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Structural Basis for Tail-Anchored Membrane Protein Biogenesis by the Get3-Receptor Complex

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Abstract

Tail-anchored (TA) proteins are involved in cellular processes including trafficking, degradation, and apoptosis. They contain a C-terminal membrane anchor and are posttranslationally delivered to the endoplasmic reticulum (ER) membrane by the Get3 adenosine triphosphatase interacting with the hetero-oligomeric Get1/2 receptor. We have determined crystal structures of Get3 in complex with the cytosolic domains of Get1 and Get2 in different functional states at 3.0, 3.2, and 4.6 angstrom resolution. The structural data, together with biochemical experiments, show that Get1 and Get2 use adjacent, partially overlapping binding sites and that both can bind simultaneously to Get3. Docking to the Get1/2 complex allows for conformational changes in Get3 that are required for TA protein insertion. These data suggest a molecular mechanism for nucleotide-regulated delivery of TA proteins.

Sophisticated mechanisms have evolved to target eukaryotic membrane proteins to the correct membrane-surrounded organelle and to protect them from aggregation. Of central importance are cytosolic factors that decode the targeting information of a signal sequence by transient association and escort membrane proteins to their designated location (1, 2). Tail-anchored membrane proteins (TA proteins) consist of a N-terminal soluble domain and a single C-terminal transmembrane domain (TMD) (3–5). The C-terminal localization of their targeting signal requires release of the synthesized protein from the ribosome before interaction with an insertion machinery (6). This precludes TA proteins from cotranslational

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Supporting Online Material

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insertion mediated by the signal recognition particle (SRP)–Sec61 translocon system (7–9), which mainly targets membrane proteins with a N-terminal signal sequence. Although some studies suggest an unassisted insertion of TA proteins into the mitochondrial outer membrane (10, 11), most endoplasmic reticulum (ER)–destined TA proteins are thought to insert by an energy-dependent process, which involves several cytosolic factors (2, 12–17). In yeast, it was shown that the adenosine triphosphatase (ATPase) Get3 is necessary for the biogenesis of TA proteins (2, 18). Get3 is a dimeric protein with each subunit comprising a nucleotide-binding domain (NBD) and a methionine-rich α -helical domain that has been implicated in TA protein binding (TA binding domain, TABD). Crystal structures of Get3 have shown that the protein switches between an open and closed state, depending on its nucleotide load. Whereas in the apo and magnesium-free adenosine diphosphate (ADP) forms the open state is favored (19–23), ADP-Mg²⁺ and the nonhydrolyzable ATP analog 5'-adenylyl- β , γ -imidodiphosphate-Mg²⁺ (AMPPNP-Mg²⁺) induce the closed state (19), which is further tightened up in the transition state of adenosine triphosphate (ATP) hydrolysis (20). The hydrophobic groove responsible for TA binding seems fully assembled only in the transition state (20). At the membrane, Get3 interacts with the two receptor proteins, Get1 and Get2, which are essential for TA protein insertion (2). In order to understand the molecular mechanisms underlying Get3-dependent membrane insertion of TA proteins, we determined crystal structures of Get3 in complex with the soluble domains of its membrane-bound Get1 and Get2 receptors and functionally characterized the interaction in different nucleotide-bound states.

