying that the Ssa1-Ydj1 interaction is important in maintaining efficient GPI-anchored protein translocation. See also Figure S4A.

(B) RFP-Gas1 was visually assayed in the absence of cytosolic Hsp40s (lens 100×). The deletions of Apj1 or Jjj3 resulted in RFP-Gas1 accumulating in cytosolic aggregates, indicating that they have a role in chaperoning preinserted Gas1. A mutation in Ram1, required for the farnesylation of Ydj1, also resulted in aggregate formation, suggesting that the ER localization of Ydj1 is important for its preinsertional cochaperoning role. See also Figure S4B.

(C) The effects of mutations in Apj1, Jjj3, and Ram1 on the localization of the SRP-dependent and -independent substrates Hxt2 and CPY (Prc1) were visually analyzed (lens 100×). None of the mutations affected the cell-surface staining of Hxt2, indicating that this chaperoning network is specific to SRP-independent substrates. Vacuolar Prc1-GFP (CPY) was mislocalized in the absence of Jjj3 and Ram1, indicating that non-GPI SRP-independent substrates engage a subset of this network.

anchoring sequence has a previously unappreciated cytosolic role in the targeting of its preinserted GPI-anchored proteins to the cytosolic surface of the ER.

A Network of Specific Cytosolic Hsp40s Is Required for the Efficient Chaperoning of GPI-Anchored Proteins

Both the SS and the anchor sequence contain a hydrophobic core, which could lead to their aggregation should these domains not be chaperoned prior to translocation. Furthermore, the dimensions of the translocon pore dictate that proteins must insert as secondary structured polypeptides, requiring the SRP-independent substrate to maintain a loosely folded conformation while in the cytosol (Rapoport, 2007). Previous work has implicated the cytosolic yeast Hsp70 protein Ssa1 in such preinsertional chaperoning (Chirico et al., 1988; Deshaies et al., 1988). The promiscuous chaperoning capacity of Hsp70s is regulated by the Hsp40 cochaperones, which induce higher-affinity binding to the unfolded substrate by promoting the Hsp70's ATPase activity (Walsh et al., 2004). As their two hydrophobic motifs must require extensive preinsertional shielding, we hypothesized that GPI-anchored proteins could be utilized as a powerful tool to study cytosolic Hsp40s as modulators of SRP-independent chaperoning.

Ydj1, a yeast Hsp40 that is tethered to the ER membrane via a farnesyl modification, has been shown to promote the binding of Ssa1 to preinserted ppαf and proteinase A (Becker et al., 1996; Caplan et al., 1992). We sought to test whether this interaction is also important for the efficient ER insertion of GPI-anchored proteins. We utilized a temperature-sensitive form of Ydj1, *ydj1-151*, which is defective in promoting the ATPase activity of Ssa1 (Caplan et al., 1992). Indeed, in the mutant Ydj1 strain imaged at restrictive temperature, GPI-anchored proteins partially accumulated in cytosolic aggregates (Figure 4A), phenocopying the absence of the SRP-independent translocation pathway. These findings indicate that Ydj1 and Ssa1 play a part in the cytosolic chaperoning of a wide range of SRP-independent substrates.

We also analyzed RFP-Gas1 in the absence of ER-localized Ydj1 by deleting its farnesyl transferase, *Δram1* (Flom et al., 2008). Indeed, in the absence of *RAM1*, RFP-Gas1 was mislocalized to cytosolic aggregates (Figure 4B, right). Moreover, when RFP-Gas1 is expressed in Ydj1-GFP cells, where the C′-terminal farnesylation of Ydj1 is blocked by the fluorophore, a similar mislocalization is apparent (Figure S4A). Thus, it appears that Ydj1 must be ER bound in order to function in preinsertional chaperoning.

