Bat3 promotes the membrane integration of tail-anchored proteins

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Summary

The membrane integration of tail-anchored proteins at the endoplasmic reticulum (ER) is post-translational, with different tail-anchored proteins exploiting distinct cytosolic factors. For example, mammalian TRC40 has a well-defined role during delivery of tail-anchored proteins to the ER. Although its Saccharomyces cerevisiae equivalent, Get3, is known to function in concert with at least four other components, Get1, Get2, Get4 and Get5 (Mdy2), the role of additional mammalian proteins during tail-anchored protein biogenesis is unclear. To this end, we analysed the cytosolic binding partners of Sec61β, a well-defined substrate of TRC40, and identified Bat3 as a previously unknown interacting partner. Depletion of Bat3 inhibits the membrane integration of Sec61β, but not of a second, TRC40-independent, tail-anchored protein, cytochrome b5. Thus, Bat3 influences the in vitro membrane integration of tail-anchored proteins using the TRC40 pathway. When expressed in Saccharomyces cerevisiae lacking a functional GET pathway for tail-anchored protein biogenesis, Bat3 associates with the resulting cytosolic pool of non-targeted chains and diverts it to the nucleus. This Bat3-mediated mislocalisation is not dependent upon Sgt2, a recently identified component of the yeast GET pathway, and we propose that Bat3 either modulates the TRC40 pathway in higher eukaryotes or provides an alternative fate for newly synthesised tail-anchored proteins.

Key words: Asna-1, SGTA, Get3, Sec61β, TRC40

Introduction

Tail-anchored proteins are a distinct class of integral membrane proteins, distinguished by the presence of a single C-terminal transmembrane region that targets the polypeptide for membrane integration and anchors the protein in the lipid bilayer (Borgese et al., 2007; Kutay et al., 1993; Rabu et al., 2009). Tail-anchored proteins destined for locations within the eukaryotic secretory pathway are all synthesised at the endoplasmic reticulum (ER) and can then be retained or sorted to various subcellular compartments (Behrens et al., 1996; Borgese et al., 2007; Kutay et al., 1995; Linstedt et al., 1995). The biogenesis of tail-anchored (TA) proteins at the ER has been of particular interest because the process is post-translational, and hence quite distinct from the classical signal recognition particle dependent, co-translational, pathway associated with protein synthesised at this location (Cross et al., 2009). In vitro systems have revealed several different pathways that can deliver TA proteins to the mammalian ER, with different TA protein substrates preferentially using distinct cytosolic factors to assist their biogenesis (for reviews, see Borgese et al., 2007; Rabu et al., 2009). Pathway selection is influenced by the relative hydrophobicity of the tail-anchor region, presumably by mediating the recruitment of specific cytosolic factors (Rabu et al., 2008; Rabu et al., 2009). Several recent studies have focussed on the role of mammalian TRC40 (Asna-1), and its Saccharomyces cerevisiae equivalent, Get3, during the post-translational targeting of TA proteins to the ER (Favaloro et al., 2008; Schuldiner et al., 2008; Stefanovic and Hegde, 2007). TRC40 was shown to promote the membrane integration of a number of model TA proteins with comparatively hydrophobic tail-anchor regions, including Sec61β and RAMP4 (Favaloro et al., 2008; Stefanovic and Hegde, 2007). By contrast, perturbation of the TRC40 pathway appears to have little or no effect on cytochrome b5 (Cytb5) integration at the ER membrane, as judged by in vitro assays (Colombo et al., 2009; Stefanovic and Hegde, 2007). This correlates with data suggesting that the cytosolic molecular chaperones Hsc70 and Hsp40 can facilitate the ER integration of proteins with moderately hydrophobic tail-anchor regions, including Cytb5 (Rabu et al., 2008). Alternatively, the lack of TRC40 dependency for Cytb5 integration might reflect a role for new cytosolic components, or even an unassisted mechanism (Colombo et al., 2009).

TRC40 and Get3 are conserved ATPases, which mediate ATP-dependent TA-protein integration at the ER membrane (Favaloro et al., 2008; Favaloro et al., 2010; Rabu et al., 2009; Schuldiner et al., 2008; Stefanovic and Hegde, 2007). Furthermore, several recent studies of Get3 provide structural insights into the mechanisms that underlie its substrate binding and release, and provide models for how ATP binding and hydrolysis might influence these steps (Bozkurt et al., 2009; Hu et al., 2009; Mateja et al., 2009; Suloway et al., 2009). Studies of S. cerevisiae have shown that several components function in concert with Get3: Get1 and Get2 are ER-localised membrane receptors for the GET pathway of TA-protein delivery (Schuldiner et al., 2008), whereas Get4 and Get5 are cytosolic components that appear to act in concert with Get3 before membrane delivery (Jonikas et al., 2009; Rabu et al., 2009). It is assumed that higher eukaryotes possess functional equivalents of these additional components (Rabu et al., 2009), and indeed TRC40 appears to be part of a larger cytosolic complex (Stefanovic and Hegde, 2007). To address the identity of other components that might contribute to this pathway, we analysed the cytosolic binding partners of Sec61β – a well-defined TRC40 substrate (Stefanovic and Hegde, 2007).

