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ABSTRACT

Hundreds of eukaryotic membrane proteins are anchored to membranes by a single transmembrane domain at their C-terminus. Many of these tail-anchored (TA) proteins are post-translationally targeted to the endoplasmic reticulum (ER) membrane for insertion by the Guided-Entry of TA protein insertion (GET) pathway. In recent years most of the components of this conserved pathway have been biochemically and structurally characterized. Get3 is the pathway targeting factor that utilizes nucleotide-linked conformational changes to mediate the delivery of TA proteins between the GET pre-targeting machinery in the cytosol and the transmembrane pathway components in the ER. Here we focus on the mechanism of the yeast GET pathway and make a speculative analogy between its membrane insertion step and the ATPase-driven cycle of ABC transporters.

INTRODUCTION

The mechanism of membrane protein insertion into the endoplasmic reticulum (ER) has been extensively studied for many years (Shao and Hegde, 2011). From this work, the signal recognition particle (SRP)/Sec61 pathway has emerged as a textbook example of a co-translational membrane insertion mechanism (Grudnik et al., 2009). The SRP binds a hydrophobic segment (either a cleavable N-terminal signal sequence or a transmembrane domain) immediately after it emerges from the ribosomal exit tunnel. This results in a translational pause that persists until SRP engages its receptor in the endoplasmic reticulum (ER) and delivers the ribosome-nascent chain complex to the Sec61 channel. Lastly, the Sec61 channel enables protein translocation into the ER lumen along with partitioning of hydrophobic transmembrane domains into the lipid bilayer through the Sec61 lateral gate (Rapoport, 2007).

Approximately 5% of all eukaryotic membrane proteins have an ER targeting signal in a single C-terminal transmembrane domain that emerges from the ribosome exit tunnel following completion of protein synthesis and is not recognized by the SRP (Stefanovic and Hegde, 2007). Nonetheless, because hydrophobic peptides in the cytoplasm are prone to aggregation and subject to degradation by quality control systems (Hessa et al., 2011), these tail-anchored (TA) proteins still have to be specifically recognized, shielded from the aqueous environment, and guided to the ER membrane for insertion. In the past five years, the Guided Entry of TA proteins

(GET) pathway has come to prominence as the major machinery for performing these tasks and the enabler of many key cellular processes mediated by TA proteins including vesicle fusion, membrane protein insertion, and apoptosis. This research has rapidly yielded biochemical and structural insights (Tables 1 and 2) into many of the GET pathway components (Chartron et al., 2012a; Denic, 2012; Hegde and Keenan, 2011). In particular, Get3 is an ATPase that uses metabolic energy to bridge recognition of TA proteins by upstream pathway components with TA protein recruitment to the ER for membrane insertion. However, the precise mechanisms of nucleotide-dependent TA protein binding to Get3 and how the GET pathway inserts tail anchors into the membrane are still poorly understood. Here, we provide an overview of the budding yeast GET pathway with emphasis on mechanistic insights that have come from structural studies of its membrane-associated steps and make a speculative juxtaposition with the ABC transporter mechanism.

TA protein capture by the GET pathway

The first step in all membrane protein insertion pathways is the selective capture of substrates. The C-terminal hydrophobic anchors of TA proteins don't interact efficiently with SRP after they emerge from the ribosome exit tunnel following completion of protein synthesis (Stefanovic and Hegde, 2007). Instead, they are shielded from the aqueous environment by the pre-targeting complex of the GET pathway, which comprises Sgt2 (a small glutamine-rich tetratricopeptide repeat (TPR)-containing protein;), Get4, and Get5 (Figure 1). Sgt2 binds directly to tail