Although Ydj1 has been previously studied in the context of translocation, it is unknown what role, if any, other Hsp40s have in this pathway. Although all Hsp40s share the J domain

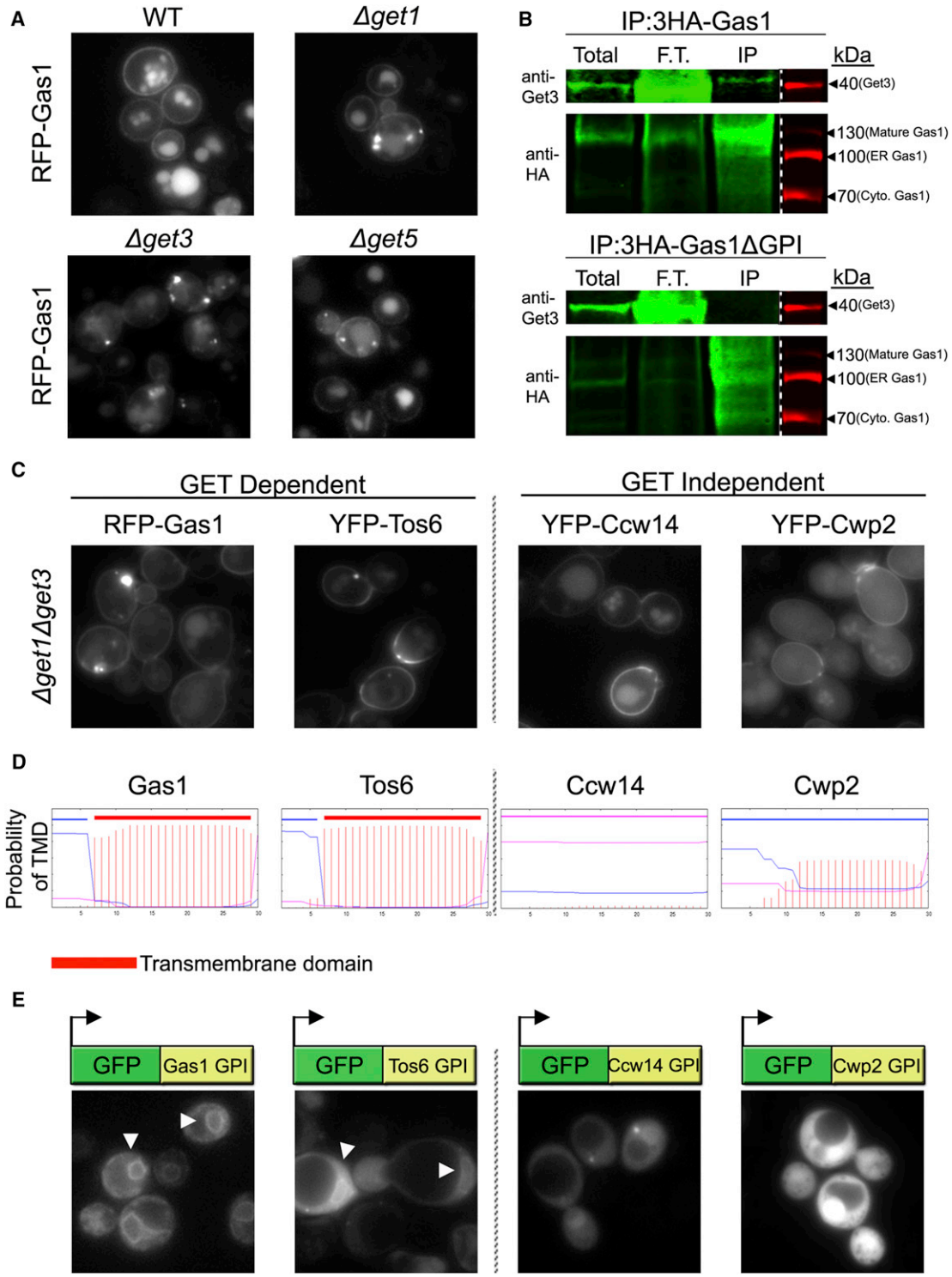

Figure 5. The GET Pathway Targets a Subset of Preinserted GPI-Anchored Proteins to the ER

(A) Mutations in various proteins of the GET pathway (*Δget1*, *Δget3*, and *Δget5*) affect the correct localization of RFP-Gas1 (lens 100×). See also Figure S5.

(B) The binding of Get3 to an HA-tagged form of Gas1, either bearing (3HA-Gas1) or lacking its C′-terminal GPI-anchoring domain (3HA-Gas1ΔGPI), was analyzed following IP with an anti-HA antibody and probing against endogenous Get3. Get3 binds to the full HA-Gas1 protein, but not to the anchor-sequence-deleted protein, although 30% more 3HA-Gas1ΔGPI is found in the cytosol.

(C) Whereas Gas1 and Tos6 accumulate cytosolically in the *Δget1 Δget3* mutant, Ccw14 and Cwp2 are not affected by these mutations and exhibit normal surface localization (lens 100×).