We identified Bat3 (Kabbage and Dickman, 2008) as a new interacting component that binds to both Sec61β and RAMP4, but not to a version of Sec61β that lacks the hydrophobic TA region.
Strikingly, Bat3 depletion from reticulocyte lysate inhibited the membrane integration of recombinant Sec61β but did not affect Cytb5 insertion, specifically implicating Bat3 in the TRC40 pathway. When biosynthetic intermediates were analysed, the Sec61β chains that co-fractionated with Bat3 appeared distinct from the integration-competent population associated with TRC40. When expressed in *S. cerevisiae* lacking a functional GET pathway, mammalian Bat3 associated with the resulting cytosolic pool of non-targeted TA proteins and diverted it to the nucleus. Sgt2 has recently been identified, both biochemically and genetically, as an additional component of the yeast GET pathway (Chang et al., 2010; Costanzo et al., 2010). Its mammalian equivalent, SGTA is known to associate with Bat3 (Winnefeld et al., 2006), suggesting that these components function in concert during TA-protein biogenesis. We found that SGTA was preferentially associated with tail-anchor regions in a similar fashion to Bat3. However, the Bat3-dependent relocalisation of TA proteins occurs in the absence of Sgt2, confirming a role for Sgt2 in the GET pathway but ruling out any requirement for this component to enable Bat3 to redirect non-targeted TA proteins to the nucleus in yeast. *S. cerevisiae* lack an obvious Bat3 equivalent, and we propose that Bat3 either modulates the TRC40 pathway in higher eukaryotes, or provides an alternative fate for newly synthesised TA proteins that complements the role of the TRC40 complex.

**Results**

**Recombinant Sec61β requires cytosolic factors and ATP for membrane integration**

Previous studies of TA-protein biogenesis have successfully analysed the behaviour and binding partners of in vitro synthesised polypeptides to identify key components and to understand the pathways that mediate TA-protein delivery to the ER membrane (for a review, see Rabu et al., 2009). As an alternative strategy, we have now exploited recombinant polypeptides expressed in *Escherichia coli* to identify novel cytosolic factors that bind the TA region of Sec61β and contribute to its membrane integration. To this end, we expressed human Sec61β, with a short C-terminal extension bearing an N-glycosylation site (Abell et al., 2007; Colombo et al., 2009; Kutay et al., 1995; Rabu et al., 2008), as a polyhistidine-tagged fusion protein in *E. coli* (supplementary material Fig. S1A). The recombinant polypeptide was purified by nickel affinity chromatography and released from the affinity tag by proteolysis (Colombo et al., 2009), generating full-length recombinant Sec61β and a small amount of truncated material, which probably lacks a few residues at the N-terminus (supplementary material Fig. S1B).

To ensure that our recombinant Sec61β was capable of binding relevant cytosolic factors, we confirmed that the protein was efficiently integrated into ER-derived microsomes as judged by a well-established N-glycosylation assay (Abell et al., 2007; Kutay et al., 1995; Rabu et al., 2008) (supplementary material Fig. S1C). Efficient membrane integration of recombinant Sec61β requires the addition of cytosol (Fig. 1A), and membrane insertion is enhanced when the proportion of lysate is increased (Fig. 1B). As expected from previous studies (Abell et al., 2007; Favaloro et al., 2008; Stefanovic and Hegde, 2007), the membrane integration of recombinant Sec61β was also exquisitely sensitive to the presence of nucleotide triphosphate (Fig. 1C). Hence, the requirements for the membrane integration of recombinant Sec61β appear to be identical to those previously defined using in vitro synthesised polypeptides. Thus, the recombinant protein provides a viable tool for the biochemical analysis of the cytosolic factors that promote membrane integration (Colombo et al., 2009).

