Localisation of a family of complex-forming β-barrels in the T. vaginalis hydrogenosomal membrane

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ABSTRACT

Crucial to organellogenesis was the development of membrane translocases responsible for delivering proteins to new cellular compartments. This investigation examines the Trichomonas vaginalis hydrogenosome, a mitochondrially derived organelle. We identify an expanded family of putative β-barrel proteins (THOM A–I) comprising nine related sequences. Sub-cellular localisation by immunofluorescence and biochemical fractionation is consistent with THOMs being localised to the hydrogenosomal membrane. Native gel electrophoresis and chemical cross-linking support the ability of THOM proteins to be components of membrane-bound oligomeric protein complexes, consistent with a role in protein translocation.

1. Introduction

In the development of endosymbiont derived organelles there has occurred a movement of genes from the endosymbiont to the nucleus [1–3]. A necessary consequence of this has been the evolution of membrane transporters capable of importing cytosolically expressed proteins back into the organelle [4,5]. Moreover, the establishment of outer membrane translocases would have enabled the host to independently access the endosymbiont [5,6]. In the mitochondrion, outer membrane translocases are derived from endosymbiont membrane translocases [4,5], sharing a common anti-parallel β-barrel pore domain [7].

In mitochondria two β-barrel preprotein translocases are present and are functionally distinct. The best characterised with respect to its descent is Sam50, the eukaryotic homologue to Omp85, which functions as a β-barrel insertase [4,8]. The Tom40 β-barrel protein is an another translocase, and although the origins of this protein are not as clearly determined, structural studies of porins and Tom40 show similarity [7]. Indeed, outer membrane β-barrels are proposed to have functional overlap in some organisms [9–11].

The nature of pre-protein transport systems in mitochondrially divergent organelles remains an important challenge and is key to determining the evolution of the mitochondrion. Most hydrogenosomes and mitosomes have lost their genomes and so have frustrated direct determination of their descent by comparison with mitochondrial DNA. Investigations have subsequently focused on characterising highly conserved mitochondrial proteins in these organelles.

This investigation focuses on Trichomonas vaginalis, the first organism to be identified with a hydrogenosome [12]. We examine whether mitochondrial homologues to the Tom40 β-barrel translocase exist in the T. vaginalis hydrogenosome, and determine the nature of their complexes. If the development of endosymbiont organelles does follow the timeline proposed [5,6] then the presence of a mitochondrial Tom40 like system in Trichomonas would suggest that this organelle descended from an ancestral organism already possessing a recognisable preprotein import system. This investigation identifies candidates from genomic analyses and examines these proteins using biochemical and microscopy techniques.
2. Materials and methods

2.1. Culture of T. vaginalis

*T. vaginalis* strains ATCC30001 (C1) and G3 were maintained in Diamond’s medium (TYM) supplemented with iron as previously described [13,14].

2.2. Stable transfection PCR of *T. vaginalis* with HA-tagged THOM proteins

Genomic DNA was extracted from *T. vaginalis* G3 as previously described [14] and DNA sequences for candidate THOM proteins were amplified using primers listed in Supplementary Table 1. THOM PCR products were inserted into a modified pTagVag2 [15] vector (a double haemaglutinin tag (HA–HA) being inserted in frame with the start codon) by restriction enzyme mediated subcloning. All constructs were validated by DNA sequencing across the entire coding region. *T. vaginalis* was transfected and selected with G418 as previously described [14].

2.3. Cell lysis and fractionation

Dense cultures of *T. vaginalis* C1 were pelleted by centrifugation, washed in SH buffer (250 mM sucrose, 20 mM HEPES, pH7.4) supplemented with 10 mM β-mercaptoethanol, and re-pelleted. The cell pellet was resuspended (700 µl/g pellet weight) in SH supplemented with 5 mM DTT, and protease inhibitors TLCK (25 µg/ml) and leupeptin (10 µg/ml) (SHDI buffer). The suspension was passed repeatedly through a 23-gauge needle until >90% of cells were lysed (fraction WC in figures). Cellular debris was removed by centrifugation (1000 × g, 5 min, 4 °C), and the supernatant was further centrifuged (8000 × g, 10 min, 4 °C) to produce an organelle pellet. The supernatant from this step was re-centrifuged (16000 × g, 10 min, 4 °C) to produce crude cytosol (fraction C). Purified hydrogenosomes (fraction H) were obtained by resuspending the crude hydrogenosome pellet to a final volume of 6 ml in SHDI (10% (v/v) iodoxan). The suspension was then loaded on top of an SHDI/iodixanol gradient (20–40%) and ultracentrifuged (70,000 × g, 2 h, 4 °C). The fraction corresponding to isolated hydrogenosomes (identified by a light brown hue) was extracted, diluted tenfold in SH containing protease inhibitors and then re-pelleted by centrifugation (8000 × g, 10 min, 4 °C). Organelles were resuspended in SH buffer containing protease inhibitors and glyceral (10% (v/v)) before freezing at −80 °C. Membrane and soluble protein fractions were generated by resuspending 1 mg purified hydrogenosomes per ml of 0.1 M sodium carbonate (pH 11.5). The suspension was incubated on ice for 90 min with periodic vortexing, before ultracentrifugation (30 min, 100,000 × g, 4 °C) resulting in a pelleted hydrogenosomal membrane fraction (P) and a soluble protein fraction (S).

