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Preliminary X-ray crystallographic studies of yeast Get3

Tail-anchored (TA) proteins contain a single transmembrane domain (TMD) at the C-terminus. The post-translational insertion of TA proteins into the ER membrane requires the cooperation of the Golgi ER-trafficking (GET) complex, which contains Get1, Get2 and Get3. Get3 is a cytosolic ATPase which can recognize and bind the TMD of the TA proteins. Get1 and Get2 are ER transmembrane proteins which can recruit and form a complex with TA-bound Get3. The GET complex carries out an energy-dependent process that facilitates the insertion of the TA-protein TMD into the ER membrane. In order to investigate the mechanism by which the GET complex functions to promote protein insertion into the ER membrane, yeast Get3 has been crystallized. The crystals diffracted to 2.7 Å resolution using a synchrotron X-ray source. The crystals belonged to space group $P2_12_12$, with unit-cell parameters $a = 220.26$, $b = 112.95$, $c = 48.27$ Å. There is one Get3 dimer in the asymmetric unit, which corresponds to a solvent content of approximately 65%.

1. Introduction

How membrane proteins insert their transmembrane domains (TMD) into membranes is a fundamental question in membrane biology. Tail-anchored (TA) proteins represent a unique family of transmembrane proteins which contain a single transmembrane helix at the C-terminus. The N-terminal fragments of TA proteins are exposed to the cytosol. TA proteins can be found in all secretory pathways in the nuclear envelope, peroxisomes and mitochondria. Most TA proteins target their C-terminal TMD into the ER and mitochondria. TA proteins are present in all eukaryotic systems ranging from yeast to humans (Borgese *et al.*, 2007) and it has been estimated that the human genome encodes more than 400 TA proteins (Kalbfleisch *et al.*, 2007). The TA proteins localized at the ER membranes play central roles in protein secretion, folding, translocation and degradation (Borgese *et al.*, 2007; Osborne *et al.*, 2005). Well known TA protein examples include ER translocon member Sec61 β , SNAREs involved in vesicle trafficking, apoptosis-related protein Bcl-2 and PTP1B involved in signal transduction (Anderie *et al.*, 2007; Abell *et al.*, 2007; Kutay *et al.*, 1995; Kalbfleisch *et al.*, 2007; Walter *et al.*, 2001; Shi *et al.*, 2007; Setoguchi *et al.*, 2006; Kim *et al.*, 2004). Despite the diverse functional importance of the TA proteins, the mechanisms by which TA proteins are targeted or inserted into membranes are poorly understood.

The mechanisms by which TA proteins insert their TMD into membranes are distinct from the well studied co-translational insertion pathway, which is mediated by the cytosolic signal recognition particle (SRP), the ER-localized SRP receptor and the ER translocon formed by the Sec61 complex (Osborne *et al.*, 2005; Shan & Walter, 2005; Wickner & Schekman, 2005). Because TA proteins contain the TMD at the C-terminus, the TA proteins will be released into the cytosol from the ribosome, while the TMD remain in the ribosomal channel. This will prevent the TA protein utilizing the traditional co-translational membrane-insertion pathway.

It has been shown that TA-protein insertion into the ER membrane is ATP-dependent (Kutay *et al.*, 1995). A soluble cytosolic

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ATPase TRC40/Asna-1 has been identified to interact with the newly synthesized TA protein Sec61 β in the mammalian cell line by biochemical studies (Stefanovic & Hegde, 2007). The complex can then translocate to the ER membrane, where the TMD of Sec61 β can be incorporated into the ER membrane in an ATP-dependent fashion. Recently, yeast genetic and biochemical studies have shown that post-translational insertion of the TA proteins into the ER membrane requires the cooperation of the Golgi ER-trafficking (GET) complex, which contains Get1, Get2 and Get3 (Schuldiner *et al.*, 2008; Auld *et al.*, 2006). Get3 is the yeast homologue of TRC40 in mammals. Get3 can recognize and bind the TMD of the TA proteins. Get1 and Get2 are ER transmembrane proteins which can recruit and form a complex with the TA-bound Get3. The GET complex carries out an energy-dependent process that facilitates the insertion of the TA-protein TMD into the ER membrane. Complex formation by Get1, Get2 and Get3 ensures specific TA-protein insertion into the ER membranes.

Other cytosolic factors, such as the molecular chaperones Hsp40 and Hsp70, have been suggested to play roles in TA-protein insertion into ER membranes (Rabu & High, 2007; Rabu *et al.*, 2008). Hsp40 and Hsp70 may interact directly with the TMD of TA proteins and protect the aggregations of these hydrophobic proteins. However, no receptors have been identified on the ER membrane for Hsp40 and Hsp70, which indicates that Hsp40 and Hsp70 may only function as a backup system for the GET complex owing to the lack of specificity of Hsp40 and Hsp70 for ER membranes.

The yeast cytosolic ATPase Get3 and its mammalian counterpart TRC40 play a broad range of important roles in cell biology. Get3 has been shown to function as a molecular chaperone that renders metal and heat tolerance in yeast (Shen *et al.*, 2003). Get3 can also interact directly with G-protein and facilitate guanine-nucleotide exchange (Lee & Dohlman, 2008). Mammalian TRC40 has been shown to be highly expressed in pancreatic β cells and may function to regulate insulin secretion (Kao *et al.*, 2007).