**The tail-anchor of Sec61β recruits several cytosolic factors**

To identify novel cytosolic components that might contribute to the biogenesis of TA proteins, we looked for proteins that bound preferentially to recombinant polypeptides with an intact tail-anchor region (supplementary material Fig. S1A). To this end, recombinant opsin-epitope-tagged versions of Sec61β (Sec61β-OPG) with and without the tail-anchor region were coupled to an Ultralink resin and then used as bait for binding to components present in reticulocyte lysate. Upon analysis of the bound material, we noted that the binding of several proteins was substantially enhanced by the presence of the tail anchor (Fig. 2A). Strikingly, when a second recombinant protein with an intact tail-anchor region, RAMP4-OPG, was immobilised, an almost identical pattern of binding partners was detected (Fig. 2A). The behaviour of these components was consistent with a role in TA-protein biogenesis (Favaloro et al., 2008; Stefanovic and Hegde, 2007) and we investigated their identity using a combination of mass spectrometry and immunoblotting. In the case of cytosolic factors previously implicated in TA-protein biogenesis, we found that the recovery of SRP54, TRC40, Hsp/Hsc70 and Hsp40 were all enhanced by the presence of the hydrophobic tail-anchor regions of Sec61β and RAMP4 (Fig. 2B). The binding of an additional component of ~175 kDa was only apparent when the tail anchor was present (Fig. 2A, lanes 2 and 3, see asterisk). We were able to identify this protein as Bat3 by mass spectrometry, and confirmed its preferential binding to recombinant proteins with an

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**Fig. 1. Sec61β-OPG membrane integration requires cytosol and nucleotide triphosphates.** (A) A membrane-integration reaction of recombinant Sec61β[OPG] was performed in the presence of sheep microsomes and either rabbit reticulocyte lysate (RRL) or a buffer control. The membrane fraction was isolated and analysed by immunoblotting with a monoclonal antibody recognising the OPG tag. N-glycosylated (+gly) and non-glycosylated (−gly) forms of Sec61β[OPG] are labelled. The lower product is a truncated version of Sec61β[OPG] (supplementary material Fig. S1B,C). (B) Three 50 μl membrane-integration reactions containing −1.1 μM Sec61β-OPG and −2.1 OD_{280}/ml sheep pancreatic microsomes supplemented with increasing amounts of rabbit reticulocyte lysate (% of total reaction volume shown) were performed and analysed by EndoH treatment and immunoblotting. (C) A standard membrane-integration reaction carried out with untreated rabbit reticulocyte lysate in the presence or absence of an energy regenerating system, as indicated.
The biogenesis of the TA-protein cytochrome b5 (Cytb5) is not dependent upon the canonical TRC40-mediated pathway (Favaloro et al., 2008; Favaloro et al., 2010; Rabu et al., 2008; Stefanovic and Hegde, 2007), and its membrane insertion is unaffected by the immunodepletion of this component (Colombo et al., 2009). In contrast to the clear reduction in Sec61β insertion, depletion of Bat3 had no effect upon the membrane integration of Cytb5 (Fig. 3C). Likewise, TRC40 immunodepletion had no effect on Cytb5 integration (Fig. 3C). This assay confirms that Bat3 depletion does not affect N-glycosylation per se. More importantly, the substrate specificity of Bat3 depletion for inhibiting the integration of Sec61β, but not Cytb5 (Fig. 3), combined with the tail-anchor-dependent association of Bat3 with Sec61β and RAMP4 (Fig. 2), suggests that Bat3 influences the TRC40 mediated route for TA-protein integration (Rabu et al., 2009).

A Bat3-enriched fraction rescues Sec61β membrane integration
As an alternative approach for identifying cytosolic factors that facilitate TA-protein biogenesis, we developed a strategy to selectively deplete candidate components on the basis of their differential binding to various resins (Gorlich et al., 1994). After an empirical screening of a range of matrices, Cibacron Blue agarose was identified as a resin that substantially reduces the levels of Bat3 and SRP54 present in reticulocyte lysate, whilst leaving both TRC40 and Hsp/Hsc70 unaffected (Fig. 4A). When Cibacron-treated lysate was tested in a membrane-integration assay, its ability to promote the membrane integration of Sec61β was substantially impaired, although comparable treatments with other resins did not perturb this process (Fig. 4B). Thus, we found a correlation between Bat3 levels and the ability of reticulocyte lysate to promote Sec61β integration. We detected a substantial amount of Bat3 in the mixture of proteins that could be eluted from the Cibacron resin using a high-salt wash (Fig. 4C; supplementary material Fig. S2). Furthermore, when this eluted material was added back to the previously depleted reticulocyte lysate, Sec61β membrane integration was restored (Fig. 4D). Additional fractionation using nickel-NTA agarose reiterated a clear correlation between the presence of Bat3 and the ability of an eluted fraction to promote membrane integration (supplementary material Fig. S2). In summary, although TRC40 remains in reticulocyte lysate following either Bat3 immunodepletion (Fig. 3A) or Cibacron Blue agarose treatment (Fig. 4A), efficient Sec61β integration is only observed when Bat3 and TRC40 are both present in the lysate (Fig. 3A,B; Fig. 4).