(legend continued on next page)

through which they interact with Hsp70s, the 22 yeast Hsp40 proteins are not functionally identical. Genetic studies in yeast have demonstrated that some Hsp40s are redundant, whereas others appear to be specialists, whether due to structure or localization (Sahi and Craig, 2007). We therefore wondered whether a specific subset of cytosolic Hsp40s aids in the chaperoning of preinserted SRP-independent proteins. To this end, we imaged the GPI-anchored protein RFP-Gas1 in the absence of the 11 nonessential cytoplasmic Hsp40s (Figure S4B). RFP-Gas1 accumulated in cytosolic puncta on the background of only two mutations: *Δapj1* and *Δjjj3* (Figure 4B). Under the same conditions, the SRP-dependent substrate Hxt2 was not mislocalized (Figure 4C, top) demonstrating that both Hsp40s are specific for SRP-independent translocation. Neither one of these Hsp40s have been previously characterized in mediating efficient SRP-independent translocation, although their effect on RFP-Gas1 was similar to mislocalized Ydj1.

We further validated the effects of these Hsp40s on a non-GPI-anchored SRP-independent substrate, CPY (Prc1), which localizes to the vacuole (Figure 4C, bottom). Although the absence of Jjj3 and Ram1 disturbed the efficient insertion of CPY, no effect was witnessed in the *Δapj1* mutant. It appears that the cochaperoning function of the Hsp40s is not uniform for all SRP-independent substrates; SS-bearing proteins require only a subset of these partners. Together, these results indicate that SRP-independent ER insertion relies on a network of cochaperones that maintain the translocational competence of its many varied substrates.

The GET Pathway Functions in the ER Targeting of a Subset of GPI-Anchored Proteins

Although cytosolic chaperoning mediated by Ydj1, Apj1, and Jjj3 plays a significant role in preserving the insertional competence of SRP-independent substrates, we speculated that rapid ER targeting might also promote efficient translocation of these proteins. Genetic interaction studies (Costanzo et al., 2010; Jonikas et al., 2009; Schuldiner et al., 2005) revealed that the deletion of Ydj1 was synthetically lethal when combined with deletions of the GET pathway (Figure S5), hinting that the two might have parallel cellular functions. The GET pathway has been characterized to take part in the SRP-independent ER targeting and insertion of TA proteins, which contain a single TM domain at their C′ terminus (Favaloro et al., 2008, 2010; Jonikas et al., 2009; Leznicki et al., 2010; Schuldiner et al., 2008; Stefanovic and Hegde, 2007). As GPI-anchored proteins also contain a hydrophobic C′-terminal element, we hypothesized that they could also be candidate substrates for the GET pathway.

To ascertain whether the GET pathway takes part in the translocation of GPI-anchored proteins, we imaged RFP-Gas1 in cells lacking Get1, Get3, or Get5 (Figure 5A), which represent the three stages of this pathway: ER docking, ER targeting, and ribosomal handoff of substrates, respectively (Jonikas et al., 2009; Schuldiner et al., 2008). In the absence of all of these various GET components, RFP-Gas1 aggregated in the cytoplasm, suggesting that the GET pathway might also play a role in the ER targeting of GPI-anchored proteins.

Next, we wished to further verify that the effect of the GET pathway is direct. Thus, we probed for a physical interaction in vivo between Get3 and a hemagglutinin (HA)-tagged Gas1 either containing or lacking its GPI-anchoring sequence (3HA-Gas1 and 3HA-Gas1ΔGPI) (Figure 5B). Following pull-down on the HA tag, we found that Get3 is bound to the full-length HA-Gas1 protein, but not to HA-Gas1 lacking its anchor sequence, despite the fact that there is 30% more 3HA-Gas1ΔGPI in the cytosol than 3HA-Gas1. Thus, it seems that Get3 interacts with Gas1 in vivo in an anchor-sequence-dependent manner in accordance with its previously characterized binding preferences.