Get1 and Get2 are integral membrane proteins, and each comprises three TMDs (Fig. 1A). In order to analyze the Get3-receptor interaction in more detail, we performed flotation assays of Get3 with microsomal membranes (RMs) (Fig. 1B) and insertion assays with the TA protein Sec22 [a soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein] in the presence of different nucleotides (Fig. 1C) (2, 14, 15). Efficient TA protein insertion was observed in presence of ATP and ADP, but not with adenosine 5'-*O*-(3-thiotriphosphate) (ATP- γ -S)—in line with Get3 recruitment observed in the flotation assays. Get1-CD (residues 19 to 103) is the most conserved region of Get1 and predicted to adopt a coiled-coil structure (24) with two long helices (α 1 and α 2) extending the flanking transmembrane regions (Fig. 1A and fig. S1A), similar to its human homolog, the tryptophan-rich basic protein, WRB (25, 26). Using a pull-down approach, we found that detergent-solubilized Get1 efficiently binds to Get3 (fig. S2A). Additional truncation constructs demonstrated that Get1-CD is both necessary and sufficient for Get3 binding (fig. S2A). Furthermore, size-exclusion chromatography, combined with multiangle light scattering, established that the Get3/Get1-CD complex has a 2:2 stoichiometry (fig. S2B). To quantitatively monitor effects of nucleotide binding to Get3 on the stability of the Get3/Get1-CD complex, we used surface plasmon resonance (SPR) with wild-type Get3 and an ATP hydrolysis-deficient mutant (D57N) in different nucleotide loads (Fig. 1D). Get3 displays high affinity for Get1-CD in both the apo (K_d = 17 nM) and ADP (K_d = 31 nM) states; the affinity is dramatically decreased for the ATP- γ -S-bound state (Fig. 1D, table S1, and fig. S3). Taken together, these data confirm the nucleotide dependence of the interaction and show that TA protein insertion requires the presence of nucleotides that are able to induce the closed state and allow the transition to the open state (Fig. 1, B to D). To dissect the contribution of the two receptor proteins, membrane insertion assays were performed with membranes derived from yeast strains harboring Get1 and Get2 variants mutated in highly conserved regions (Fig. 1E). Membranes derived from a Get1/2 deletion strain did not support insertion of Sec22, and mutations in either Get1 or Get2 severely reduced the insertion efficiency, which indicates that both receptor proteins are required for membrane insertion.

To elucidate the molecular mechanisms by which Get1-CD binding to Get3 enables TA protein insertion, we determined the crystal structure of the Get3/Get1-CD* complex at 3.0 Å resolution. All data collection and refinement statistics are given in table S2. For simplicity, the structure of Get1-CD* is referred to as Get1. Get3/Get1 is a symmetric heterotetramer with two Get1 molecules bound at the interface of the Get3 homodimer (Fig. 2, A and B, and fig. S4), and Get3 is in the open state (fig. S5) (22). Get1 inserts like a wedge into the homodimer and complements the dimer interface, interacting with both Get3 monomers. Unlike most other reported Get3 structures (19–23), the flexible TABD is almost fully defined (Fig. 2A). Although crystals were grown in presence of ADP-Mg²⁺ or AMPPNP-Mg²⁺, nucleotide is not present in the structure.

Get1 helices $\alpha 1b$ and $\alpha 2$ form a typical coiled-coil stabilized by a hydrophobic (leucine) zipper (Fig. 2A and fig. S1). The tip region of the coil includes an α -helical turn (αt , tip helix) (Fig. 2D) and contains a conserved S(A/S)QD(N/E) sequence motif (Fig. 2C). Get1 contacts both Get3 monomers. However, the two contacts differ substantially (Fig. 2B). One Get3 monomer shares an extensive interface with Get1 (interface I), which is primarily formed between $\alpha 2^{Get1}$ and helices $\alpha 10$ and $\alpha 11$ of the Get3 NBD. Conserved and predominantly aromatic residues cluster between helix $\alpha 2^{Get1}$ (Y65, A66, and W68) and helices $\alpha 10$ (F246, L249, and Y250) and $\alpha 11$ (Y298 and L305) of Get3, and a salt bridge between the invariant R73^{Get1} and E253 is stabilized by L305 and Y306 from the conserved (D/E)ELYE(D/E) motif (residues 303 to 308, subsequently termed DELYED) contributed by helix $\alpha 11$ (Fig. 2C). Mutagenesis studies underline the importance of interface I, as alanine replacement of the two glutamates within the Get3 DELYED motif and of arginine 73 in Get1 disrupted both the Get3/Get1 interaction (fig. S3 and table S1) and Sec22 insertion into microsomes (Fig. 1E and fig. S6, A and B).

