FEBS Letters 586 (2012) 4038–4045

journal homepage: www.FEBSLetters.org

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

Christopher Kay [a], Karen Lawler [a], Tim J. Self [a], Sabrina D. Dyall [b], Ian D. Kerr [a,*]

[a] School of Biomedical Sciences, University of Nottingham Medical School, Queen's Medical Centre, Nottingham, NG7 2UH, United Kingdom
[b] Department of Biosciences, University of Mauritius, Reduit, Mauritius

ARTICLE INFO

Article history:
Received 31 August 2012
Revised 1 October 2012
Accepted 1 October 2012
Available online 13 October 2012

Edited by Stuart Ferguson

Keywords:
β-Barrel
Trichomonas
Hydrogenosome
Tom40
Organelle
Membrane

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.

Abbreviations: gDNA, genomic DNA; aa, amino acids; BN PAGE, blue native PAGE; THOM, translocase of hydrogenosome outer membrane; RT, room temperature; DSP, Dithiobis [succinimidyl propionate]; MBS, m-maleimidobenzoyl-*N*-hydroxysuccinimide ester

* Corresponding author.
E-mail address: ian.kerr@nottingham.ac.uk (I.D. Kerr).

0014-5793/$36.00
http://dx.doi.org/10.1016/j.febslet.2012.10.004

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) iodixanol. 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 glycerol (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 $MgCl_2$, 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-aminocaproic 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% (w/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 1×10^7/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 RNAase (100 μg/ml) at 37° 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.