To discern whether the GET pathway affects other GPI-anchored proteins, we visualized fluorescently tagged Tos6, Ccw14, and Cwp2 in the absence of Get1 and Get3 (Figure 5C). Interestingly, we saw that these proteins were differentially impacted—whereas RFP-Gas1 and YFP-Tos6 showed GET dependence, YFP-Ccw14 and YFP-Cwp2 localization was not altered in the GET mutants. This dependence on the GET pathway correlated with the hydrophobicity of the C′-terminal GPI-anchoring sequence (Figure 5D). Gas1 and Tos6 contain a GPI-anchoring sequence that is markedly hydrophobic and TM like, making them structurally similar to the previously described GET substrates, the TA proteins. In contrast, Ccw14 and Cwp2 have a mildly hydrophobic anchoring sequence. When we analyzed the last 30 aa of all predicted GPI-anchored proteins, we saw that ~80% of them have a TM-like GPI-anchoring sequence (Table S4). These findings would imply that the GET pathway plays a role in targeting GPI-anchored proteins to the ER surface. It is likely that additional mechanisms for targeting exist for SRP-independent proteins that do not contain a highly hydrophobic GPI-anchoring sequence.

If the GET pathway is indeed responsible for anchor-sequence-dependent ER targeting, then we expected that only TM-like GPI-anchoring sequences would induce targeting to the ER. We therefore visualized the GPI-anchoring sequence of Gas1, Tos6, Ccw14, and Cwp2 when fused to the C′ terminus of GFP (Figure 5E). As expected, the GET-dependent Gas1 and Tos6 GPI-anchoring sequences generated a ring-like ER pattern (arrows), whereas the anchoring sequences of Ccw14 and Cwp2 did not. Furthermore, swapping the TM domain of a TA protein for the GPI-anchoring sequence of Gas1 also resulted in an ER pattern (Figure S6). Thus, it seems that the hydrophobic core of the GPI-anchoring sequence has a role in the ER targeting of the GPI-anchored proteins through the recruitment of the GET pathway.

(D) The GPI-anchoring sequence of Gas1, Tos6, Ccw14, and Cwp2 were analyzed by TMHMM. GET dependence correlated with C′-terminal hydrophobicity, as Gas1 and Tos6 encode for a GPI-anchoring sequence that is as hydrophobic as a TM domain, whereas Ccw14 and Cwp2 only contain a mildly hydrophobic GPI-anchoring sequence. See also Table S4.

(E) Constructs bearing GFP-tagged C′-terminally with Gas1 or Tos6 GPI-anchoring sequence generate a ring-like ER pattern (arrows), whereas the GPI-anchoring sequences of Ccw14 and Cwp2 do not (lens 100×), indicating that a hydrophobic GPI-anchoring sequence directs ER targeting. See also Figure S6.

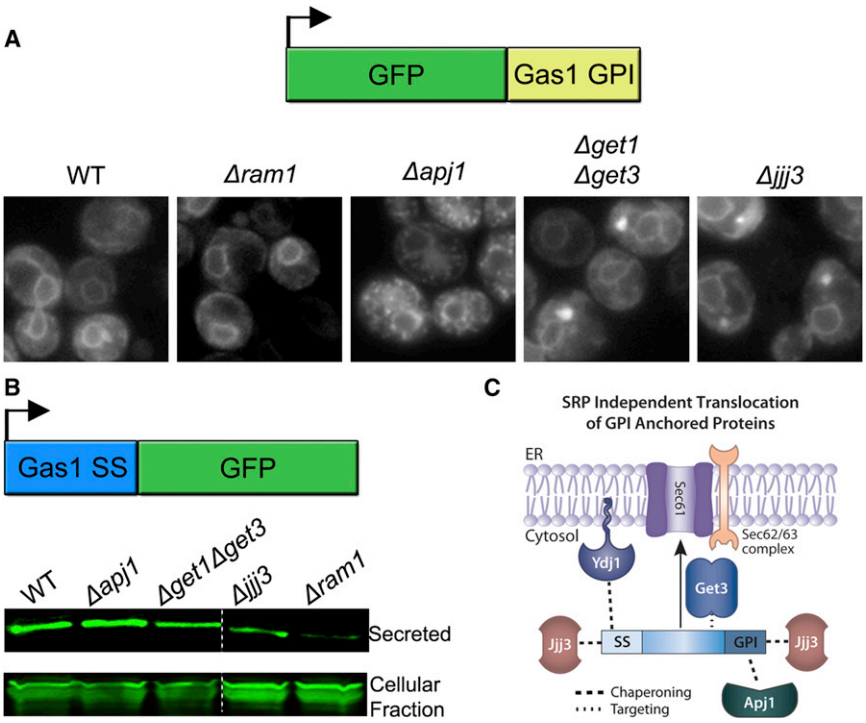

Figure 6. Preinserted ER-Targeting Domains Recruit Different Cytosolic Proteins

(A) GFP-Gas1GPI was visualized in the absence of the various cytosolic chaperones that we have identified to affect GPI-anchored protein translocation (lens 100×). Apj1, Jjj3, and the GET pathway affected the ability of the GFP-Gas1GPI construct to reach the ER, whereas the mislocalization of Ydj1 (*Δram1*) had no impact on its localization.

