Charles University, Faculty of Science

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Ph.D. study program: Parasitology

Doktorský studijní program: Parazitologie

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Protein translocation into hydrogenosomes of *Trichomonas vaginalis*

Translokace proteinů do hydrogenosomů *Trichomonas vaginalis*

Ph.D. Thesis

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Prague, 2019

ABSTRACT

Mitochondria carry out several important functions in eukaryotic cells such as energy metabolism, iron-sulfur cluster assembly, apoptosis, signaling pathways, protein quality control etc. Most mitochondrial proteins are synthesized on the cytosolic ribosomes and transported to the organelles by the cytosolic chaperones and mitochondrial protein import machinery based on specific targeting signals. Although, the basic principles of protein import have been explained, many questions remain unanswered, particularly for highly modified mitochondria such as hydrogenosomes. The aim of the study was to investigate protein translocation into hydrogenosomes of a human parasite, *Trichomonas vaginalis* (Tv) with a focus on the composition, function and structure of protein translocases and the role of targeting signals.

The translocase of the outer membrane (TOM) is responsible for the import of most proteins into the organelle. Even though, the presence of a TOM complex in trichomonad hydrogenosomes was predicted, its components were not known. Moreover, the generic structure of the mitochondrial TOM complex was not resolved. This study showed that the TvTOM complex is highly divergent consisting of two modified core subunits – channel-forming TvTom40 isoforms and a Tom22-like protein, and two lineage-specific subunits – Tom36 and Tom46 that most likely, function as receptors. Additionally, TvTOM forms a stable supercomplex with Sam50 that is involved in the biogenesis of β-barrel proteins. Electron microscopy revealed that the translocase has a triplet-pore structure with a unique skull shape.

Mitochondrial matrix preproteins carry an N-terminal targeting sequence (NTS). Interestingly, a glycolytic enzyme, ATP-dependent phosphofructokinase (ATP-PFK) that does not contain a predictable NTS localizes to hydrogenosomes. Localization experiments suggested that TvATP-PFK and its homologous ATP-PFKs from yeast and *E. coli* possess unknown internal targeting signal (ITS) that is possibly recognized by the protein import machinery. From an evolutionary perspective, the ability of mitochondria and hydrogenosomes to recognize proteins such as ATP-PFK may represent an ancient mechanism from the early phases of organelle evolution whereas, NTS-dependent import might have evolved later. *T. vaginalis* has several unique tail-anchored (TA) proteins, a class of integral membrane proteins that localize to the hydrogenosomal outer membrane, including the newly characterized TvTOM subunits. Analyses of physico-chemical properties and localization experiments identified new traits for hydrogenosomal TA protein targeting such as higher net positive charges in the C-terminal segment which, otherwise are primarily for peroxisomal TA proteins

in aerobic eukaryotes, and a slightly longer transmembrane domain when compared to mitochondrial TA proteins.

Taken together, these studies show that the protein import into hydrogenosomes is rather divergent compared to that of mitochondria. The triplet-pore TOM complex, composed of conserved core subunits was present in the last common eukaryotic ancestor while, the peripheral receptors evolved independently in different eukaryotic lineages. The changes observed in the protein translocases and the targeting signals most likely reflect the adaptation of hydrogenosomes to anaerobic conditions, particularly, the loss of respiratory chain complexes that resulted in low or absence of membrane potential.

ABSTRAKT (CZECH)

Mitochondrie plní řadu významných funkcí v eukaryotických buňkách, jako je energetický metabolismus, syntéza železo-sirných center, apoptóza, bunečná signalizace, kontrola kvality proteinů atd. Většina mitochondriálních proteinů je syntetizována na cytosolických ribozomech a transportována do organel za pomocí cytosolických chaperonů a mitochondriálních membránových translokáz, které rozpoznávají specifické adresové sekvence. Přestože základní principy importu proteinů jsou známé, mnoho otázek zůstává nezodpovězeno, zejména u vysoce modifikovaných mitochondrií, jako jsou hydrogenosomy. Cílem této studie bylo prozkoumat translokaci proteinů do hydrogenosomů lidského parazita *Trichomonas vaginalis* (Tv), se zaměřením na složení, funkci a strukturu proteinových translokáz a roli adresových sekvencí.

