

Invited review

Protein targeting in parasites with cryptic mitochondria

Lena Burri, Patrick J. Keeling *

Canadian Institute for Advanced Research, Department of Botany, University of British Columbia, 3529-6270 University Boulevard, Vancouver, BC, Canada V6T 1Z4

Received 4 October 2006; received in revised form 5 December 2006; accepted 11 December 2006

Abstract

Many highly specialised parasites have adapted to their environments by simplifying different aspects of their morphology or biochemistry. One interesting case is the mitochondrion, which has been subject to strong reductive evolution in parallel in several different parasitic groups. In extreme cases, mitochondria have degenerated so much in physical size and functional complexity that they were not immediately recognised as mitochondria, and are now referred to as ‘cryptic’. Cryptic mitochondrion-derived organelles can be classified as either hydrogenosomes or mitosomes. In nearly all cases they lack a genome and all organellar proteins are nucleus-encoded and expressed in the cytosol. The same is true for the majority of proteins in canonical mitochondria, where the proteins are directed to the organelle by specific targeting sequences (transit peptides) that are recognised by translocases in the mitochondrial membrane. In this review, we compare targeting sequences of different parasitic systems with highly reduced mitochondria and give an overview of how the import machinery has been modified in hydrogenosomes and mitosomes.

© 2006 Australian Society for Parasitology Inc. Published by Elsevier Ltd. All rights reserved.

Keywords: Parasites; Mitochondria; Hydrogenosome; Mitosome; Targeting sequence; Presequence processing

1. Introduction

Mitochondria are double-membrane bounded organelles that originated through the endosymbiosis of an α -proteobacterium in the last common ancestor of all known eukaryotes (Fig. 1) (Gray et al., 1999). One of the critical steps in this integration process was the movement of genetic information from the endosymbiont to the host cell nucleus. To ensure the proto-mitochondrion retained all the proteins required for its maintenance, this movement of genes required a complementary system to ensure the endosymbiont-derived proteins now translated on cytosolic ribosomes were correctly identified and re-imported into the evolving organelle. This involved the evolution of mitochondrial targeting sequences in these proteins and the assembly of a protein import machinery in the membranes of the endosymbiont.

Ultimately mitochondria have become involved in many important cellular processes: they are best known for their role in Krebs cycle, electron transport and oxidative phosphorylation, but they are also involved in maturation of iron–sulphur (Fe–S) proteins, urea cycle, heme biosynthesis, metabolism of fatty-acid and certain amino acids, and programmed cell death (Reichert and Neupert, 2004). There is great diversity in mitochondrial metabolism and it probably varies from species to species, so it is difficult to call one mitochondrion ‘normal’ and another ‘strange’, but for simplicity we will refer to mitochondria involved in aerobic metabolism ‘canonical’. This is useful because in many lineages of anaerobic or microaerophilic eukaryotes, predominantly parasites, mitochondria have lost oxidative phosphorylation and associated activities and have been reduced and remodelled structurally and functionally to such an extent that they became cryptic, or difficult to recognise as mitochondria. Indeed, several such lineages were for some time thought to be primitively amitochondriate (Cavalier-Smith, 1987). It is now clear that all ‘amitochondriate’ eukaryotes actually evolved after the

* Corresponding author. Tel.: +1 604 822 4906; fax: +1 604 822 6089.
E-mail address: pkeeling@interchange.ubc.ca (P.J. Keeling).