(B) The secretion of the Gas1SS-GFP construct was analyzed by immunoblot, probing for secreted and cellular GFP in the above strains. The secretion of this construct is attenuated in the *Δjjj3* and *Δram1* strains.

(C) A model summarizing the chaperoning and targeting pathways for preinserted GPI-anchored proteins. While in the cytosol, SRP-independent proteins present hydrophobic sequences that engage the chaperoning and targeting machinery. The N′-terminal SS can be recognized by the Hsp40 Ydj1, whereas the C′-terminal GPI-anchoring sequence engages the Hsp40 Apj1. Both sequences can also be recognized by the Hsp40 Jjj3. Furthermore, highly hydrophobic GPI anchors complex with the GET pathway for ER targeting.

The Two Hydrophobic Domains of GPI-Anchored Proteins Differentially Recruit Targeting and Chaperoning Factors

The SS- and GPI-anchoring sequences appear to signal that the relevant preinserted protein requires chaperoning, while also mediating ER targeting. We therefore sought to map the sequence dependence of the various chaperoning and targeting factors. To identify the proteins recruited by the GPI anchor sequence, we imaged GFP-Gas1GPI on the background of all mutants that we have characterized thus far to mediate efficient translocation (Figure 6A). While in the absence of Apj1, Jjj3 or the GET pathway this fusion protein accumulated in cytosolic aggregates, mislocalized Ydj1 (*Δram1*) did not affect its localization. No mutation completely abolished the ER targeting of the GFP-Gas1GPI construct, implying that these proteins, and potentially others, have overlapping roles in this capacity. We then assayed the secretion of the Gas1SS-GFP construct in these same mutants by western blot, probing for secreted and cellular GFP (Figure 6B). Whereas mutations in Apj1 or the GET pathway did not affect the levels of secreted Gas1SS-GFP, mutants of Jjj3 and Ram1 exhibited lower levels of secreted GFP. This correlates with the finding that the non-GPI-anchored SRP-independent substrate, CPY, was mislocalized only in the absence of Jjj3 and Ram1, but not Apj1 (Figure 4C).

These results map the contribution of each hydrophobic signal in recruiting the cytosolic machinery (Figure 6C). The Hsp40 Ydj1 recruits the cytosolic chaperoning machinery to the SS, common to most SRP-independent substrates, whereas the GPI-anchoring sequence engages the Hsp40 Apj1 for chaperoning and the GET pathway for ER targeting. Additionally, both of these hydrophobic sequences recruit Jjj3, which appears to act as a general chaperoning factor for the SRP-independent pathway.

DISCUSSION

Although the mechanism of SRP-dependent translocation has been studied in detail from bacteria to mammals, our understanding of the respective contribution and the cytosolic aspects of SRP-independent translocation remains partial. Recently, three studies have demonstrated that SRP-independent translocation is more common in mammals than originally thought (Johnson et al., 2012; Lakkaraju et al., 2012; Shao and Hegde, 2011). Our findings as to the prevalence of SRP-independent translocation in yeast, alongside these mammalian studies, highlight the need for a better understanding of this lesser-studied route.

Using hydropathy analysis, we distinguish between yeast TM-bearing proteins, which are predicted to rely partially or fully on SRP-mediated translocation, and soluble SS-containing proteins that are predicted to rely on SRP-independent translocation for the most part. This would imply that, in yeast, the "signal recognition particle" is, in fact, predominantly a "signal-anchor recognition particle." This nonrandom distribution suggests that structurally diverse secretory pathway proteins have evolved to utilize different insertional routes.