Bat3 and TRC40 associate with distinct populations of Sec61β polypeptides
TRC40/Get3 forms a stable association with newly synthesised TA proteins (Favaloro et al., 2008; Stefanovic and Hegde, 2007), and functions at a late stage of the ER-delivery process (Bozkurt et al., 2009). We investigated the relationship of Bat3 with newly synthesised Sec61β-OPG chains generated by cell-free translation. Radiolabelled Sec61β chains were generated using a reticulocyte-lysate translation system and then fractionated by centrifugation through a sucrose gradient (Favaloro et al., 2008; Stefanovic and Hegde, 2007). Each fraction was analysed for the presence of both radiolabelled Sec61β polypeptides and a variety of cytosolic factors including Bat3 and TRC40 (Fig. 5A). In parallel, equivalent samples were analysed for their capacity to support...
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The distribution of Bat3 is clearly distinct from that of TRC40, showing a partial overlap but peaking at a lower part of the gradient from fractions 7 to 11 (Fig. 5A). In particular, we noted that although fraction 9 contains the strongest Bat3 signal, it supports rather low levels of Sec61β membrane integration (Fig. 5A,B; ~4% of total N-glycosylated material). Hence, although fraction 9 contains levels of Sec61β chains comparable with fractions 7 and 8 (Fig. 5A), the population of TA proteins found in the same fraction as the peak of Bat3 appears far less competent for membrane integration (Fig. 5B, compare fraction 7 with fraction 9).

**Bat3 can influence the fate of a tail-anchored protein in S. cerevisiae**

We found no evidence to suggest that a stable complex is formed between the bulk of Bat3 and TRC40 present in reticulocyte lysate (Figs 3-5); therefore, to gain further insight into the role of Bat3 during TA-protein biogenesis, we exploited an experimental system originally designed to characterise components of the equivalent GET pathway in S. cerevisiae. This assay relies on the observation that the deletion of components of the GET pathway inhibits the ER integration of a model TA protein, GFP-Sed5 (Weinberger et al., 2005), reducing its trafficking to the Golgi and concomitantly increasing the level of cytosolic GFP-Sed5 that has failed to become membrane integrated (Jonikas et al., 2009; Schuldiner et al., 2008).

Although quite clearly distinct, mammalian Bat3 and yeast Mdy2 (Get5) both contain a ubiquitin-like domain (Hu et al., 2006; Kabbage and Dickman, 2008) and we tested the hypothesis that Bat3 is a functional equivalent of one of the two novel cytosolic components, Get4 and Mdy2 (Get5), which have been recently identified in S. cerevisiae (Jonikas et al., 2009) and are presumed to have mammalian homologues (Rabu et al., 2009). Full-length human Bat3 could be expressed in both wild-type and Δmdy2 (Δget5) deletion strains (Fig. 6A,B), and immunofluorescence microscopy showed that Bat3 was readily detected in the nucleus of both strains (Fig. 6C and supplementary material Fig. S3A). This is consistent with studies in cultured mammalian cells where populations of Bat3 in both the nucleus and the cytosol can be observed (Desmots et al., 2008). Bat3 expression had no effect on the location of GFP-Sed5 in wild-type cells, where numerous punctae were seen, consistent with efficient membrane integration and Golgi localization (Fig. 6D,E and supplementary material Fig. S3C, wild-type panels). Bat3 expression had no effect on the location of GFP-Sed5 in wild-type cells, where numerous punctae were seen, consistent with efficient membrane integration and Golgi localization (Fig. 6D,E and supplementary material Fig. S3C, wild-type panels). Bat3 expression had no effect on the location of GFP-Sed5 in wild-type cells, where numerous punctae were seen, consistent with efficient membrane integration and Golgi localization (Fig. 6D,E and supplementary material Fig. S3C, wild-type panels). Bat3 expression had no effect on the location of GFP-Sed5 in wild-type cells, where numerous punctae were seen, consistent with efficient membrane integration and Golgi localization (Fig. 6D,E and supplementary material Fig. S3C, wild-type panels).
localisation signal (NLS). Hence, although an N-terminal fragment of Bat3 and a full-length version with the NLS rendered non-functional were both efficiently expressed (Fig. 6A,B and supplementary material Fig. S3E), neither Bat3 variant supported the nuclear relocalisation of GFP-Sed5 (Fig. 6F).

Immunofluorescence microscopy confirmed the cytosolic localisation of the two Bat3 mutants (supplementary material Fig. S3B,D), and we conclude that full-length Bat3 associates with GFP-Sed5 and redirects this TA protein to the nucleus by virtue of its previously defined NLS (Manchen and Hubberstey, 2001).