anchors via its C-terminal domain, which is able to discriminate between TA proteins destined for the ER and those destined for the mitochondria (Wang et al., 2010). Defining the structural basis of substrate recognition by this Sgt2 domain has been hampered by its poorly-folded nature (Chartron et al., 2011) but we have more structural information on the other Sgt2 domains and components of the pre-targeting complex. The central TPR domain of Sgt2 has a canonical structure that enables it to associate with a variety of chaperones (Chartron et al., 2011; Kohl et al., 2011; Wang et al., 2010). Sgt2's N-terminal domain mediates both homodimerization and binding to the central, ubiquitin-like domain of Get5 (Chang et al., 2010; Chartron et al., 2011; Liou and Wang, 2005). The C-terminal domain of Get5 assumes a novel, helical bundle fold that mediates homodimerization (Chartron et al., 2012b). Get4 is an elongated, α-helical solenoid (Bozkurt et al., 2010; Chang et al., 2010; Chartron et al., 2010) that forms a tight complex with the N-terminus of Get5 at one end and a more labile complex with Get3 at the other (Chang et al., 2010; Chartron et al., 2010). In addition to this wealth of piecemeal structural information, a key insight into the function of Get4/5 came from the observation that these components of the GET pathway facilitate transfer of TA proteins from Sgt2 to Get3 by a dual mechanism (Wang et al., 2010): they increase the local concentration of Get3 near TA proteins held by Sgt2 and make Get3 more receptive for substrate binding. The precise stoichiometry and mechanistic details of the pre-targeting step in the GET pathway remain to be worked out. For example, do Get4/5 enable a TA protein "hand-off" mechanism between Sgt2 and Get3 during which the substrate's hydrophobic anchor is transiently bound to both TA protein

chaperones? What mechanism makes Get3 more receptive to bind TA proteins when it becomes transiently recruited to the pre-targeting complex? Regardless of these details, the baroque nature of the pre-targeting step in the GET pathway suggests that this mechanism is being used to rapidly and selectively channel the appropriate substrates from the ribosome to Get3.

TA protein recognition by the Get3 ATPase

Get3 is an ATPase of the SIMIBI class of NTP binding proteins (Leipe et al., 2002). The function of Get3 is to shuttle TA proteins between the pre-targeting complex in the cytoplasm and the transmembrane components of the GET pathway in the ER (Schuldiner et al., 2008). Numerous crystal structures of fungal Get3 proteins in different nucleotide-bound states have yielded a plausible structural model for substrate recognition by Get3 (Simpson et al., 2010). Specifically, Get3 is a homodimer with subunit interactions stabilized by a coordinated zinc ion. Each monomer comprises a core nucleotide-binding domain (NBD) with interspersed α-helical insertions. Several pieces of evidence argue that these helices comprise the TA protein-binding domain (TABD). First, the TABD helices are amphipathic and enriched in methionine and hydrophobic residues, a structural feature shared with the M-domain of the SRP, which is involved in signal sequence binding (Hainzl et al., 2011; Janda et al., 2010; Keenan et al., 1998; Rosendal et al., 2003). Moreover, hydrophilic mutations at these residues result in reduced TA protein binding to Get3 (Mateja et al., 2009). Second, in certain nucleotide bound states (see below),

the TABD becomes structured into a large, composite hydrophobic groove with contributions from both monomers and the appropriate dimensions to receive an alpha helical tail anchor (Mateja et al., 2009). Lastly, hydrogen-deuterium exchange mass spectrometry experiments have revealed that specific parts of the TABD are protected from exchange by TA protein binding (Bozkurt et al., 2009).

While the TABD is undoubtedly the site of TA protein binding to Get3, the precise stoichiometry of Get3-TA protein complexes is a matter of debate. Small-angle x-ray scattering and analytical ultracentrifugation studies with fungal Get3 homologs have found that Get3 bound to TA proteins is a tetramer (Bozkurt et al., 2009; Suloway et al., 2012). This view is further supported by the crystal structure of an archaeal Get3 homolog showing a tetramer with a head-to-head arrangement of TABDs (Suloway et al., 2012). It is worth pointing out that tetrameric Get3 species bound to TA proteins come from proteins co-overexpressed in *E. coli* in the absence of the pre-targeting pathway components. Future studies should establish the stoichiometry of Get3-TA protein complexes assembled following TA protein delivery by Get4/Get5/Sgt2 (Wang et al., 2010).

The crystal structures of the Get3 dimer have also revealed how nucleotide-dependent conformational changes in the NBD are propagated to the TABD (Figure 2). Specifically, in the apo and Mg^{2+}-free ADP states, the Get3 dimer is “open”, while $ADP{\cdot}Mg^{2+}$ and $AMPPNP{\cdot}Mg^{2+}$ induce a “closed” conformation. Furthermore, in the transition state of ATP hydrolysis (mimicked by $ADP{\cdot}AlF_4^{-}{\cdot}Mg^{2+}$), Get3 assumes a

"fully-closed" state in which the hydrophobic groove of the TABD is assembled. A recent free energy calculation study of the Get3 opening and closing pathway (Wereszczynski and McCammon, 2012) corroborates these findings and, in addition, postulates that Get3 can adopt a "wide open" apo state, as well as a "semi-open," asymmetric nucleotide conformation with one ADP and one ATP. This latter conformation might be reminiscent of the one observed in a crystal structure of Get3 complexed with a piece of its ER receptor (see below). In sum, these experimental and computational studies have revealed a plausible mechanistic framework for how the Get3 nucleotide-linked conformational changes coordinate the pre-targeting and membrane-associated steps of the GET pathway.