2.4. Solubilisation of hydrogenosomes

Purified hydrogenosomes were resuspended in a solubilisation buffer (10% glycerol (v/v), 50 mM HEPES, 5 mM MgCl₂, 5 mM DTT, 5 mM EDTA, 1% Roche protease inhibitor cocktail V, pH 7.4) with concentrations of NaCl and detergents indicated in Figures. Suspensions were rotated at 4 °C for 90 min, before the solubilised protein was separated from insoluble material by centrifugation (16,000 × g, 15 min, 4 °C).

2.5. Blue native PAGE

Solubilised hydrogenosomal proteins were examined by blue native electrophoresis [16]. Samples were mixed with a 10 × BN PAGE sample buffer (5% (w/v) Coomassie Brilliant Blue G250, 0.75 M 6-aminopropionic acid, 100 mM Bis-Tris, pH7.0) and loaded onto 12% uniform BN PAGE gels with a 4% stacking region, with buffers described previously [16].

2.6. SDS–PAGE and western blotting

One-dimensional denaturing electrophoresis was carried out according to Laemmli [17] on 10 or 15% (v/v) polyacrylamide gels. Gels were electro-blotted onto PVDF membranes which were blocked in TBS containing 0.1% (v/v) Tween (TBS-T) and 5% (w/v) milk powder prior to incubation with primary and secondary antibodies (mouse monoclonal anti-HA, 1:5000 and goat anti-mouse-HRP, 1:20000, both from Sigma). Membranes were developed using chemiluminescence (EZ-ECL; Geneflow) and imaged using a Fujifilm LAS-1000 imager.

2.7. Crosslinking

Purified hydrogenosomes were resuspended in SH buffer to a protein concentration of 5 mg/ml. 200 µl crosslinking reactions were assembled on ice with either MBS or DSP (both from Pierce) to a final crosslinker concentration of 0.5 mM. Reactions were quenched after 20 min with the addition of Tris pH7.4 to a final concentration of 10 mM. Protein samples were resuspended in SH (+)/– 0.1 M DTT to reverse DSP crosslinking, prior to electrophoretic analysis.

2.8. Co-immunoprecipitation

One milligram of solubilised hydrogenosomal protein produced as described previously was incubated with 2 µl mouse monoclonal anti-HA antibody (Sigma) overnight with rotation at 4 °C. Immunocomplexed protein was recovered with incubation of 50 µl Protein-A Sepharose beads for 1 h at RT. Subsequent to bead washing in additional solubilisation buffer (3 washes of 20 × bead volume), captured protein was eluted with 50 µl 0.1 M glycine pH 2.1.

2.9. Confocal microscopy

Cells were separated from media by centrifugation and resuspended to a density of 1 × 10⁷/ml in phosphate buffered saline (PBS) and transferred to silane covered microscope slides (Sigma) and left to adhere for 30 min at RT. Non-adhered cells were washed from the slide with PBS and remaining cells fixed with 4% w/v paraformaldehyde, 0.1% (v/v) Triton X-100 for 20 min at RT. Permeabilised cells were washed with PBS before being incubated in PBS supplemented with 0.25% (w/v) BSA and 0.25% (w/v) fish scale gelatin (blocking buffer). The cells were then incubated in primary anti-HA antibody (1:2500 in blocking buffer) for 1 h at RT. The slides were then washed with PBS twice before incubation of the secondary Alexafluor 488 coupled secondary (1:1000 in blocking buffer). To stain the nuclei slides were incubated with PBS containing RNase (100 µg/ml) at 37 °C for 20 min, and then with PBS supplemented with 3.3 µg/ml propidium iodide (PI) for 5 min. Excess PI was removed with PBS washes before slides were mounted in 50% glycerol (v/v)/PBS. Slides were analysed on a Zeiss 710 confocal laser scanning microscope, using a 63 ×/1.4 oil-immersion objective. Alexa488 and PI were excited using a 488 nm argon and a diode pumped solid state 562 nm laser respectively and emission was collected between 500 and 530 nm (Alexa488) and between 565 and 700 nm (PI). Confocal images were processed in the Carl Zeiss Zen 2009 Light Edition and Carl Zeiss LSM Image Browser software.
2.10. Phylogenetic analysis