Translokáza vnější mitochondriální membrány (TOM) je zodpovědná za import většiny proteinů do mitochondrií. Ačkoliv přítomnost komplexu TOM v hydrogenosomech trichomonád byla predikována na základě analýzy genomu, jednotlivé složky komplexu nebyly známy. Navíc ani celková struktura mitochondriálního komplexu TOM nebyla zcela vyřešena. Tato studie ukázala, že komplex TvTOM je velmi divergentní, sestávající se ze dvou modifikovaných základních podjednotek – TvTom40, který tvoří translokační kanál a protein podobný Tom22, a dále ze dvou podjednotek specifických pro linii trichomonád – Tom36 a Tom46, které pravděpodobně fungují jako receptory. TvTOM navíc tvoří stabilní superkomplex se Sam50, který se podílí na biogenezi β-barelových proteinů. Elektronová mikroskopie odhalila, že studovaná translokáza obsahuje triplet pórů a má neobvyklý tvar připomínající lebku.

Preproteiny, které jsou určeny pro transport do mitochondrialní matrix obvykle nesou N-terminální adresovou sekvenci (NTS). Je proto zajímavé, že glykolytický enzym, ATP-dependentní fosfofruktokináza (ATP-PFK) je importován do hydrogenosomů bez predikovatelné NTS. Lokalizační experimenty prokázaly, že TvATP-PFK a homologní ATP-PFK z kvasinek a *E. coli* mají neznámé interní adresové signály (ITS), které jsou rovněž rozpoznávány importní mašinerií. Z evolučního hlediska může být schopnost mitochondrií a hydrogenosomů rozpoznávat proteiny jako je ATP-PFK na základě ITS původním mechanismem, zatímco import, který závisí na NTS se objevil později. *T. vaginalis* má řadu proteinů kotvených C-terminální doménou (tail-anchored, TA) ve vnější hydrogenosomální membráně, včetně nově charakterizovaných podjednotek TvTOM. Analýzy fyzikálně-chemických vlastností a lokalizační experimenty ukázaly specifické vlastnosti hydrogenosomálních TA proteinů, jako je vyšší kladný náboj v C-terminálním segmentu, který je v ostatních eukaryotických buňkách charakteristický pouze pro peroxisomální TA proteiny, a ve srovnání s mitochondriálními TA proteiny, hydrogenosomální TA proteiny mají delší transmembránovou doménu.

Celkově tyto studie ukazují, že import proteinů do hydrogenosomů je ve srovnání s mitochondriemi v mnoha aspektech odlišný. Ačkoliv komplex TOM s tripletem pórů složených ze tří centrálních podjednotek, byl patrně přítomen již u posledního společného předka eukaryotických organismů, periferní receptory se vyvíjely nezávisle v různých eukaryotických liniích. Změny pozorované ve struktuře translokáz a adresových sekvencích u hydrogenosomů s největší pravděpodobností odrážejí adaptaci hydrogenosomů k anaerobním podmínkám, zejména ztrátu respiračních komplexů, která vedla ke snížení nebo ztrátě membránového potenciálu.

INTRODUCTION

Mitochondria perform numerous important functions in eukaryotic cells such as energy metabolism, combat oxidative stress, protein quality control and cellular signaling, iron-sulfur cluster assembly etc. Mitochondrion originated from a proteobacterium that formed an endosymbiotic relationship with an early-eukaryotic cell or an archaebacterium [1,2]. During the transition from an endosymbiont to the organelle, the endosymbiont genes were either transferred to the host nucleus or lost. Consequently, the proteins required were synthesized on the cytoplasmic ribosomes and transported to the evolving organelle [3]. The protein import

into mitochondria are mainly mediated by the translocase of the outer membrane (TOM), sorting and assembly machinery (SAM), translocases of the inner membrane (TIM23 and TIM22 complexes), presequence-associated motor (PAM) and other lineage-specific import machinery [4,5]. The mitochondrial proteins are targeted and sorted to their final destination based on specific targeting signals [6]. The mechanism of protein import has been well studied in opisthokonts while, many aspects remain unknown in organisms of other groups especially those which carry highly modified anaerobic forms of mitochondria such as hydrogenosomes. Additionally, the structure of mitochondrial protein translocases has not been resolved completely.