Although the expression of full-length Bat3 did not restore a wild-type phenotype for GFP-Sed5 localisation in the mdy2-deletion strains, the protein had a clear effect on the fate of the TA substrate. To establish whether this effect simply resulted from some perturbation of the remaining cytosolic GET complex, we looked at the outcome of Bat3 expression in yeast strains lacking other components of the GET pathway. A strikingly similar effect, namely a reduction in cytosolic labelling and the appearance of a nuclear GFP-Sed5 signal, was also observed in strains lacking either Get3 or Get4 (Fig. 6G,H). Clearly Bat3 is not compensating for the loss of a specific component of the GET pathway in yeast, but rather providing a non-physiological alternative under conditions where the GET pathway is perturbed. This conclusion was further supported by the lack of any apparent perturbation of GFP-Sed5 localisation following Bat3 expression in wild-type cells (Fig. 6E). Since Bat3 is able to redirect GFP-Sed5 to the nucleus in all three of the GET-pathway mutants tested, the association of Bat3 with a TA protein does not require Get3, Get4 or Mdy2 (Get5).

Yeast Sgt2 has recently been implicated in the GET pathway for TA-protein biogenesis (Chang et al., 2010; Costanzo et al., 2010) and its mammalian equivalent, SGTA (SGT), is a known interacting partner of Bat3 and Hsp/Hsc70 in mammalian cells (Winnefeld et al., 2006). We therefore addressed the possibility that any role for Bat3 during TA-protein biogenesis might also involve SGTA and/or its yeast equivalent. When the mammalian cytosolic components that bind tail-anchor regions were re-analysed by immunoblotting, we found that SGTA was enriched in fractions eluted from full-length TA proteins (Fig. 7A), consistent with a potential role in TA-protein biogenesis (Fig. 2). However, the ability of Bat3 to relocalse GFP-Sed5 to the nucleus of a Δget5 strain was not dependent upon the presence of Sgt2 (Fig. 7B). Furthermore, the loss of Sgt2 alone proved sufficient to enable Bat3-mediated
the subcellular localisation of GFP-Sed5 was determined by live-cell imaging immunofluorescence microscopy. (Hubberstey, 2001). These data are shown is altered to KRSL to disrupt nuclear targeting of Bat3 (Manchen and Hubberstey, 2001). Although the peak of integration-competent Sec61β chains co-migrate with the bulk of TRC40, strongly supporting a role in promoting membrane integration (Bozkurt et al., 2009; Favaloro et al., 2008; Stefanovic and Hegde, 2007), the bulk of Bat3 co-migrates with a pool of Sec61β chains that are poorly membrane integrated. Thus, although Bat3 can influence the fate of TA-protein substrates of the TRC40 pathway, its function might be spatially and temporally distinct from the TRC40-mediated delivery step that precedes membrane integration (Bozkurt et al., 2009; Favaloro et al., 2010). Interestingly, a recent proteomic analysis that used the ubiquitin-like-domain-containing protein UBL4A as bait identified a protein network containing both Bat3 and TRC40 (Asna-1), together with SGTA and C7orf20 [see table S4 in Sowa et al., 2009]. Hence, Bat3 and TRC40 might associate transiently or share common interacting partners.

To place the role of Bat3 into a cellular context, we exploited S. cerevisiae mutants lacking various components of the GET pathway that is functionally equivalent to the mammalian TRC40 pathway, but is at present far better defined (Jonikas et al., 2009; Rabu et al., 2009; Schuldiner et al., 2008). The very clear outcome of this approach is that the expression of Bat3 in a variety of GET-pathway mutants results in the relocalisation of a model GET-pathway substrate, GFP-Sed5, to the nucleus. We show that the ability of Bat3 to redirect GFP-Sed5 to the nucleus relies on a previously defined nuclear-localisation signal present in Bat3, which is known to be functional in mammalian cells (Desmots et al., 2008; Manchen and Hubberstey, 2001). These data are reminiscent of the PEX19-mediated nuclear relocalisation of several peroxisomal membrane protein substrates, in this instance elicited by the introduction of an artificial NLS motif into PEX19, which was used to help identify class 1 peroxisomal-membrane proteins (Jones et al., 2004). The effect of Bat3 expression in S. cerevisiae strongly supports a model where the association of TA proteins with Bat3 influences their subsequent fate in vivo.

We found that the effect of Bat3 is comparable in the absence of any of the known soluble proteins of the yeast GET pathway, including Sgt2.

