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Knockout of the abundant *Trichomonas vaginalis* hydrogenosomal membrane protein *Tv*HMP23 increases hydrogenosome size but induces no compensatory up-regulation of paralogous copies

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ABSTRACT

The *Trichomonas vaginalis* genome encodes up to 60 000 genes, many of which stem from genome duplication events. Paralogous copies thus accompany most *T. vaginalis* genes, a phenomenon that limits genetic manipulation. We characterized one of the parasite's most abundant hydrogenosomal membrane proteins, *Tv*HMP23, which is phylogenetically distinct from canonical metabolite carriers, and which localizes to the inner hydrogenosomal membrane as shown through sub-organellar fractionation and protease protection assays. Knockout of *Tv*hmp23 through insertion of the selectable neomycin marker led to a size increase of hydrogenosomes, the first knockout-induced phenotypes reported for *Trichomonas*, but no growth impairment. The transcriptional response of its four paralogous copies then analyzed revealed that they are not up-regulated, and hence do not compensate for the *Tv*hmp23 knockout.

1. Introduction

Trichomonas vaginalis is an anaerobic protist and the causative agent of trichomoniasis, a widespread sexually transmitted infection of the urogenital tract of humans, causing symptoms particularly in women where the parasite feeds on vaginal epithelial cells [13,15] in a low oxygen environment [12,17,44]. Like all trichomonads, *T. vaginalis* possesses hydrogenosomes, anaerobic relatives of mitochondria, that generate ATP via substrate level phosphorylation through the fermentative breakdown of pyruvate to acetate, CO_2, and H_2 [25,27]. Like oxygen-respiring mitochondria, hydrogenosomes are surrounded by two membranes. Their matrix is granular and electron-dense [3], and because they lack a genome and translation machinery, trichomonad hydrogenosomes must import all their proteins from the cytosol [5,37].

Reports investigating the molecular machineries behind protein import and metabolite exchange across hydrogenosomal membranes are scarce. Bioinformatic analyses identified homologs of Tom40, Sam50, Tim17/22/23, Tim44 and Pam18 in *T. vaginalis* [8,36]. Some of these components have since been localized to the hydrogenosomal membranes [9,20] and proteomic analysis focusing on hydrogenosomal membrane proteins (HMPs) have identified additional components of the translocases of the outer and inner mitochondrial membrane (TOMs and TIMs, respectively; [36]). Multiple paralogs of the core component TOM40 and uniquely modified small TIM chaperones were detected. Other HMPs characterized include HMP35, a potential pore forming protein of the porin family, whose exact function remains unknown [11]. The only other identified metabolite carriers of *Trichomonas* are five genes belonging to the otherwise much larger family of mitochondrial carrier proteins (MCF; [36]). One MCF protein, HMP31, was characterized in detail and phylogenetic analysis and heterologous studies in yeast mitochondria demonstrated the protein to be an ATP/ADP carrier [10,41].

Information about protein and substrate transporters in *Trichomonas* is still limited. Functional analysis of novel components is often hampered by the many paralogous copies of a gene that can be present due to genome duplication events [6]. The lack of tools to genetically manipulate *Trichomonas* further complicates matters. The knockout of a gene through homologous

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recombination has only been reported once, for a hydrogenosomal ferredoxin [22]. The knockout produced no detectable phenotype, most likely because at least six potentially redundant copies of ferredoxin were later found encoded when the genome was sequenced [6]. The knockdown technique using antisense RNA generally seems feasible in *Trichomonas*, as indicated by two separate reports [28,30]. These however also remain isolated, suggesting this technique, too, has its limits.

To revisit the possibility of insertional gene deletion in *T. vaginalis* and in particular to determine whether the knockout of a gene results in the transcriptional up-regulation of its paralogous copies, we focused on *Tv*HMP23 (TVAG_455090). This protein was found to be among the most abundant proteins of the hydrogenosomal membranes [36], its function however remains unknown. Through homologous recombination we successfully knocked out *Tv*hmp23 and characterize the first knockout-induced phenotype reported for the parasite.

2. Materials and methods

2.1. Culturing and cloning

T. vaginalis strain T1 (JH Tai, Institute of Biomedical Sciences, Taipei, Taiwan) was cultivated in TYM medium at 37 °C as described previously [14]. *Tv*HMP23 (TVAG_455090) was tagged with a dihemagglutinin-tag (HA-tag) at the amino- and at the carboxy-terminus independently into pTagVag2 [18]. The knockout of the gene was based on homologous recombination. We generated a construct, in which the resistance cassette (Neo^R, with *Kpn*I and *BamH*I restriction sites) was flanked by 1182 base pairs of the 5′ upstream (containing *Sac*II and *Kpn*I restriction sites) and 974 base pairs of the 3′ downstream sequence of the *hmp23* encoding locus (containing *BamH*I and *Apa*I restriction sites). The construct was ligated into the *Sac*II/*Apa*I restricted plasmid pα-SCSB-NEO [7]. All constructs were verified through sequencing before transfecting *T. vaginalis* T1 as described previously [7,22] with 50 μg plasmid DNA for localization studies and with 100 μg of *Sac*II and *Apa*I digested plasmid p*Tv*HMP23-NEO. The restriction of the latter plasmid was verified through gel analysis prior to transfection. A list of all primers used is found in the supplementary material (Table S1). Positive transformants were selected for two weeks before further analysis with 100 μg/ml G418 (Roth) in TYM medium for pTagVag2 transfected cells and with 80 μg/ml G418 for p*Tv*HMP23-NEO transfected cells. Cell density was determined using a hemocytometer Thoma New (Superior-Marienfeld). Counts were made of quadruple samples from each strain, and 12 ml of TYM medium (w/o G418) were initially inoculated with 20 000 cells.