Hydrogenosomes are present in many anaerobic free-living and parasitic eukaryotes including the human parasite, *Trichomonas vaginalis* [7,8]. The trichomonad hydrogenosomes metabolize mostly pyruvate and/or malate to carbon dioxide, acetate and hydrogen with concomitant synthesis of ATP by substrate-level phosphorylation. They are devoid of genome, protein synthesis machinery, membrane-bound respiratory chain complexes, cristae in the inner membrane and tricarboxylic acid cycle. However, they play vital roles in iron-sulfur cluster assembly, amino acid metabolism and detoxification etc. As per the proteomic data, the organelle has around 600 proteins with 70 of them predicted to be in the outer and inner membranes [9,10].

The TOM complex is the general import pore through which most mitochondrial proteins are imported [6]. In yeast, this complex is composed of a channel-forming Tom40, primary receptors Tom20 and Tom70, central receptor Tom22 that also tethers two Tom40 molecules to form an oligomer and small Toms - Tom5, Tom6 and Tom7 that are involved in the maintenance of TOM. Tom40, Tom22 and Tom7 are conserved in most eukaryotes and thus, are likely to have been present in the proto-TOM complex of the last common eukaryotic ancestor [4,11]. The small Toms Tom5 and Tom6 are present in animals, fungi and plants. The canonical Tom20 is present in animals and fungi while, a tail-anchored Tom20 is found in plants [4,12]. Tom70 is present in animals, fungi and members of Stramenopiles, Alveolata and Rhizaria supergroup (SAR) [13]. Some protists have lineage-specific receptors such as Tom60 in amoebozoans (Entamoeba species), and ATOM69 and ATOM46 in *Trypanosoma brucei* [14,15]. However, the *T. vaginalis* hydrogenosomal proteome showed that other than putative Tom40-like proteins, none of the known TOM components was present [9]. Our knowledge on the structure of the TOM complex is exclusively based on experiments conducted in two fungal model organisms. Cryo-electron microscopy studies showed a TOM

with three pores in *Saccharomyces cerevisiae* and a TOM with two pores in *Neurospora crassa* [16,17]. Hence, the generic structure of TOM was still unresolved.

The mitochondrial proteins carry a cleavable N-terminal targeting sequence (NTS) or an internal targeting signal (ITS) for their efficient delivery to the correct sub-compartment of mitochondria [5]. The NTS is present on the matrix proteins and in some cases, intermembrane space and inner membrane proteins. However, the NTS in many hydrogenosomal proteins of *T. vaginalis* is shorter and has lower positive charge compared to the mitochondrial NTS [18]. Even when the NTS was removed, certain hydrogenosomal proteins were targeted to *T. vaginalis* hydrogenosomes and yeast mitochondria [19,20]. Tail-anchored (TA) proteins are a class of integral membrane proteins with a transmembrane domain (TMD) near the C-terminus. These proteins are present in the outer membrane of mitochondria and chloroplast, membrane of endoplasmic reticulum and peroxisomes, and in bacteria. The mitochondrial TA proteins are imported independent of TOM channel and their targeting signal is present in the TMD and its flanking regions [21]. Mitochondrial TA proteins have a TMD of moderate hydrophobicity that is flanked with positively charged residues [21,22]. Changes made to the TMD and its flanking regions by deleting the TMD, increasing the TMD length, lowering the net positive charge of the CTS, inserting a linker between the TMD and the CTS have impaired the localization of mitochondrial TA proteins [21]. So far, the mechanism of targeting of mitochondrial TA proteins has been mainly studied in yeast and mammalian cell lines. The proteome of *T. vaginalis* hydrogenosomes had shown the presence of 12 unique putative TA proteins [9]. Previously, what factors are important for the targeting of TA proteins in *T. vaginalis* was not known.