Membrane recruitment of Get3-TA protein complexes

Following the pre-targeting events in the GET pathway, Get3-TA protein complexes are recruited to the ER membrane by Get1 and Get2 (Figure 1, steps 2 and 3) (Schuldiner et al., 2008). These two membrane proteins each have three transmembrane domains that mediate formation of a stable transmembrane complex (Mariappan et al., 2011). The intimate nature of this association is well illustrated by the observation that deletion of either Get1 or Get2 leads to a significant reduction in the concentration of the partner protein (Mariappan et al., 2011; Schuldiner et al., 2008; Wang et al., 2011). Two recent structural studies argue that the N-terminal cytoplasmic domain of Get2 mediates the initial contact between the Get3-TA protein complex and Get1/2 (Mariappan et al., 2011; Stefer et

al., 2011). Specifically, even though the isolated cytoplasmic domain of Get2 (~140 amino acids) is for the most part unstructured, it can form a 2:2 complex with Get3. Crystal structures of this complex have revealed a pair of short Get2 helices (α1 and α2) that interact with Get3. Importantly, the Get3 dimer can interact with Get2 in a closed, nucleotide-bound state that is compatible with TA protein binding. This is because the Get2 binding site on Get3 is a monomeric epitope located away from the homodimerization interface. Binding is for the most part mediated by electrostatic interactions between the conserved RERR sequence on the Get2 α1 helix and the negatively charged surface patch on Get3's NBD that includes the conserved DELYED motif. Importantly, point mutations in the RERR sequence abolish Get3/Get2 complex formation and TA protein insertion into ER-derived membranes (microsomes).

TA protein release from Get3

Like Get2, Get1 has a large cytoplasmic domain (~80 amino acids) that in isolation forms a stable 2:2 complex with Get3 (Mariappan et al., 2011; Stefer et al., 2011). Structural studies of this complex support the idea that Get1 induces TA protein release from Get3 (Figure 1, steps 3 and 4) (Kubota et al., 2012; Mariappan et al., 2011; Stefer et al., 2011). In particular, the cytoplasmic domain of Get1 is a coiled coil of two α-helices (α1b and α2) stabilized by hydrophobic contacts similar to leucine zippers. In striking contrast to the Get3/Get2 complex, Get3 is bound to the coiled coils in an open conformation that lacks nucleotide (or has ADP, see Figure 2)

and has a disrupted TABD. This is because each coiled coil binds to both Get3 monomers via a composite epitope that is largely occluded in the closed Get3 conformation. Few interactions occur between Get1 α1b and the TABD helix α4 on one Get3 monomer but more extensive contact is established by interactions between Get1 α2 and NBD helices α10 and α11, which include the Get3 DELYED motif recognized by Get2, on the opposing monomer. Notably, Get1 mutations that disrupt interactions with the DELYED motif abolish Get3/Get1 complex formation and TA protein insertion into microsomes. The overlap between the primary Get3/Get1 interface and the Get2 binding site implied that Get1 and Get2 compete for binding to Get3. This hypothesis was confirmed by an NMR analysis showing that Get1 can displace the Get2 α2 helix from its binding site on Get3 while still allowing the Get2 α1 helix to remain bound in the ternary complex (Stefer et al., 2011). Attempts to crystallize this ternary complex revealed instead the structure of a new Get3/Get1 complex with a novel Get3 conformation: a semi-open state with respect to the closed AMPPNP-$Mg^{2+}$and ADP-Mg^{2+} bound structures and the open apo form (Stefer et al., 2011) (Figure 2). Interestingly, the semi-open and open Get3 makes similar contacts with the Get2 α2 helix. Taken together with a related semi-open Get3/Get1 structure (Kubota et al., 2012), these studies suggest that the Get1 α2 helix initiates TA protein release from Get3 tethered to Get2.