Collection of sequence data was performed by taking alignments from NBR's Conserved Domain Database (CDD) [18] for VDAC and Tom40 proteins, which were then used to create Hidden Markov Models (HMMs) [19]. These models enabled searches for homologues in *Trichomonas vaginalis* as well as other selected Excavate organisms. Results were assembled with selected reference sequences from Homolgene [20] and aligned with ClustalX2 [21] using a Gnonet substitution matrix. Phylogenetic analysis was performed using both Neighbour-Joining [19] and Bayesian analysis. Neighbour-Joining was performed within the ClustalX2 GUI, and repeated to 1000 bootstraps, whereas Bayesian analysis was performed with MrBayes [22] using 100,000 iterations of the Markov-Chain Metropolis-Coupled Monte-Carlo algorithm, at a subsampling frequency of 1 in 200, and an invgamma distribution.

![Fig. 1](image_url)

A family of β-barrel protein sequences in *T. vaginalis* is related to mitochondrial Tom40. Homologues to Tom40 and VDAC proteins detected through HMM models were aligned with Clustal and phylogenetic analysis performed with Neighbour-Joining and Bayesian probability techniques. Figures at nodes show NJ boot-strap values (% right), and Bayesian consensus (% left). The dash sign (−) indicates that the two methods produce alternative consensus topologies at these nodes. Dendrogram distances shown were derived from the MrBayes analysis, with scale bar shown.
model and WAG rate matrix [23]. The consensus tree was constructed from dendrograms corresponding to the last 80,000 iterations. Phylogenetic trees were visualised in Dendroscope[24].

3. Results

3.1. Bioinformatic analysis

HMMs constructed from alignments of Tom40 and VDAC were used to screen the *T. vaginalis* genome. Using HMMsearch [19], the Tom40 HMM was able to detect five *T. vaginalis* proteins (THOMs A–E), whereas the VDAC HMM only retrieved a single *T. vaginalis* protein sequence (THOM-A). Iterations of the HMM search (jackHMMer, [19]) revealed sequences with much weaker similarity to the original profile, and in this case, the Tom40 HMM detected two further candidate sequences from *T. vaginalis* (THOMs F, G), whereas the VDAC HMM identified a further six (THOMs B-F). HMMs built from these results identified a further two candidates, THOMs H, I. Similar search strategies enabled the identification of putative Tom40 homologues from other ‘Excavates’ [25]- Leishmania major, Trypanosoma brucei, Euglena gracilis, Naegleria gruberi, Giardia intestinalis. Phylogenetic analyses performed using Neighbour-Joining and Bayesian algorithms clearly resolve the Tom40 and VDAC families and demonstrate the *T. vaginalis* homologues to belong to a single family, which is more closely related to the Tom40 reference sequences than it is to the VDAC reference sequences (Fig. 1). These results indicate the potential for multiple Tom40 homologues in *T. vaginalis*. Additionally,

![Confocal microscopy of THOM proteins within *T. vaginalis*.](image)

Fig. 2. Confocal microscopy of THOM proteins within *T. vaginalis*. Proteins were immunodetected by an anti-HA primary antibody, with an Alexa fluor488 tagged secondary. Nuclei were stained with propidium iodide. THOMs(A–E) are localised to the periphery of discrete spherical organelles, whereas the luminal marker protein, frataxin (Fr), is expressed throughout the organelle.
the C-terminal region of the *T. vaginalis* homologues showed considerable homology to β-barrel signatures sequences [26] (Supplementary Fig. 1).