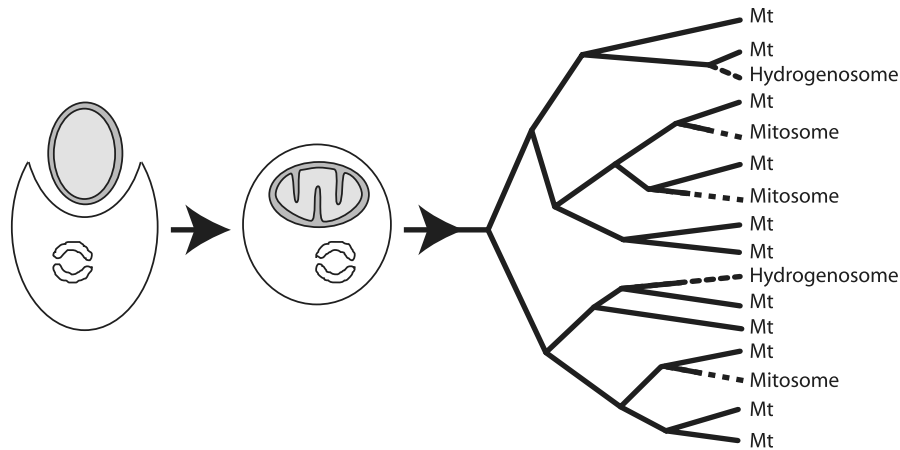


Fig. 1. Schematic representation of the endosymbiotic origin of mitochondria. An ancestor of all extant eukaryotes engulfed an α -proteobacterium, which was retained in the cytosol. Most of the endosymbiont genes were subsequently lost or transferred to the host nuclear genome. For these transfers to take place without disrupting organelle function, a system had to be created to target specific proteins to the organelle using targeting sequences on those proteins and an import machinery to recognise them, translocate the proteins, and sort them within the organelle. Increasing evidence indicates that hydrogenosomes and mitosomes evolved multiple times, independently from mitochondria, and that they retain core components of this targeting system.

endosymbiotic event and retain a relic organelle (Roger, 1999; Williams and Keeling, 2003; Van Der Giezen and Tovar, 2005; Van Der Giezen et al., 2005). Among these are some that are a conspicuous part of the cell and still play an important role in energy generation, in particular the hydrogenosomes, which are an adenosine 5'-triphosphate (ATP)- and hydrogen-generating organelle (Müller, 1993). Others are referred to as mitosomes and are small, non-descript cryptic organelles that do not appear to play a major role in the generation of ATP anymore (Katinka et al., 2001; Abrahamsen et al., 2004; Xu et al., 2004). Instead, the main function of mitosomes (and one role of hydrogenosomes) seems to be Fe-S cluster assembly (Tovar et al., 2003). Interestingly, although Fe-S cluster assembly is not the first thing we typically associate with mitochondria, it is the only essential process in yeast mitochondria and is conserved in all mitochondria and mitochondria-derived organelles observed to date (Lill and Kispal, 2000), with the apparent exception of *Entamoeba histolytica* where the enzymes are derived by a more recent lateral gene transfer and are apparently cytosolic due to their lack of targeting signals (Ali et al., 2004; Van Der Giezen et al., 2004).

In canonical mitochondria, only a small genome is retained, most proteins are encoded by the host nucleus and are targeted to mitochondria by specific targeting sequences that are recognised by a protein import machinery. The targeting information resides in either N-terminal extensions (presequences) or is contained internally in the protein itself (Schatz and Dobberstein, 1996; Neupert, 1997; Pfanner and Geissler, 2001; Wattenberg and Lithgow, 2001; Rapaport, 2003). Presequences are a short stretch of basic amino acids (Von Heijne et al., 1989) forming an amphipathic α helix with one positively charged and one hydrophobic side (Schatz and Dobberstein, 1996; Neupert, 1997; Abe et al., 2000; Pfanner and Geissler, 2001). They are recognised by the TOM (Translocase of the Outer

Membrane) complex and the TIM (Translocase of the Inner Membrane) 23 complex before being sorted to the matrix, inner membrane or intermembrane space. Once in the matrix, most presequences are removed by the mitochondrial processing peptidase (MPP) (Hawlitschek et al., 1988; Arretz et al., 1994). On the other hand, proteins with internal targeting signals are transported to the inner membrane, intermembrane space or outer membrane. They are not well defined and can be distributed throughout the whole protein (Brix et al., 1999; Wiedemann et al., 2001).

The purpose of this review is to summarise recent findings of the impact of reductive evolution on protein targeting and presequence processing systems in mitochondria of parasites and in the mitochondria-derived organelles, hydrogenosomes and mitosomes.