The contact with the second monomer is rather small (interface II) and involves helix $\alpha 4$ of the Get3 TABD (Fig. 2B). The tip of Get1 protrudes into the active site, which suggests a function of Get1 in regulation of the Get3 nucleotide interaction. Compared with the open state of Get3 (22), the tip of Get1 reconfigures both switch I (57-DPAHN) and switch II (166-DTAPTGH) (Fig. 2D). Switch I is shifted by about 3 Å toward the nucleotide-binding site, and switch II moves by about 2 Å toward the P-loop. The observed conformations of switch I and II are incompatible with bound nucleotide-Mg²⁺, which could be released by a lateral exit tunnel observed in the Get3/Get1 complex (Fig. 2E). Thus, interface I seems to provide the observed high affinity and specificity to the interaction, whereas interface II seems to be involved in regulation. This structural analysis is consistent with the SPR and flotation experiments described above. In addition, a release experiment revealed that Get3 dissociates from Get1 when ATP or ATP- γ -S is added (fig. S8B). Taken together, these characteristics of the Get3/Get1 interaction are reminiscent of a nucleotide exchange factor and suggest that binding of ATP prohibits rebinding of Get1.

The receptor proteins Get1 and Get2 are both necessary for TA protein insertion (Fig. 1E), and in the absence of Get2, the cellular level of Get1 is decreased (fig. S6C) and vice versa (2). Get2 also contains a cytosolic domain (residues 1 to 151) (Fig. 1A), which is predominantly unfolded (fig. S7), and only a short stretch at the N terminus is conserved (residues 1 to 35) (Fig. 3A). Pull-down assays and nuclear magnetic resonance (NMR) data show that Get1-CD and Get2-CD can bind to Get3 individually as well as simultaneously (fig. S8A and fig. S10). We attempted to crystallize the ternary complex, but structure determination at 3.2 Å resolution showed that the crystals were made up of only the Get3/Get1 complex without any nucleotide bound, even though ADP or AMPPNP was present during crystallization (Fig. 3B). Compared with the closed AMPPNP-Mg²⁺- and ADP-Mg²⁺-bound structures (19) and the open form (20–23), Get3 here is in a semiopen state, which was not observed before. Interface I, described above for the open Get3/Get1

complex, stays intact, whereas interface II is different (Fig. 3C). The tip of Get1 does not reach into the active site of the second monomer, as seen in the open state (Fig. 2, B and D). In the semiopen Get3/Get1 complex, the active site is shielded within the Get3 dimer interface, and the switch regions are not perturbed. Analysis of the two Get3/Get1 structures suggests that Get1 can stay bound to Get3 by interface I during the transition from the fully closed to the open state (fig. S11).

To visualize the interaction of Get3 with Get2, we solved the structure of the Get3/Get2-CD* complex at 4.6 Å resolution with ADP-Mg²⁺ bound (the structure of Get2-CD* is referred to as Get2) (Fig. 1A). Get3/Get2 is also a symmetric heterotetramer with Get2 bound to the closed Get3 homodimer (Fig. 3D). In contrast to Get1, Get2 binds away from the dimer interface such that each Get2 contacts only one Get3 subunit. Get2 binds to a negatively charged surface patch on Get3, including the DELYED motif, which also forms distinct contacts with Get1, but in a different manner (Figs. 2E and 3E). Get2 comprises two amphipathic, positively charged helices connected by a flexible glycine linker (Fig. 3A and fig. S7). In particular, the conserved 14-RERR motif in helix $\alpha 1^{\text{Get2}}$ forms ionic interactions with conserved negatively charged residues (D265, E307, and D308) in Get3. Mutagenesis studies underline the importance of this interface as reversed-charge mutations within the 14-RERR motif (to EREE) or an alanine substitution in the DELYED motif abolished TA protein insertion (Fig. 1E and fig. S6, A and B). The binding site of helix $\alpha 2^{\text{Get2}}$ overlaps with interface I described above for the Get3/Get1 complex, which also involves the DELYED motif and E253, which interacts with both Get1 and Get2 (fig. S9). We were able to identify Get3 mutations in the DELYED motif that selectively disrupt the Get2 interaction without an impact on Get1 binding (fig. S9A). As Get1 and Get2 can bind simultaneously to Get3 and share a partially overlapping binding site (fig. S8A and fig. S9), Get1 must displace helix $\alpha 2^{\text{Get2}}$, which is connected to helix $\alpha 1^{\text{Get2}}$ by the flexible glycine linker. NMR analyses revealed that Get1 binding indeed causes some Get2 interactions with Get3 to disappear. Specifically, interaction with Get2 was observed in the region of L4 to A49, and upon addition of Get1, residues G24 to A49 no longer interacted with Get3 (fig. S10). This shows that helix $\alpha 2^{\text{Get2}}$ is no longer bound to Get3 in the ternary complex.