### 3.2. Organelle localisation by confocal microscopy

To investigate the sub-cellular localisation of *Trichomonas* Tom40 candidates we ligated cDNAs for the five strongest homologues to *Saccharomyces cerevisiae* and *Plasmodium falciparum* Tom40 (as determined by BLAST [20], viz THOM A–E) into a modified pTagVag2 vector [15], to enable N-terminal fusion of a HA-tag. These constructs were then transfected into *T. vaginalis* C1 in parallel with a well characterised lumenal hydrogenosomal marker protein, frataxin (also bearing a HA tag) [15]. The expression of these HA-tagged proteins did not affect doubling time, cell morphology or maximum cell density (data not shown). Confocal microscopy of the transformant cells showed a distinct pattern of fluorescence between the lumenal (frataxin) and the proposed membrane localised (THOM) proteins (Fig. 2). Frataxin expressing cells demonstrated small (sub-micron diameter) punctate features consistent with localisation of this protein to hydrogenosomes [15]. The number of these punctate bodies per cell (approximately 200–400) is consistent with the number of hydrogenosomes per cell as determined by electron microscopy [27]. Transformants expressing any of the five THOM proteins displayed fluorescence that was localised to the periphery of discrete, spherical structures. Again, the number and size of these (hundreds/cell; typical diameter 0.5–1.0 μm) are consistent with the argument that the THOM proteins localise to the membrane of the hydrogenosome. Visual demonstration of co-localisation of THOM and frataxin to the same organelle is not possible as both are HA-tagged.

### 3.3. Subcellular localisation of THOM proteins

Transformant cells expressing THOM proteins were processed by disruption and differential centrifugation to generate fractions representing cytosol, hydrogenosome and lysosome compartments. The localisation of these THOM proteins are shown in Fig. 3A, where equivalent quantities of hydrogenosomes (H) and lysosomes (L), as well as preparatively equivalent quantities of cytosol (C) are shown with a sample of whole cell lysate (WC). THOM proteins are found to localise most strongly to the hydrogenosomal fraction, though trace quantities of protein are detected in lysosomal fractions, possibly resulting from limited contamination from the preparative ultracentrifugation. Notably, THOM proteins are not observed in the cytosolic fraction.

Hydrogenosomes were further treated with sodium carbonate to create a membrane fraction (containing integral membrane proteins, P) and a soluble fraction (containing matrix and peripheral membrane proteins, S). The presence of THOM proteins was analyzed in these fractions and the results are shown in Fig. 3B. All THOM proteins exhibit a similar pattern of localisation with the vast majority of protein detected in membrane enriched fractions. The trace amounts in the soluble fraction may either reflect...
incomplete membrane pelleting of membranes subsequent to carbonate “stripping”, or possible limited solubility of THOM proteins in aqueous buffers as has been seen for bacterial porins [28].

3.4. Characterising the complexes of the THOM proteins

To determine whether the membrane localised THOM proteins formed complexes within the membranes of hydrogenosomes two different approaches, blue native (BN) PAGE and chemical crosslinking, were used to characterise potential intermolecular and intramolecular interaction. The results of both these approaches are shown in Fig. 4. For BN PAGE, THOM proteins were solubilised from purified hydrogenosomes employing three different detergents, DDM, digitonin and Triton X-100. Solubilised protein was separated on uniform BN-PAGE gels (Fig. 4A). THOM proteins exhibited some variation with respect to their solubility in the tested detergent conditions - presumably reflecting the sequence divergence across the five THOMs (Fig. 1B). Triton X-100 appeared the best tolerated detergent and organelles solubilised under these conditions produced \( \approx 90 \text{ kDa} \) complexes with THOMs A, D and E (Fig. 4A). THOM proteins B–E were also able to produce a complex of higher molecular weight \( \approx 140 \text{ kDa} \) when solubilised with digitonin. DDM was able to solubilise THOMs B and E which produced complexes of comparable weight to Triton X-100, though THOM B produces a doublet signal with two complexes (\( \approx 70, \approx 90 \text{ kDa} \)) visible on DDM solubilisation. Irrespective of the detergents employed, all five THOM proteins were able to form higher molecular weight (presumably oligomeric) assemblies on native PAGE in contrast to the faster migrating species (ca. 40 kDa) observed in the presence of SDS.

Chemical crosslinking was also employed to investigate the membrane complexes formed by the THOM proteins. In this