2. Protein targeting to mitochondria in parasites

One example of parasites where mitochondrial presequences have been functionally characterised are kinetoplastids. They are a group of flagellate protists responsible for serious human and animal diseases. Their single unusual mitochondria is distinguished by the presence of a kinetoplast, a DNA-containing granule composed of maxicircles and minicircles, RNA editing and tRNA import (Simpson et al., 1989; Hancock and Hajduk, 1990; Simpson, 1990; Borst, 1991; Mottram et al., 1991). Many, but not all, trypanosomatid mitochondrion-targeted proteins include a small but predictable presequence (Clayton et al., 1995), and targeting has been shown to be both ATP- and membrane potential-dependent (Hauser et al., 1996; Priest and Hajduk, 1996). Many other features of the general matrix import pathway also seem to be conserved as it has been shown that yeast mitochondrial alcohol dehydrogenase (ADHIII) and an artificial precursor protein containing the nine-amino acid presequence of the trypanosome dihydrolipoamide dehydrogenase fused to

mouse dihydrofolate reductase (LDH-DHFR), were recognised and imported into mitochondria and processed using in vitro systems of both *Trypanosoma brucei* and *Leishmania tarentolae* (Hauser et al., 1996). Nonetheless, some elements of the assembly pathway of the cytochrome *c* reductase complex in trypanosomes are unconventional. One subunit, the Rieske iron–sulfur protein (ISP), has a 17-residue presequence with characteristics predicted for a mitochondrial targeting sequence that is processed in two steps like its fungal homologue. In contrast to fungal ISP, however, which is always first processed in the matrix by MPP and then by the mitochondrial intermediate peptidase (MIP), trypanosome ISP precursor is processed by metalloproteases that reside on opposite sides of the mitochondrial membrane (Priest and Hajduk, 1996). Another subunit of the cytochrome *c* reductase complex, cytochrome *c*₁, which has a long presequence in most eukaryotic organisms (60–80 amino acids), has no presequence in *T. brucei* (Priest et al., 1993). Priest et al. used crude mitochondria isolated from *T. brucei* procyclic cells to further show that labeled cytochrome *c*₁ is imported in the absence of a membrane potential and without matrix ATP hydrolysis, similar to the non-conservative pathway described for the inner membrane carrier proteins of other organisms. The C-terminus region of this protein is predicted to form a transmembrane domain and an amphipathic α -helix, which might direct the insertion of cytochrome *c*₁ into the inner mitochondrial membrane. These observations indicate that the assembly of the reductase complex in trypanosomes is different than in other eukaryotes (Priest and Hajduk, 2003).

3. Protein targeting to hydrogenosomes

Hydrogenosomes are anaerobic, hydrogen-producing organelles that are evolutionarily related to mitochondria but are metabolically and structurally reduced in comparison. They have been identified in all parabasalids, certain chytrid fungi, certain ciliates, and the heterolobosean *Psaltteriomonas* (Embley et al., 2003; Boxma et al., 2005; Hackstein et al., 2006). Hydrogenosomes lack DNA, with the exception of the ciliate *Nyctotherus* (Akhmanova et al., 1998), so all proteins have to be imported into the double membrane-bounded organelle. The targeting system has attracted some attention due to the debate over the evolutionary relationship between parabasalid hydrogenosomes and canonical mitochondrion. It has been reasoned that demonstrating that their import machineries are homologous would be strong evidence in favour of the homology of the organelles, since the import machinery would not likely have been inherited directly from the endosymbiont and therefore is unlikely to have evolved twice in the same way independently. Several studies have confirmed import information used for targeting to the matrix of hydrogenosomes is similar to that used in canonical mitochondria (Bradley et al., 1997; Hausler et al., 1997; Van Der Giezen et al., 1998). In the human parasite, *Trichomonas vaginalis*, short, conserved N-terminal presequenc-

es have been identified in 12 major hydrogenosomal proteins and these were also found to be absent from the mature proteins (Johnson et al., 1990, 1993; Lahti et al., 1992; Lange et al., 1994; Hrdy and Muller, 1995a,b; Bui et al., 1996). Bradley and colleagues developed an in vitro import assay using radiolabeled precursor proteins and purified hydrogenosomes to confirm that targeting to the matrix depends on cleavable N-terminal presequences and is temperature- and ATP-dependent. As in mitochondrial protein import, a membrane potential is essential for import into hydrogenosomes (Bradley et al., 1997).