A recent work that systematically identified mRNAs associated with SRP-bound ribosomes in yeast demonstrated that this ribonucleoprotein complex is recruited to ribosomes translating a wide range of nascent secretory pathway proteins (del Alamo et al., 2011). In agreement with our work, the pool of enriched SRP-binding mRNAs was predominantly made up

of TM-bearing proteins (67%, 621 proteins). This enriched group also contained proteins lacking either SS or TM domain (23%, 213 proteins) and soluble SS-bearing proteins (10%, 91 proteins), which might represent proteins with highly hydrophobic SS, such as Kar2. Furthermore, SRP appeared to be recruited in the strongest or more sustained manner to ribosomes translating TM proteins, as the TM proteins were markedly enriched in the SRP-bound versus -unbound fractions. However, it should be noted that SRP recruitment and SRP dependence are not synonymous, as the SRP has been shown to bind ribosomes translating known SRP-independent substrates, although this recruitment is not fruitful (Plath and Rapoport, 2000). Therefore, our study and that of del Alamo et al. (2011) pertain to different stages of SRP function.

One prominent protein group—which we uniformly predicted and later verified—to undergo SRP-independent translocation is the GPI-anchored protein family. Our results complement previous findings in the parasite *Trypanosoma brucei*, whose numerous GPI-anchored proteins also depend on the SRP-independent pathway for ER entry (Goldshmidt et al., 2008). Furthermore, the model yeast GPI-anchored protein Gas1 has previously been shown to rely on the SRP-independent pathway (Ng et al., 1996), whereas recent work has demonstrated that knocking down the mammalian Sec63 homolog attenuates the translocation of the GPI-anchored prion protein PrP (Lang et al., 2012). Taken together with our results, this implies that the dependence of GPI-anchored proteins on the SRP-independent machinery might be conserved from yeast, through trypanosomes, to mammals. This conservation raises the intriguing question as to why the GPI-anchored proteins have uniformly maintained their translocational partners throughout evolution.

GPI-anchored proteins contain both an N′-terminal SS and a C′-terminal GPI-anchoring sequence, which has, until now, only been studied in the context of its posttranslocational modification (Orlean and Menon, 2007). However, we now show that this domain has a preinsertional role in targeting up to 80% of GPI-anchored proteins to the ER membrane. It remains to be uncovered how the remaining 20% of GPI-anchored proteins, as well as other SRP-independent substrates, are actively targeted to the ER membrane.

Our findings highlight that both the SS- and GPI-anchoring sequences recruit molecular chaperones while in the cytosol, which facilitates their efficient translocation. Previous studies suggested that the Hsp70 Ssa1 and also the Hsp40 Ydj1 play a role in preinsertional chaperoning of SRP-independent substrates, although the generality of this process remained unclear (Becker et al., 1996; Caplan et al., 1992; Chirico et al., 1988; Deshaies et al., 1988). Our work demonstrates that the interaction between Ydj1 and Ssa1 is also important for the translocation of GPI-anchored proteins, indicating that it might indeed be a central component in maintaining the preinsertional competence of a large number of SRP-independent substrates.

Apart from Ydj1, other cytosolic Hsp40s were never studied in the context of translocation (Walsh et al., 2004). Our work now maps the in vivo effects of each of the cytosolic Hsp40 proteins on translocational efficiency, highlighting the importance of Apj1 and Jjj3 in this process. Apj1 has previously been associated with the propagation of yeast prion proteins (Hines et al., 2011), a cellular role also shared by Ydj1 (Perrett and Jones, 2008), whereas Jjj3 has been implicated in the biosynthesis of diphthamide, iron storage, and endosomal recycling (Shi et al., 2011; Thakur et al., 2012; Webb et al., 2008). It does not appear that Jjj3 or Apj1 are indirectly affecting the fidelity or efficiency of translation or translocation, as they do not affect the localization of an SRP-dependent substrate (Figure 4C) or the amount and length of preinserted GFP fusion constructs (Figure 6B). Neither one of these three Hsp40s is exclusively necessary for the ER insertion of GPI-anchored proteins, as cell-surface staining was still apparent in the absence of each Hsp40 (Figures 4A and 4B). This most likely stems from the fact that each ER-targeting sequence is recognized by at least two factors, generating a "double-safe" network for this essential insertional process in whose absence the cytoplasm is burdened with mislocalized aggregates.