**Discussion**

To better understand the pathways and components responsible for the biogenesis of TA proteins we used an affinity-binding approach to identify candidate cytosolic factors. A number of proteins preferentially associate with both Sec61β- and RAMP4-bearing intact tail-anchors, including all of the cytosolic factors previously implicated in TA-protein biogenesis (Abell et al., 2004; Abell et al., 2007; Favaloro et al., 2008; Rabu et al., 2008; Stefanovic and Hegde, 2007). We found an additional component of ~175 kDa that was substantially enriched in the presence of a tail anchor, and this protein was identified as Bat3. Bat3 has been implicated in a variety of biological processes, including the regulation of apoptosis (Desmots et al., 2008), Hsp70 stability (Corduan et al., 2009; Sasaki et al., 2008) and function of natural killer cells (Simhadri et al., 2008). Bat3 has no clear homology with TRC40, and its most obvious features are an N-terminal ubiquitin-like domain, a nuclear-localisation signal (NLS) and a C-terminal BAG domain (Kabbage and Dickman, 2008).

Strikingly, Bat3 depletion results in a substantial inhibition of the reticulocyte-lysate-dependent membrane integration of Sec61β, indicating that this component can influence the biogenesis of TA protein. Whether Bat3 removal also depletes other, as yet unidentified, cytosolic components that are important for TA-protein biogenesis remains to be established. However, the removal of Bat3 does not substantially alter TRC40 levels, nor is the integration of the TRC40-independent substrate Cytb5 affected by Bat3 removal. We conclude that the role of Bat3 is most likely restricted to TA proteins that are TRC40 clients (Colombo et al., 2009; Rabu et al., 2009; Rabu et al., 2008; Stefanovic and Hegde, 2007). Although the peak of integration-competent Sec61β chains restricted to TA proteins that are TRC40 clients (Colombo et al., 2009; Rabu et al., 2009; Rabu et al., 2008; Stefanovic and Hegde, 2007), the bulk of Bat3 co-migrates with a pool of Sec61β chains that are poorly membrane integrated. Thus, although Bat3 can influence the fate of TA-protein substrates of the TRC40 pathway, its function might be spatially and temporally distinct from the TRC40-mediated delivery step that precedes membrane integration (Bozkurt et al., 2009; Favaloro et al., 2010). Interestingly, a recent proteomic analysis that used the ubiquitin-like-domain-containing protein UBL4A as bait identified a protein network containing both Bat3 and TRC40 (Asna-1), together with SGTA and C7orf20 [see table S4 in Sowa et al., 2009]. Hence, Bat3 and TRC40 might associate transiently or share common interacting partners.

relocalisation (Fig. 7B). Thus, Bat3 can associate with TA proteins in the absence of any of the known soluble proteins of the yeast GET pathway, including Sgt2.

![Image](https://example.com/image.png)

**Fig. 6. Bat3 relocalises GFP-Sed5 in S. cerevisiae GET mutants.** (A) Outline of full-length Bat3 (isoform 2), and the N-terminal fragment used in this study. The locations of the ubiquitin-like domain, NLS and BAG domains and the antibody-binding region are indicated. In the ΔNLS mutant, the KRRK motif shown is altered to KRSL to disrupt nuclear targeting of Bat3 (Manchen and Hubberstey, 2001). (B) Immunoblot showing Bat3 and phosphoglycerate kinase 1 (Pgk1) levels in wild-type or Δget5 transformed S. cerevisiae cells. (C) Subcellular localisation of full-length Bat3 expressed in wild-type S. cerevisiae and DAPI staining of nuclei visualised by immunofluorescence microscopy. (D-H) The effect of Bat3 expression upon the subcellular localisation of GFP-Sed5 was determined by live-cell imaging in wild-type, Δget3, Δget4 or Δmdy2 (Δget5) cells, as indicated. Full-length Bat3, an N-terminal fragment or the ΔNLS mutant, were used as indicated. See also supplementary material Fig. S2C for fixed and immunostained cells of the same genotype demonstrating co-localisation of Bat3 and GFP-Sed5 immunoreactivity with DAPI staining of the nucleus in Δmdy2 (Δget5) cells. Scale bar: 5 μm.
between Bat3 and syntaxin 5A, the human homologue of Sed5, using a yeast two-hybrid approach (Stelzl et al., 2005). Two recent publications identify the yeast protein Sqt2 as a component of the GET pathway for TA-protein biogenesis (Chang et al., 2010; Costanzo et al., 2010), providing an alternative model for our observations when Bat3 is expressed in yeast. Specifically, Bat3 interacts with the mammalian equivalent of Sqt2, Sgta (Winnefeld et al., 2006), providing a potential intermediary between the TRC40/GET pathway and Bat3 (Fig. 7C). Although we found that mammalian Sgta preferentially associates with hydrophobic TA segments, the Bat3-dependent relocation of TA proteins in yeast does not require Sqt2, and in fact the loss of Sqt2 alone is sufficient to enable Bat3-mediated relocation to occur. These data strongly support a role for Sqt2 in the yeast GET pathway (Chang et al., 2010; Costanzo et al., 2010), and indicate that Sgta might have a similar role in higher eukaryotes (Fig. 5A; Fig. 7C). However, the association of Bat3 with yeast Sqt2 observed in yeast is direct, as previously suggested (Stelzl et al., 2005), or that the process involves some other functionally conserved component associated with or regulated by Bat3, for example Hspa/Hsc70 (Corduan et al., 2009; Sasaki et al., 2008).