AIMS AND OBJECTIVES:

1. To identify and characterize the components of TOM complex of hydrogenosomes in *T. vaginalis*
2. To investigate the interaction between the hydrogenosomal proteins (substrates) and the subunits of TvTOM complex
3. To elucidate the structure of TvTOM complex
4. To understand the evolution of TOM complex
5. To examine the NTS-independent transport of proteins to *T. vaginalis* hydrogenosomes
6. To study the targeting of TA proteins in *T. vaginalis*

LIST OF PUBLICATIONS AND CONTRIBUTIONS

1. Makki A, Rada P, Žárský V, Kereïche S, Kováčik L, Novotný M, Jores T, Rapaport D, Tachezy J (2019) **Triplet-pore structure of a highly divergent TOM complex of hydrogenosomes in *Trichomonas vaginalis***. PLOS Biol 17(1):e3000098.
Contributions: Design of the project and experiments, cloning and preparation of Trichomonas and yeast strains, immunofluorescence microscopy, subcellular fractionation, protease protection assay, alkaline carbonate extraction, blue native-PAGE, complementation study in yeast, immunoprecipitations, mass spectrometry (MS) data analyses and curation of MS data in PRIDE, bioinformatics, isolation of TvTOM complex and sample preparation for electron microscopy, cloning and synthesis of radiolabeled precursors, *in vitro* protein import experiments, expression and purification of His-tagged Trichomonas proteins from *E. coli*, binding assay for TvTOM receptor candidates, data analyses and interpretation, manuscript preparation and submission

2. Rada P, **Makki A**, Zimorski V, Garg S, Hampl V, Hrdý I, Gould SB, Tachezy J (2015) **N-terminal presequence-independent import of phosphofructokinase into hydrogenosomes of *Trichomonas vaginalis***. *J Eukaryot Cell* 14(12):1264–1275.
Contributions: Cloning and preparation of Trichomonas strains, immunofluorescence microscopy, subcellular fractionation, protease protection assay, figures for the manuscript

3. Rada P, **Makki A**, Žárský V, Tachezy J (2019) **Targeting of tail-anchored proteins to *Trichomonas vaginalis* hydrogenosomes**. *Mol Microbiol* 111(3):588–603.
Contributions: Cloning and preparation of Trichomonas cell lines, immunofluorescence microscopy, subcellular fractionation, protease protection assay, figures for the manuscript

4. Dolezal P, **Makki A**, Dyall SD (2019) **Protein import into hydrogenosomes and mitosomes. Hydrogenosomes and Mitosomes: Mitochondria of Anaerobic Eukaryotes**, ed Tachezy J (Springer International Publishing, Cham), pp 31–84.
Contribution: Co-author of the book chapter.

RESULTS AND CONCLUSIONS:

1. The TOM complex of *T. vaginalis* hydrogenosomes (Makki et al. 2019)

In this project, different subunits of TvTOM were identified and functionally characterized, and the structure of the translocase was elucidated. Among the seven putative Tom40-like proteins, TvTom40-2 was found to be the most conserved as per bioinformatic searches and modelling. TvTom40-2 was present in the hydrogenosomal outer membrane in high molecular weight complexes of ~570 and ~330 kDa [23]. Further, TvTom40-2 could partially complement the function of yeast Tom40 suggesting that the basic characteristics of Tom40 channel in different eukaryotes are similar [23,24].

The composition of TvTOM complex was investigated using co-immunoprecipitations (coIP) coupled with proteomic and bioinformatics analyses. TvTom40-2 interacts with other isoforms of TvTom40s, four TA proteins – Tom22-like protein, Tom36, Tom46 and Homp19, and the core subunit of sorting and assembly machinery, Sam50 [23]. The TA proteins were present in high molecular weight complexes in the hydrogenosomal outer membrane similar to TvTom40-2 [23]. Tom22-like protein has a short cytosolic *cis* domain and a conserved Tom22 transmembrane segment but, lacks an intermembrane space-localized *trans* domain [23]. Tom36 and Tom46 have an N-terminal Hsp20 chaperone-like domain followed by three TPRs, similar to the TOM receptor ATOM69 of *T. brucei* [15,23]. The cytosolic domains of both Tom36 and Tom46 which have Hsp20 chaperone-like and TPR domains can bind to hydrogenosomal preproteins frataxin and α-subunit of succinyl coA synthetase but, not cytosolic control protein cytochrome b_5 as observed through binding assays. Based on overall data, Tom36 and Tom46 most likely function as TvTOM receptors [23].