The structural model for the membrane-associated steps of the GET pathway is supported by biochemical reconstitution studies (Mariappan et al., 2011; Wang et al., 2011). In particular, TA proteins remain bound to Get3 upon Get2 binding, but

Get1 binding triggers substrate release from Get3. Importantly, even though high concentrations of the Get1 coiled coil can drive substrate release, under physiological conditions, the Get2 cytoplasmic domain is essential to increase the local concentration of Get3-TA protein complexes near Get1. Moreover, quantitative measurements of Get3 binding to Get1 have shown that in the ATP-bound state Get3 does not interact with Get1 while the apo form binds Get1 with high affinity (Kubota et al., 2012; Mariappan et al., 2011; Stefer et al., 2011). Indeed, in the crystal structures of the Get3-Get1 complex, the tip helix of the Get1 coiled-coil protrudes into the nucleotide-binding site of Get3 suggesting that Get1 is a nucleotide exchange factor for Get3. Regardless of the details, ATP binding drives dissociation of Get3 from the membrane (Figure 1, step 5) and prepares it for another round of substrate loading by the pre-targeting complex.

The insertion step of the GET pathway: a speculative analogy to ABC transporters

The membrane insertion step remains the least well-understood step in the GET pathway. Proteoliposomes with purified Get1/2 afford the minimal membrane machinery necessary for TA protein insertion (Mariappan et al., 2011; Wang et al., 2011) but whether the transmembrane domains of Get1 and Get2 play an active role during this step is not known. A variety of mechanisms for Get1/2-mediated tail anchor insertion have been hypothesized (Chartron et al., 2012a; Denic, 2012; Hegde and Keenan, 2011) and we add to this a speculative analogy to the substrate

transport mechanism of ATP-binding cassette (ABC) transporters (Figure 3). ABC transporters couple the energy of ATP hydrolysis to the transport of diverse substrates across the membrane. In general, they consist of two sets of transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs) that bind and hydrolyze ATP. Most ABC transporter NBDs are monomers in the absence of their TMDs but become dimerized in the presence of non-hydrolyzable ATP analogs. At first glance, the mechanistic requirements of ABC transporters and Get1/2/3 membrane machinery might seem disparate. However, closer inspection reveals a striking commonality: ATP binding drives rotation of both types of NBDs towards each other to create an extensive dimer interface. In ABC transporters, changes in the NBD conformation induced by binding and hydrolysis of ATP are transmitted to the neighboring TMDs via coupling helices. The resulting flipping of TMDs from an inward to an outward facing conformation drives substrate movement across the membrane. For TA protein insertion one could envisage a similar scenario whereby Get1 coiled-coils act as coupling helices that translate movements associated with Get3 dimer opening into conformational changes in the flanking transmembrane domains (Figure 4). Future structural and functional analysis of the Get1/2 transmembrane complex should establish if its transmembrane domains carry out the insertion step of the GET pathway.

A working model for the GET pathway

In summary, in a relatively short period of time, a detailed mechanistic framework for the GET pathway has emerged (Figure 1). ATP·Mg^{2+}-bound Get3 receives a newly synthesized TA protein from the Get4/Get5/Sgt2 pre-targeting complex. ATP hydrolysis, which probably occurs upon substrate binding to Get3, ensures the formation of a stable, closed Get3-TA protein complex containing the hydrolyzed nucleotide (step 1). This complex is recruited to the ER membrane by the interaction with Get2, which tethers it into proximity with Get1 (step 2). Binding of Get1 to Get3 displaces Get2 and induces Get3 dimer opening to release the TA protein substrate and hydrolyzed nucleotide (steps 3 and 4). These changes in the Get3 dimer conformation might be directly coupled to the Get1/2 transmembrane domains to facilitate the membrane insertion step. Lastly, binding of ATP to Get3 weakens the Get3-Get1 interaction to recycle Get3 for a new round of pre-targeting (step 5).

Arguably, the most challenging problem that remains in the field is to define the structural and biochemical basis of the insertion step. For example, does the release of substrate and hydrolyzed nucleotide from Get3 generate a “power stroke” that enables Get1/2 transmembrane domains to catalyze the insertion step. Furthermore, detailed biophysical and modeling studies will also be needed to turn the structural snapshots of the Get3 ATPase cycle into a movie of TA protein insertion. Together with characterization of conserved pathway components in higher eukaryotes and archaea, studies of the yeast GET pathway are well on their

way to adding another textbook example of how cells chaperone membrane proteins into lipid bilayers.