Although we can only speculate about the precise role(s) of Bat3 during TA-protein biogenesis, the following scenarios provide a basis for further experimentation. Our current results suggest that Bat3 is not stably associated with other components of the TRC40 pathway (Fig. 5A; Fig. 7C); however, Bat3 might modulate or enhance particular steps in the TRC40 cycle for TA-protein delivery to the ER (Rabu et al., 2009). Hence, Bat3 could facilitate the formation of a productive TRC40-substrate ER-delivery complex (Rabu et al., 2009), or promote the interaction of the assembled complex with the ER membrane. If so, then the role of Bat3 can be circumvented by the co-expression of TRC40/Get3 and a TA-protein substrate in E. coli, because this results in a functional ER-delivery complex (Bozkurt et al., 2009; Favaloro et al., 2010). Alternatively, Bat3 could contribute to the recycling of the TRC40 complex by facilitating its release from the, as yet unidentified, mammalian ER-membrane receptor after delivery of TA protein has occurred (Rabu et al., 2009). Equally, the association of selected TA proteins with Bat3 might in fact represent an alternative and complementary fate to TRC40 binding. In this scenario, association with Bat3 could provide aberrant or misfolded TA proteins with an opportunity for refolding and return to the TRC40 pathway (Fig. 7C), most likely via the actions of Hspa/Hsc70 chaperones (Corduan et al., 2009; Sasaki et al., 2008). Removal of Bat3 from our in vitro system could cause the TRC40 pool to be titrated out by binding aberrant TA proteins to form complexes that are incapable of authentic ER delivery. Bat3 might also regulate the entry of aggregated or terminally misfolded TA proteins into a degradative pathway (Fig. 7C) (Auld et al., 2006), which is consistent with the presence of its N-terminal ubiquitin-like domain (Kabbage and Dickman, 2008).

Whatever the precise role of Bat3 during TA-protein biogenesis, the presence of a C-terminal BAG domain provides an opportunity for its regulation via several cellular components, including Hspa/Hsc70 (Kabbage and Dickman, 2008). Intriguingly, the so-called BAG domain, found in various proteins, including Bat3, was first identified via its interaction with Bcl-2, a TA protein (Janik et al., 1994) implicated in the regulation of apoptosis (Heath-Engel et al., 2008). Hence, one could speculate that the modulation of apoptosis and proliferation ascribed to Bat3 (Scythe) (Desmots et al., 2005) might reflect a BAG-domain-dependent interaction with TA proteins such as Bcl-2. Interestingly, our previous studies suggest that, similarly to Cytb5, Bcl2 is not an obligatory client of the generic TRC40-mediated pathway (Rabu et al., 2008), and on this basis it seems unlikely that Bat3 will influence the membrane integration of Bcl2 in a similar fashion to Sec61B. It is becoming increasingly apparent that the biogenesis of TA proteins is a remarkably complex and multifaceted process. Defining the precise function of Bat3 during this process and understanding this in the context of its various other cellular roles will be a major focus of our future efforts.

Materials and Methods

Materials

Bacterial expression vector, pHisTrx, was a gift from Richard Kammerer (University of Manchester, Manchester, UK). Rabbit polyclonal antiserum recognising TRC40 (Asna-1) was a gift from Bernhard Dobberstein (ZMBH, Heidelberg, Germany) and the monoclonal anti-opsin tag antibody (Adams et al., 1991) was provided by Paul Hargrave (Department of Ophthalmology, University of Florida, FL). Commercial antibodies were used to detect Hspa/Hsc70 and Hspa40 (Stressgen), SRP56 (BD Biosciences), Bat3 (Abcam), yeast Pgp1 (Invitrogen) and GFP (ab290, Abcam). Nuclease-treated rabbit reticulocyte lysate for in vitro translation was from Promega, rabbit reticulocyte untreated lysate was from Green Hectares.