2.2. Localization analysis

Hydrogenosomes of *T. vaginalis* were isolated as described by Bradley and colleagues [4] with slight modifications [23,34]. All centrifugations were performed at 4 °C. *T. vaginalis* cells were lysed by grinding them in a mortar for 20 min on ice with glass beads (∅ 4–6 μm). Cell lysis was verified through microscopic examination. Glass beads were removed through centrifugation at 755×*g* for 10 min and crude cell lysate obtained from the supernatant. A cytosolic fraction was collected from the supernatant after subsequent centrifugation at 7500×*g* for 10 min of the initial lysate. Hydrogenosomes were separated from the remaining cell components by isopycnic centrifugation with 45% (v/v) Percoll. To separate hydrogenosomal membranes from the matrix, 2 mg of hydrogenosomes (wet paste) were incubated with 30 ml 0.1 M Na_2CO_3 pH 11.5 for 45 min on ice, including a 10 s shake every 10 min. The membrane fraction was then pelleted by ultracentrifugation at 208 000×*g* for 1 h at 4 °C. To reduce cross contaminations the procedure was repeated for the two individual fractions. 10 ml of the resulting supernatant was precipitated with acetone/TCA (7.5:1) and resuspended in 70 μl resuspension buffer [7 M urea, 2 M thiourea, 4% (w/v) CHAPS] and 10 μl concentrated Tris solution to neutralize the pH. The membrane pellet was washed with 100 μl ddH_2O and resuspended in 40 μl ddH_2O.

100 μg of each fraction collected were separated by a 12% SDS–PAGE and blotted onto nitrocellulose membranes (Hybond™-C Extra, GE Healthcare). Membranes were blocked for 1 h in TBS [20 mM Tris–HCl pH 7.4, 150 mM NaCl] containing 5% (w/v) dried milk powder and then incubated for 1 h at RT, or over night at 4 °C with mouse anti-HA monoclonal antibody (Sigma) diluted 1:5000 in blocking solution. After three TBS washes the membrane was incubated for 1 h at RT, or over night at 4 °C with goat anti-mouse horseradish peroxidase-conjugated antibody (ImmunoPure, Pierce) diluted 1:10 000 in blocking solution. After three TBS washes signals were detected using 3 ml solution A [1.25 mM luminol (Sigma) in 0.1 M Tris–HCl pH 8.6], 300 μl solution B [6 mM para-hydroxycoumaric acid (Sigma) in DMSO], 0.9 μl 30% (v/v) H_2O_2 and Lumi-Film chemiluminescent detection films (Roche). Original blots were stripped as described previously [47] and reprobed with anti-ASCT [42] and treated as above, but using a goat anti-rabbit horseradish peroxidase-conjugated as secondary antibody. Stripping was performed with two 10 min incubations with mild stripping buffer [1.5% glycine, 0.1% (w/v) SDS, 1% (v/v) Tween 20; pH 2.2], 2 × 10 min washes with PBS [0.8% (w/v) NaCl, 0.02% (w/v) KCl, 0.144% Na_2HPO_4, 0.024% (w/v) KH_2PO_4; pH 7.4] and 2 × 5 min washes with TBST (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% (v/v) Tween 20). Protease protection assays were performed with freshly isolated hydrogenosomes following a previously established protocol [47].

Immunofluorescent images of the expressed HA-tagged proteins and ASCT (hydrogenosomal matrix marker) were performed using fixed *T. vaginalis* cells and mouse anti-hemagglutinin monoclonal antibody (Sigma–Aldrich) and rabbit anti-ASCT polyclonal antibody [42] as primary antibodies and secondary Alexa Fluor-488 donkey anti-mouse and Alexa Fluor-633 donkey anti-rabbit antibodies (Invitrogen) as described previously [47]. Images were obtained using a LSM 510 Meta confocal laser scanning microscope (Zeiss). For determination of potential transmembrane-spanning regions using TMHMM, HMMTOP and TMPred, the default settings were used.

2.3. Isoelectric focusing (IEF) and SDS–PAGE electrophoresis

IEF, equilibration and SDS–PAGE electrophoresis were performed as described by Pütz and colleagues [35]. IEF was run on the Ettan IPGphor 3 system (GE Healthcare) using an 18 cm immobilized pH gradient (IPG) (Immobiline DryStrip, GE Healthcare) with a linear gradient pH 6–11, followed by a standard 2nd dimension SDS–PAGE using a 12% gel. Protein spots were picked from gels and tryptic digested in-gel. Eluted protein spots were digested in-gel with trypsin (Promega) as described previously [19]. Peptides were sequenced through mass spectrometry at the BMFZ (HHU Düsseldorf) and a MASCOT search used to identify the proteins [33].

2.4. Transmission electron microscopy

T. vaginalis cells were pelleted at 1000×*g* for 10 min and washed three times with PBS [0.8% (w/v) NaCl, 0.02% (w/v) KCl, 0.144% Na_2HPO_4, 0.024% (w/v) KH_2PO_4; pH 7.4]. After fixation over night at 4 °C in fixation buffer [2.5% (v/v) glutaraldehyde in 0.1 M Na-cacodylate buffer pH 7.3] the cells were washed four times for 10 min with 0.1 M Na-cacodylate buffer pH 7.3. Post fixation was done within two hours incubation with 2% (w/v) osmiumtetroxide diluted in