Very little is known about the structures of the GET-complex members. A BLAST search revealed that the crystal structure of *Escherichia coli* ATPase ArsA, which shares about 25% sequence identity to yeast Get3, is available (Zhou *et al.*, 2000). However, ArsA (583 amino acids) is a much larger protein that contains two nucleotide-binding domains, while Get3 (354 amino acids) only possesses one nucleotide-binding domain. ArsA plays very different cellular roles from Get3. ArsA contains metal-binding sites in its structure and functions to pump arsenite and antimonite out of *E. coli*. In contrast, no metal-binding site is found in Get3. Moreover, the structure of *E. coli* ArsA is not sufficient to explain how Get3

Figure 1
Yeast Get3 crystals.

Table 1

Statistics of the data set from the Get3 crystals.

Resolution shell (Å)	$I/\sigma(I)$	$R_{\text{merge}}^{\dagger}$	Completeness (%)	Redundancy
50.00–5.16	48.5	0.040	86.3	4.5
5.16–4.10	37.7	0.059	90.4	3.6
4.10–3.58	25.3	0.060	91.1	3.2
3.58–3.25	14.4	0.093	91.7	3.0
3.25–3.02	7.1	0.168	90.5	2.8
3.02–2.84	4.0	0.288	85.9	2.7
2.84–2.70	2.4	0.421	75.1	2.4
Overall	20.7	0.058	87.3	3.2

$$^{\dagger} R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}.$$

interacts with the TMD of TA proteins. No structural information is available for Get1 and Get2 proteins. Therefore, the crystal structure of the GET complex is urgently needed in order to illustrate the mechanisms by which Get1 and Get2 recruit TA-protein-bound Get3 to the ER membranes for TMD insertion. The crystal structure of the Get3–TA protein complex may provide information on how Get3 specifically recognizes and binds the hydrophobic TMD of TA proteins for ER targeting.

2. Experimental and discussion

2.1. Cloning, expression and purification of yeast Get3

The gene encoding *Saccharomyces cerevisiae* Get3 (residues 1–354) was amplified by PCR using the yeast genome as the template. The PCR product was digested by restriction enzymes and ligated into pET28b (Novagen). The Get3 sequence was confirmed by DNA sequencing. The plasmid was then transformed into *E. coli* strain BL21 (DE3) for protein expression.

The *E. coli* cells were harvested 20 h later after induction with 0.5 mM IPTG at 291 K. The recombinant yeast Get3 was expressed in a soluble form. Because the recombinant Get3 was N-terminally histidine-tagged, the protein could be purified by passage through a metal-chelating Ni column. The N-terminal His tag was removed by thrombin treatment. The Get3 was then further purified using a Superdex 200 gel-filtration column (GE Healthcare) to remove the protein aggregates. The apparent molecular mass of Get3 was shown to be about 80 kDa, which indicated that Get3 may form a homodimer in solution. The purified Get3 was kept in 10 mM Tris pH 8.0, 150 mM NaCl, 1 mM MgCl₂. We typically obtained ~10 mg purified Get3 (above 95% purity) from 1 l of *E. coli* culture.

2.2. Crystallization, data collection and processing

The Get3 protein was concentrated to 20 mg ml^{−1} in 10 mM Tris buffer pH 8.0, 150 mM NaCl, 1 mM MgCl₂ by centrifugation using Centricon (Millipore). The hanging-drop vapor-diffusion method was used for the crystallization trials. 2 μ l protein solution was mixed with 2 μ l mother liquor to constitute the hanging drop. Large plate-shaped crystals (0.3 $\times$ 0.3 $\times$ 0.05 mm) were obtained by the hanging-drop vapor-diffusion method using Linbro plates at room temperature (Fig. 1). The well solution consisted of 1 ml 100 mM MES buffer pH 6.0, 18% PEG 3350, 0.2 M ammonium sulfate. SDS-PAGE analysis and mass-spectrometric measurement of the crystals showed that the crystal samples contained Get3 protein. The Get3 crystals grew to full size overnight.

Diffraction data were collected on SER-CAT beamline 22-BM at the APS. The crystal was flash-frozen at 100 K in a nitrogen-gas stream in cryoprotectant consisting of 1 ml 100 mM MES buffer pH 6.0, 18% PEG 3350, 0.2 M ammonium sulfate and 20% ethylene

glycol. The crystals were soaked in the cryoprotectant for about 30 s before transfer to the cold stream.

The Get3 crystals diffracted X-rays to 2.7 Å resolution at SER-CAT. The wavelength was set to 1.0 Å. The data were collected using a MAR 300 CCD detector. During data collection, the crystal-to-detector distance was kept at 275 mm. 120 images covering an oscillation range of 120° were collected and processed using *HKL-2000* (Otwinowski & Minor, 1997). The crystals belonged to space group $P2_12_12$, with unit-cell parameters $a = 220.26$, $b = 112.95$, $c = 48.27$ Å. The R_{merge} of the data set was 5.8%. The details of the data set are given in Table 1. Crystal analysis showed that the asymmetric unit contained one dimer of Get3, which corresponds to a solvent content of 65% ($V_M = 3.7 \text{ Å}^3 \text{ Da}^{-1}$). We failed to solve the crystal structure by molecular replacement using the structure of ArsA as the search model. We are currently working to determine the Get3 crystal structure using the MAD/SAD method.

2.3. Discussion

The GET complex plays a central role in inserting the TA protein TMD into the ER membrane. Yeast Get3 can bind directly to TA-protein TMD and form a complex with the ER transmembrane proteins Get1 and Get2 to constitute the GET complex. The mechanisms by which Get3 recognizes and interacts with the TA protein TMD are currently unknown. The crystal structures of GET-complex members are critically needed in order to understand the molecular basis of its functions. In this study, yeast Get3 has been purified and crystallized. The crystals diffracted X-rays to 2.7 Å resolution on the SER-CAT beamline at the APS. Structure determination by the MAD/SAD method is under way.

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