Protein expression and purification

cDNAs encoding single cysteine variants of Sec61B, Sec61J lacking the tail anchor, RPM4 and cytochrome b5, in each case including an in-frame opsin tag at the 3′ end (see supplementary material Fig. S1A), were subcloned into the pHisTrx expression vector and all constructs confirmed by DNA sequencing. The proteins were expressed in Escherichia coli strain BLR (DE3) pLysS cells by IPTG induction, and after harvesting cells were lysed in buffer A [50 mM Tris-HCl, pH 7.4, 300 mM
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NaCl, 10 mM MgCl₂, 10 mM imidazole, 5 mM 2-ME, 1 mM PMSF, 10 % (v/v) glycerol] supplemented with 1 U/ml lysozyme, 10 μM Dnase 4, 100 μM DTT and 10 μM PMSF in a 1 hour reaction volume in vitro, as previously described. 10%收取的eluate or equivalent buffer control was mixed with 20 μl of the Cibacron-treated rabbit reticulocyte lysate, and a membrane integration-assay performed.

Sucrose-gradient centrifugation

Sucrose-gradient centrifugation was performed as a 100 μl reaction volume in vitro, as previously described (Rabu et al., 2008) and the material fractionated by centrifugation through a sucrose gradient using a modified version of the procedure described (Stefanovic and Hegde, 2007). Thirteen individual fractions were collected from the gradient (1st, 3rd, 6th, 9th, 12th, 15th) from pellet-washed and resolved by SDS-PAGE, the location of distinct cytosolic factors was determined by immunoblotting and the distribution of the radio labelled Sec61β-OPG visualised by phosphorimaging. In a parallel experiment, 50 μl of each fraction was mixed with 70 μl of 100 mM sucrose (final concentration of 2.0 OD₅₇₀ pm) incubated for 30 minutes at 4°C and the membrane fraction isolated as described for membrane integration reaction. Following SDS-PAGE and quantitative phosphorimaging, the relative proportion of the total N-glycosylated material in each fraction was determined to provide a measure of the capacity for membrane integration for the Sec61β-OPG chains present in each fraction.

Bat3 expression and analysis in S. cerevisiae

The full-length version of Bat3, isoform 2, was obtained from OriGene and cloned into the yeast expression vector p416Met25 (Mumburg et al., 1994) via the XbaI and XhoI restriction sites. For the Bat3N1 fragment that lacked the C-terminus, a stop codon was introduced in place of residue 676 of the wild-type sequence. The Bat3 ΔNLS mutant was generated as previously described (Manchen and Hubberstey, 2001). GFP-Sed5 was expressed from plasmid pRTS315 (Weinberger et al., 2005). All yeast strains used were derived from BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) (Brachmann et al., 1998). The respective deletion strains for GET3 (Aget3-KanR), GET4 (Aget4-KanR), GET5MD12 (Aget5-md12: KanR) and SG72 (Aget2-KanR) were obtained from Euroscarf (Winzeler et al., 1999). A Aget2::KanR Aget5-md12::NAT double deletion was created using standard PCR-based replacement methods with plasmid pAG25 (Goldstein and McCusker, 1999). Yeast transformation and growth in synthetic complete medium lacking uracil and/or leucine followed well-established protocols (Ausubel et al., 1997), whereas total cell lysates for western blotting analysis were prepared as described (Yaffe and Schatz, 1984). Anti-Bat3 chicken antibody was used at 1:5000 and anti-Pgk1 mouse monoclonal at 1:2000; anti-chicken and anti-mouse horseradish-peroxidase conjugated secondary antibodies were used at 1:2500 and 1:5000, respectively. Immunofluorescence followed the method described (Roberts et al., 1991), except that cells were fixed for 1 hour in 4% formaldehyde. Attached spheroplasts were then incubated in ice-cold methanol for 6 minutes, followed by ice-cold acetone for 30 seconds. Anti-GFP and anti-Bat3 primary antibodies were diluted 1:1000, the respective anti-rabbit (Alexa Fluor 488, Invitrogen) and anti-chicken (Alexa Fluor 594, Invitrogen) secondary antibodies 1:500. Stained spheroplasts were mounted in SlowFade Gold antifade reagent containing DAPI (Invitrogen). Live-cell imaging of yeast cells was performed at 20°C in a temperature controlled microscope employing a DeltaVision restoration microscope equipped with a 100X, 0.35-1.5 Uplan Apo objective and a GFP filter set (Chroma 86006). The images were collected with a Coolscope HC camera (Photometrics). The same set-up was used to image immunostained spheroplasts for which stacks with 0.2 μm spacing of 0.3 μm were acquired. Raw images were deconvolved using the additive algorithm of Softworx software.

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Note added in proof

A recent phylogenetic analysis (Borgese and Righi, 2010) suggests that the membrane insertion of TA proteins in prokaryotes either occurs through an unassisted pathway or is mediated by Hsp40 and Hsp70s.

Supplementary material available online at http://jcs.biologists.org/cgi/content/full/123/13/2170/DC1