Fig. 4. Characterising the membrane complexes of the THOM family proteins. The complexes of THOM family were investigated using BN PAGE and chemical crosslinking. (A) THOM protein complexes were extracted and resolved under native conditions using BN PAGE. Complexes were solubilised with three detergents, dodecylmaltoside (DDM), Digitonin (Dig.), and Triton X-100 (Trn) at concentrations of either 0.5 or 1.0%. These complexes were electrophoretically separated in BN PAGE alongside markers (shown for THOM-A) consisting of protein oligomers (GE Healthcare). Representative gels of multiple independent experiments are shown for each THOM. THOM-C showed qualitatively similar results but is omitted for clarity. (B) Hydrogenosomes containing THOM proteins were resuspended in solutions containing crosslinkers MBS (lane 2) or DSP (lanes 3, 4) at 0.5 mM. In addition to these crosslinked samples un-treated control (1) and DTT treated DSP reaction (4), were analyzed by SDS-PAGE to separate crosslinked species.
investigation two different crosslinking agents were employed, DSP and MBS. The stable covalent crosslinking of proteins allows for the resolution of oligomeric complexes by SDS–PAGE. The results of crosslinking on THOM expressing hydrogenosomes is seen in Fig. 4B, showing both untreated (lanes 1), and membranes cross-linked with either MBS or DSP (lanes 2, 3 respectively). Additionally, the reversibility of crosslinking was validated by reduction of DSP crosslinked samples (lanes 4). In the absence of crosslinker, THOM proteins do not exhibit much complexity above the denatured monomeric weight. With the addition of crosslinking agent to resuspended hydrogenosomes additional high molecular weight complexes are visualised. DSP appears to be more effective at capturing these complexes at this concentration. Common species appear to be present between THOM proteins, particularly an 80–90 kDa complex which might have been observed previously in BN PAGE. In addition lower molecular weight species are observed around 50 kDa for THOM A, B and E. THOM E complexes were also resolved at higher molecular weights (>100 kDa), suggesting that, under these conditions, THOM proteins are interacting with other, diverse proteins in the hydrogenosome membrane to generate additional complexes. When DSP crosslinked samples were treated with DTT, complexes were abolished indicating that the crosslinking is reversible.

3.5. Co-immunoprecipitation of THOM proteins and complexes

To investigate whether complexes identified by BN PAGE were amenable for purification and identification, we co-immunoprecipitated HA-tagged proteins from THOM expressing hydrogenosomes after solubilisation in 1% Triton X-100. Detergent-soluble, co-immunoprecipitated protein can be seen in Figs. 5A and B. Although HA-tagged THOM proteins and their complexes could be recovered by co-immunoprecipitation (Fig. 5A), with masses of complexes similar to those observed in BN-PAGE (Fig. 4), the relative abundance of these did not allow for further identification by mass spectrometry (see Coomassie stained lanes in Fig. 5B). Higher molecular weight complexes were only observed in the presence of cross-linker (Fig. 5C) and were not observed in C1 strain hydrogenosomes.

4. Discussion

Our bioinformatic analyses suggest that THOM proteins are the products of divergent evolution of an ancestral Tom40 protein, consistent with the assertion [5] that the development of the outer membrane translocases would have been an initial event in the evolution of the endosymbiont, and consistent with the hypothesis that the hydrogenosome is a product of mitochondrial evolution.

Recent eukaryotic lineages have two distinct families of β-barrels that represent VDAC and Tom40 like functions. In excavates this is not so apparent. Our analysis strongly supports the existence of an extended family of Tom40 like β-barrels in T. vaginalis, in contrast to trypanosomes which have a single ancestral β-barrel/porin-like protein that is not clearly assigned as either VDAC or Tom40 [14–15]. The THOM proteins in T. vaginalis represent a single family of translocases within the hydrogenosome, and whilst this paper supports a similarity to Tom40, the absence of strong VDAC homologues could suggest either a functional differentiation of THOM proteins to fulfill VDAC functions, or that the THOM proteins represent an ancestral group of translocases, prior to the functional divergence of outer membrane β-barrels in other lineages.

Although highly divergent in sequence, the T. vaginalis THOM proteins maintain the essential features of other eukaryotic β-barrel proteins including a well conserved β-barrel signal sequence[29]. Microscopy results show that the THOM proteins are localised to numerous discrete organelles similar to those observed in EM studies but have a localisation distinct from the lumenal marker frataxin [15]. Biochemical analysis confirms hydrogenosomal membrane localisation and supports the ability of THOM proteins to form higher molecular weight complexes. Whilst individual THOM proteins exhibit variation, complexes of around 50 kDa and ~140 kDa were common to many. Whilst this investigation was unable to identify the constituent members of these complexes it was shown that they are amenable to analysis by co-immunoprecipitation.

Recent work by two groups [30,31] has also supported the presence of multiple Tom40 candidates in the T. vaginalis hydrogenosome from proteomic data on purified organelles (see Supplementary Table 2 for a cross-reference of their results). In particular their results corroborate closely our findings for THOM-C. In the current study we have been able to provide complementary evidence for the hydrogenosomal localisation and complex-forming ability of several Tom40 candidates, bringing the total number of these in T. vaginalis to at least 9. Gene family expansion in Trichomonas is well documented [32] and further work is required to understand the redundancy of THOM proteins through identification of the protein partners in the complexes isolated here.
Acknowledgements

CK was supported by a BBSRC Doctoral Training Grant. We thank Jan Tachezy (Charles University, Prague) for the pTagVag vector, and Helen Brooks for modifications made to this vector.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2012.10.004.

References