Highly hydrophobic GPI-anchoring sequences also appear to recruit the GET pathway, which has been previously characterized in the chaperoning and targeting of proteins with one C′-terminal TM domain—the TA proteins (Favaloro et al., 2008, 2010; Jonikas et al., 2009; Leznicki et al., 2010; Schuldiner et al., 2008; Stefanovic and Hegde, 2007). These results demonstrate a striking similarity between the preinsertional machinery that is engaged by TA proteins and GPI-anchored proteins, with both families relying on cytosolic chaperones and the GET pathway for efficient insertion (Rabu et al., 2009). One marked difference between these two substrates is that GPI-anchored proteins rely on the translocon for ER insertion (Figures 2A and S3), whereas TA proteins do not (Kutay et al., 1995). It is still unclear how the GET pathway mediates both outcomes. Recently, mammalian homologs of GET components have been shown to take part in both the SRP-independent ER insertion of short secretory proteins and the degradation of untargeted secretory pathway proteins (Hessa et al., 2011; Johnson et al., 2012). We could not discern whether the GET pathway also has a role in the degradation of GPI-anchored proteins in yeast. However, as isolated GPI-anchoring sequences direct ER targeting in a GET-dependent manner (Figure 6A), in accordance with this pathway's previously established function, it appears that the GET pathway acts in a targeting capacity for this protein family.

Our results shed further light on SRP-independent translocation, demonstrating that it is a prevalent mode of ER insertion in yeast. This pathway requires complex chaperoning and targeting mechanisms for its preinserted substrates, which are recognized in the cytosol by their hydrophobic sequences. Several questions arise from this data: are there other ER targeting or insertion sequences that have been overlooked? Do other SRP-independent substrates also undergo ER targeting at the protein or mRNA level (Palazzo et al., 2007; Prilusky and Bibi, 2009)? What is the complete suite of proteins ensuring that this essential pathway will function in an accurate and tightly regulated manner?

More generally, so far the various pathways have been assumed to be different means to a similar end—ER translocation. However, our findings emphasize that the mechanism by which a protein translocates into the ER can be tightly conserved. Such conservation might imply that the various translocational pathways generate unique microdomains specialized

to their insertional clientele, emphasizing the varied and tailored nature of the translocational space.

EXPERIMENTAL PROCEDURES

Analysis of ER-Targeting Sequences

To generate a comprehensive list of the yeast secretome, the UniProtKB database was queried for proteins from the organism *S. cerevisiae* (strain ATCC 204508/S288c) that contain a sequence annotation of "signal peptide" or "transmembrane domain" but are not localized to the organelle "mitochondrion." This protein list was cross-referenced with proteins that are known to localize to the secretory pathway through the yeast GFP library data (Huh et al., 2003).

Analysis of the yeast secretome-targeting sequences analyzed both the length and maximum hydrophobicity of potential ER-targeting sequences within the first 60 aa of each secretory pathway protein, generating a compound hydropathy score by multiplying both of these values. To identify the length of potential hydrophobic regions, each protein was analyzed via Phobius (Käll et al., 2004) to identify either SS or TM domains. The maximum hydrophobicity of potential hydrophobic regions was plotted with a sliding window of 9, 11, or 19 utilizing the Kyte-Doolittle scale. Further information is available in the Extended Experimental Procedures.

To identify enriched biological themes and functional-related gene groups, the resulting SRP-dependent, -independent, and combined protein lists were analyzed via DAVID (Huang et al., 2009) using the yeast secretome protein list as background.

Yeast Strains and Strain Construction

All yeast strains in this study are based on the BY4741 laboratory strain (Brachmann et al., 1998). Manipulations were performed using a standard PEG/LiAC protocol (Gietz and Woods, 2002). General laboratory strains and strains created in this study are listed in Table S5. Unless otherwise stated, deletion or hypomorphic allele strains were taken from the yeast deletion (Giaever et al., 2002) or the DAmP (Decreased Abundance by mRNA Perturbation) libraries (Breslow et al., 2008), respectively. All deletion strains were verified using primers from within the endogenous open reading frame (ORF). Further information is present in the Extended Experimental Procedures and Table S5.

Fluorescence Microscopy

Fluorescence microscopy was performed on yeast cells grown to mid-log using an Olympus IX71 microscope controlled by the Delta Vision SoftWoRx 3.5.1 software with 100× oil lens. Images were captured by a Phoetometrics-Coolsnap HQ camera with excitation at 490/20 nm and emission at 528/38 nm (GFP) or excitation at 555/28 nm and emission at 617/73 nm (mCherry/RFP). Images were transferred to Adobe Photoshop CS3 for slight contrast and brightness adjustments.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, six figures, and five tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2013.02.003.

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