On the basis of different crystal structures of Get3, we previously proposed a model for how the Get3 ATPase regulates TA protein insertion (19). With structures of different Get3-receptor complexes as well as functional data in hand, distinct docking states can be integrated into this model (Fig. 4). Assisted by Get4/5/Sgt2, TA proteins bind to Get3-ATP-Mg²⁺ (step 1). After ATP hydrolysis, the reaction products stay trapped, and the energy gained from hydrolysis is stored in a strained conformation (19). The N terminus of Get2 tethers the Get3/TA protein complex to the ER membrane (step 2). Binding of Get1 displaces $\alpha 2^{\text{Get2}}$, and the Get3/TA protein complex is now docked to the receptor complex at the membrane (step 3). When the TA protein is released, Get3 relaxes to the closed state, and inorganic phosphate dissociates (step 4). According to the crystal structures, Get1 can stay bound to Get3 during the transition from the closed to the open state. What actually triggers opening of Get3? We favor the idea that the energy from ATP hydrolysis drives Get3 to the open state, and ADP-Mg²⁺ leaves by way of the observed tunnels. In this state, Get1 interferes with nucleotide binding and prevents closure of the dimer. Finally, binding of ATP facilitates dissociation of Get3 (step 5), which sets the stage for the next targeting cycle. As Get1-CD is rigidly linked to the TMDs, structural changes observed in the Get3/Get1 complexes can be extrapolated to the complete membrane receptor (as indicated in Fig. 4 and fig. S11). The opening of Get3 during TA protein insertion may create a force that is directly transferred to the TMDs of the receptor, which could contribute to TA protein insertion. Related structural transitions have been reported for ATP-binding cassette (ABC) transporter proteins (27, 28). In Get1, the coiled-coil domain with the tip helix may have a function similar to the coupling helix in ABC transporters and may directly communicate

nucleotide-dependent changes in Get3 to the transmembrane segments as anticipated in the model above. It is now important to dissect the precise mechanism of TA protein insertion and to see whether a general concept can be derived that is shared by different membrane transport systems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

TA protein delivery to the ER membrane by nucleotide-dependent interaction of Get3 with the Get1/2 receptor. (A) Topology models of Get1 and Get2 and an overview of receptor constructs used in this study. Truncated versions of the cytosolic domains (CDs) are marked by an asterisk. (B) Flotation assays of FLAG-tagged Get3 in the presence of different nucleotides or in the apo form with a density gradient and Get1/2/ Δ 3 microsomes. Nonhydrolyzable ATP- γ -S inhibits targeting of Get3 to the membrane receptor and results in free Get3 at the bottom of the gradient (fraction 4). (C) Membrane insertion assays with Sec22 using Get1/2/3 microsomes to analyze the nucleotide dependence. In vitro translation (IVT) of opsin-tagged Sec22 was performed in the presence of 35 S-labeled methionine. Successful insertion is indicated by cargo glycosylation (gSec22). (D) SPR studies on the interaction of Get3 with Get1-CD in the presence of different nucleotides. ATP* indicates measurements with the hydrolysis-deficient D57N mutant and therefore correspond to ATP- γ -S. (E) Analysis of Get1/2 mutants in membrane insertion. Assays were performed as described in Fig. 1C. A Get1/Get2-deletion mutant is not insertion competent (Δ get1/ Δ get2). Mutations in Get2 (14-RERR to EREE) and in Get1 (72-NR to AA) completely abolish insertion, which highlights their equal importance.