To test the function of TvTom40-2, a combination *in vitro* import assay and coIP was employed. The translocation of a hydrogenosomal ferredoxin-dihydrofolate reductase (DHFR) fusion protein used as a substrate was arrested at the hydrogenosomal protein import site via methotrexate-induced folding of the DHFR region. The translocation-arrested ferredoxin-DHFR substrate was co-purified with TvTom40-2 demonstrating that TvTom40-2 mediates protein import into hydrogenosomes [23]. This also confirmed that similar to other forms of mitochondria, hydrogenosomes import soluble preproteins in an unfolded or loosely folded manner [23,25–27].

Reciprocal coIPs showed that Sam50 is tightly associated with different isoforms of TvTom40 including TvTom40-2 and Tom36, and Sam50 is present in a ~570 kDa complex similar to TvTom40-2 implying that TvTOM and Sam50 form a stable supercomplex [23].

Sam50 is the only known subunit of SAM in *T. vaginalis* hydrogenosomes. In yeast, Sam37 interacts with Tom22 to form a transient TOM-SAM supercomplex [28,29]. Since, Sam37 is absent in trichomonads, a different mode of interaction can be expected between TvTOM and Sam50 [23].

Electron microscopic visualization of the isolated TvTOM complex revealed three types of particles – TOM with one, two and three pores [23]. The triplet-pore structures represent the mature holo complex and the particles that displayed two pores represent the TOM core complex. While some features of TvTOM are similar to TOMs in fungi [17,30], the dimensions of TvTOMs with one and three pores (70 X 125 Å and 150 X 175 Å respectively) are different because of the presence of an extra component outside the TvTom40 barrel that seems to form a stable association and thus, giving a skull shape to the triplet-pore TvTOM structure [23]. Based on this study and previous reports on TOMs in fungi, the triplet-pore structure seems to be generic for all mitochondrial TOMs.

Tom22 and Tom7 are present in many eukaryotes but, they had not been identified in many excavates questioning if, they were present in the earliest TOM. However, the recent identification of Tom22 homologues in *T. brucei*, *T. vaginalis*, *Carpediemonas membranifera*, *Naeglaria gruberi*, *Euglena gracilis* and *Stygiella incarcarata* and Tom7 homologues in *C. membranifera*, *S. incarcerata, N. gruberi*, *N. fowleri* and *E. gracilis* changed the status quo [23,31,32]. Absence of Tom7 in *T. vaginalis* suggests that Tom7 might have been secondarily lost, or an improved method is required to identify proteins encoded by small open reading frames. The TOM receptors are mostly lineage-specific and were gained later during evolution. Although, Tom36 and Tom46 resemble ATOM69 in domain features, they seemed to have evolved independently [23]. Collectively, the proto-TOM complex present in the last common eukaryotic ancestor was most likely trimeric in nature and consisted of at least three components Tom40, Tom22 and Tom7 [23].

2. NTS-independent import of phosphofructokinase into *T. vaginalis* hydrogenosomes (Rada et al. 2015)

In this project, the import of TvATP-dependent phosphofructokinase (PFK) into *T. vaginalis* hydrogenosomes was examined. Our previous study on the hydrogenosomal proteome had shown the presence of a few isoforms of ATP-PFK which is rather unusual considering that PFK is a glycolytic enzyme and none of the enzymes involved in the upstream or downstream reactions are present in hydrogenosomes [9]. TvATP-PFK1 does not have a predictable NTS nevertheless, both the full-length protein and an N-terminal 16 residue truncated version could