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FIGURE LEGENDS

Figure 1. Scheme of the steps in the yeast GET pathway

The cytosolic pre-targeting complex (Get4/Get5/Sgt2) captures a newly synthesized tail-anchored (TA) protein released from the ribosome (with differentially shaded large and small subunits) and transfers it to Get3 loaded with ATP (1). The Get3-TA protein complex is subsequently tethered to the endoplasmic reticulum (ER) membrane by the cytosolic domain of Get2 (2) and then docked to the Get1 coiled-coil (3). This latter interaction drives TA protein release by forcing open the Get3 dimer (4). Finally, rebinding of ATP dissociates Get3 from the membrane and prepares it for the next round of substrate pre-targeting (5). The precise timing of ATP hydrolysis is not known but we have several reasons to favor a model in which it occurs upon TA protein binding to Get3. First, the crystal structures of Get3 that have an assembled substrate-binding groove also have an ATP hydrolysis transition-state analog. Second, Get3 ATPase activity is essential for the TA protein release step. Third, the Get1 coiled coil preferentially binds to the apo form of Get3. Also note that for simplicity the higher-order stoichiometry of the pre-targeting complex is not shown, while the stoichiometry of the Get1/2 transmembrane complex is hypothetically drawn as a 2:2 tetramer.

Figure 2. Get3 conformations during its ATPase cycle

Get3 is a homodimeric ATPase (monomers are in blue and green) that comprises an α-helical TA protein-binding domain (TABD) and a nucleotide-binding domain

(NBD). The upper panels show cartoon representations of the Get3 dimer, while the middle and lower panels show the corresponding crystal structures from the side or top, respectively (see Table 2 for the PDB entry codes). These structures are presented as a sequence of conformational changes that might accompany the Get3 ATPase cycle. Get3 bound to ATP most likely resembles the closed dimer conformation stabilized by AMPPNP-Mg^{2+} (a non-hydrolyzable ATP analog). In the process of ATP hydrolysis, a state stabilized by the ATP transition state analog $ADP{\cdot}AlF_4^-$ -Mg^{2+}, Get3 assumes a full-closed conformation that leads to the assembly of a composite, hydrophobic groove in the TABD. Following release of inorganic phosphate (P_i), Get3 reverts to a more relaxed closed state similar to the one preceding hydrolysis. Lastly, in the absence of Mg^{2+}, Get3-ADP assumes an open conformation, which also resembles the nucleotide-free (apo) state. All Get3 structures have been superimposed by the blue monomer to illustrate that nucleotide-driven dimer opening and closing is not simply a scissor-like motion, but involves substantial rotation of the monomers.

Figure 3. ABC transporters and Get1/3 interactions: a structural comparison

The upper left panel is a cartoon of a hypothetical 2:2 Get1/2 tetrameric transmembrane complex (Get1 subunits are in yellow and red; Get2 cytoplasmic domains are eliminated for simplicity) bound to a Get3 dimer (monomers are in blue and green; nucleotide and tail-anchor binding domains, NBD and TABD, respectively, are indicated) via the cytosolic coiled coil domains of Get1. Both Get1 and Get2 are predicted to have three transmembrane domains (TMDs) each. The upper right panel is a cartoon of a generic ABC transporter with TMDs (ranging in number from 10-20) coupled to nucleotide-binding domains (ABC) by special alpha helices (cylinders). The lower panels are crystal structures of Get1 coiled-coils complexed with Get3 and the multidrug ABC transporter Sav1866 (Dawson and Locher, 2007) (the dashed line indicates the cytosolic border of the membrane) with structural elements color-coded to suggest similarities between the two structures. Note that Sav1866 is a dimer with domain swapped interactions between the coupling helices and nucleotide-binding domains. Furthermore, the ABC domains are in a closed conformation, whereas the Get3 dimer is open (see Figure 4 for further details).

Figure 4. Hypothetical structural model for the insertion step of the GET pathway

Get1 coiled coils might enable coupling between the opening of the Get3 dimer and movement of the transmembrane domains (TMDs). The pre-docking complex (left) is a hypothetical model of the Get3-Get1 interaction based on the fully-closed Get3 state (2WOJ). Get1 docking causes the opening of the Get3 dimer via a semi-open state. Also shown are the associated hypothetical movements of Get3 relative to the

membrane (from near to distant), as well as the coupled conformational changes in the Get1/2 TMDs (from wide to narrow).

Table 1: A catalog of GET pathway component structures

Table 2: An itemized list of Get3 structures and any associated co-factors

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