Fig. 2.

Structure of the open Get3/Get1 complex. **(A)** Overall structure shown in views along (left, with indicated domains) and from the membrane (right). Get3 is depicted in blue and green, Get1 in orange and red, respectively. Phosphate ions are drawn as sticks. **(B)** Close-up of the Get3/Get1 interface. Side chains of residues important for the interaction are shown. The major binding site (interface I) includes helix $\alpha 2$ of Get1 and helices $\alpha 10$ and $\alpha 11$ of Get3 (blue subunit). The DELYED motif (residues 303 to 308) is located at the C terminus of helix $\alpha 11$. The tip of the Get1 coiled-coil contacts the switch I region of the second Get3 subunit (green, interface II). **(C)** Multiple sequence alignments of the Get3 DELYED motif (top) and the conserved tip region of Get1 (bottom). The respective secondary structures are given on top of the alignments. Color coding is according to conserved charge or hydrophobicity. For species see fig. S1. **(D)** Get1 is an ATPase effector. Superposition of the Get3 active site within the Get3/Get1 complex (colored as above) with the ADP bound Get3 structure (purple, PDB code 3A37). Get1 pushes the switch regions into the active site as indicated by arrows. Glutamine Q62 within the tip helix (αt) directly binds to the P-loop. **(E)** Surface potential of the Get3/Get1 complex. Positive charges are indicated in blue, negative charges in red. The view is the same as in Fig. 2A (left). The tunnel leading to the

active site of Get3 (shown with modeled ADP) is indicated by an arrow. The prominent negative surface patch on Get3 involving the DELYED motif is marked by an oval.

Fig. 3.

Structure of the semiopen Get3/Get1 and the closed Get3/Get2 complexes. **(A)** Multiple sequence alignment of Get2 (for species, see fig. S7). The secondary structure is given above the sequences with the RERR motif highlighted. The two helices are connected by a flexible glycine linker. **(B)** Membrane view of the semiopen Get3/Get1 complex. The complex represents the state halfway between the closed and open Get3 structures. **(C)** Close-up as shown in Fig. 2B. Although interface I is identical to the open Get3/Get1 complex, interface II changed completely, and Get1 does not contact the switch I region, which is unperturbed compared with the ADP structure shown in Fig. 2D. **(D)** Membrane view of the closed Get3/Get2 complex with bound ADP-Mg²⁺ shown in sticks. Get2 (yellow) forms two helices ($\alpha 1$, $\alpha 2$) binding at the periphery of Get3 and contacting only one Get3 subunit. **(E)** Close-up of the Get3/Get2 interface. Get2 wraps around the DELYED motif (cyan) of Get3 that forms a binding platform. The conserved RERR motif (same color as in Fig. 3A) in the center of helix $\alpha 1^{\text{Get2}}$ recognizes the DELYED motif and, together with the additional positive charges, reads out the negative surface patch of Get3 shown in Fig. 2E.

Fig. 4.

Model for TA protein targeting and insertion by the Get pathway. TA protein binding (step 1) locks Get3 in the fully-closed state as inferred from the transition state structure with $\text{ADP} \cdot \text{AlF}_4^-$ (PDB code 2WOJ), and bound ATP is hydrolyzed to $\text{ADP} \cdot \text{P}_i$. Get3/TA is tethered to Get2 (step 2) as deduced from the Get3/Get2 structure and Get1 associates with the Get3/TA/Get2 complex, which results in an insertion competent complex (step 3). Release of the TA protein relaxes Get3 into the closed state, which allows for P_i release (step 4). The stored energy of ATP hydrolysis drives Get3 via the semiopen to the open state as observed in the Get3/Get1 structures and $\text{ADP} \cdot \text{Mg}^{2+}$ is released. Get3 dissociates from the membrane by ATP binding and adopts the closed conformation (step 5). The transition from closed to open Get3 reduces the distance between the two Get1 protomers, whereas the distance between the zinc ion (black dot) and the membrane increases (distances are indicated by arrows, see also fig. S11).