localize to hydrogenosomes suggesting that it carries an unknown ITS [33]. Further, when expressed in yeast, TvATP-PFK localized to mitochondria. The yeast ATP-PFK (ScPFK) is cytosolic and has three regions: an N-terminal segment, a catalytic domain that is homologous to TvATP-PFK1 and a C-terminal regulatory domain. When expressed in *T. vaginalis*, both full-length ScPFK and a mutant without the regulatory domain localized in the cytosol but, was associated with the hydrogenosomal surface. However, when the catalytic domain of ScPFK alone was expressed, it localized to hydrogenosomes. When heterologously expressed in *T. vaginalis*, *E. coli* ATP-PFK localized to hydrogenosomes. These results imply that both the catalytic domain of ScPFK, which is similar to TvATP-PFK1, and *E. coli* ATP-PFK have some targeting signal or they carry an ancient feature that facilitates their import to hydrogenosomes and perhaps, the features in the N-terminal segment of ScPFK prevents its transport to mitochondria or in this case, hydrogenosomes [33]. Despite the deletion of NTS from some mitochondrial and hydrogenosomal matrix proteins, their localization to the organelles was not affected [19]. However, TvATP-PFK is the first case of a soluble protein that localizes to hydrogenosomes (mitochondria) without a predictable NTS [33].

The loss of respiratory chain complexes in hydrogenosomes has most likely made the positive charge of NTS expendable. Most hydrogenosomal NTSs are shorter with only one positively charged residue [18], and in many cases, they are either not essential for preprotein import or simply absent [19,20,33]. Thus, the import of these proteins is based on recognition of poorly understood ITSs. Perhaps, such changes in the targeting signals are likely reflected by the presence of lineage-specific TvTOM receptors, the absence of an elongated *cis* and acidic *trans* domains in Tom22-like protein and the divergence of TIM machinery [9,23].

3. Targeting of TA proteins (Rada et al. 2018)

In this project, the targeting of TA proteins in *T. vaginalis* was investigated. *T. vaginalis* has two primary locations where the TA proteins can be localized: hydrogenosomal outer membrane and ER membrane. The proteome of *T. vaginalis* hydrogenosomes had shown the presence of 12 unique putative TA proteins [9]. First, the localization and topology of some candidates was confirmed using biochemical and microscopy experiments [34]. The elongation of the TMD of Tom5 by insertion of 3-7 valine (hydrophobic) residues had resulted in reduced targeting to mitochondria but, was never abolished, and led to their mis-localization to the ER [21]. When 9 valine residues were introduced in the TMD of *T. vaginalis* TA4, the mutant TA4 did not localize to hydrogenosomes but, instead remained in the cytosol [34]. Removal of both the TMD and the CTS of TA4 also resulted in its localization in the cytosol. However, both

removal of the CTS and replacement of five lysine residues to serine residues in the CTS caused a dual localization of the mutants to both hydrogenosomes and ER [34]. Trichomonads possess flattened ER sacs around the nucleus and interestingly, in the TA4 mutant without the CTS, the ER was dissipated into multiple vesicles [34]. Further, to understand the dynamics of TA protein targeting to hydrogenosomes and ER, domain swapping experiments were performed. A chimeric protein carrying the soluble part of hydrogenosomal TA4 fused to the C-terminal region of ER protein disulfide isomerase (flanking region 1-TMD-flanking region 2) localized to the ER. When the TMD of TA4 in this fused protein was replaced with the TMD of TA-PDI, it had no effect and was still targeted to the ER suggesting that the charges on the flanking regions of the TMD played a dominant role in the targeting [34]. The targeting of mitochondrial and hydrogenosomal TA proteins share some properties: (i) the C-terminal TMD is of a defined length, (ii) the TMD is flanked by basic residues at the N-terminus, C-terminus or both and (iii) the TMD domain is of moderate hydrophobicity. Despite these, there are notable differences in hydrogenosomal TA protein targeting: (i) the TMD is longer, (ii) the net positive charge on the CTS is higher and (iii) the difference in the net positive charge of CTS between hydrogenosomal and ER proteins is higher. In fungi and mammals, the net positive charge of CTS of TA proteins is of the decreasing order: peroxisomes followed by mitochondria and then, ER. Since, peroxisomes seem to be absent in *T. vaginalis*, the hydrogenosomal proteins have a higher range of net positive charge in the CTS [34].

Adaptation to operate under anaerobic conditions has resulted in an enormous reduction of both mitochondrial functions as well as proteome of *T. vaginalis* hydrogenosomes [9,10,35]. The following publications point out that these adaptations, particularly the loss of respiratory chain complexes that led to a low membrane potential, or its absence were seminal for the shaping of protein import into *T. vaginalis* hydrogenosomes.

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