Targeting of a hydrogenosomal membrane protein was first studied with a member of the mitochondrial carrier family, called Hmp31 (Dyall et al., 2000). It was shown that even though the protein contains a cleavable N-terminal presequence, this sequence is not necessary for import, but instead internal sequences are used for correct targeting. This is similar to targeting of other mitochondrial carriers (Pfanner et al., 1987; Smagula and Douglas, 1988). Interestingly, these signals are recognised by the translocation machinery of yeast mitochondria in an in vitro assay using the same import components as its homologue, the mitochondrial ADP–ATP carrier (AAC). Targeting sequences of mitochondrial AAC were also compatible with import into hydrogenosomes. The conserved targeting pathways to both matrix and membrane of hydrogenosomes and mitochondria therefore support the notion that these two organelles share a common evolutionary origin (Dyall et al., 2000; Embley et al., 2003).

Protein import into fungal hydrogenosome was originally debated to be either mitochondrial-like (Van Der Giezen et al., 1998) or peroxisomal-like (Marvin-Sikkema et al., 1993). Subsequent studies have shown that all fungal hydrogenosomal matrix proteins analysed thus far have cleavable presequences similar to those found on canonical mitochondrial proteins (Van Der Giezen et al., 2005). The fungal *Neocallimastix frontalis* malic enzyme (ME), for example, is targeted to the hydrogenosome via an N-terminal targeting signal (Van Der Giezen et al., 1997) and is imported into the mitochondria of the heterologous yeast host *Hansenula polymorpha* (Van Der Giezen et al., 1998). Moreover, the size of the *N. frontalis* Cpn60 protein in cell extracts matched the expected size of a mature protein if the predicted presequence was processed (Van Der Giezen et al., 2003). Lastly, the *N. frontalis* AAC has been shown to complement a yeast mutant deficient in ATP import, showing that it is correctly imported into mitochondria and therefore suggesting that the pathway for targeting inner-membrane proteins is conserved between fungal hydrogenosomes and mitochondria (Van Der Giezen et al., 2002).

4. Protein targeting to mitosomes

At the furthest extreme on the spectrum of reduction are the mitosomes. Mitosomes are typically small double membrane-bound compartments with little or no additional

structure, no known role in energy generation and a severely reduced complement of proteins. They have so far been found in the archamoeba *E. histolytica* (Tovar et al., 1999), the diplomonad *Giardia intestinalis* (Mai et al., 1999; Tovar et al., 2003), the microsporidian *Trachipleistophora hominis* (Williams et al., 2002) and are also suspected to be in close relatives of these groups such as pelobionts or retortamonads, as well as other ‘amitochondriate’ lineages like oxymonads (Williams and Keeling, 2003).

The first gene sequences for mitochondrial proteins were found in the human intestinal parasite, *E. histolytica* (Clark and Roger, 1995). These and others have been analysed for targeting sequences, and N-terminal extensions with similarity to mitochondrial presequences were found in the enzyme pyridine nucleotide transhydrogenase (PNT), the chaperonin Cpn60 and heat-shock protein Hsp70, (Clark and Roger, 1995; Mai et al., 1999; Bakatselou et al., 2000). The 15 amino acid N-terminal extension of Cpn60 was directly shown to be important for mitochondrial targeting in vivo, and correct targeting after replacement with a trypanosome mitochondrial presequence demonstrated a functional conservation of mitochondrial protein import between trypanosome mitochondria and *E. histolytica* mitochondria (Tovar et al., 1999).

Another widespread human intestinal parasite is *G. intestinalis*, where gene sequences for *cpn60* identified the likely presence of the organelle (Roger et al., 1998), which was subsequently identified by localisation of specific antibodies raised against IscS and IscU, two proteins involved in iron–sulfur cluster biosynthesis (Tovar et al., 2003). While sequence analysis suggested a putative presequence in IscU (Tovar et al., 2003), the *G. intestinalis* IscS lacks any recognizable mitochondrial targeting sequences (Tachezy et al., 2001). The functionality of the putative presequences from IscU and ferredoxin was eventually confirmed by demonstrating that their import is dependent on cleavable N-terminal domains. In contrast, IscS, Cpn60 and Hsp70 do not appear to require cleavable presequences, but instead seem to harbour multiple internal targeting signals (Dolezal et al., 2005; Regoes et al., 2005).

Organellar delivery of IscU, ferredoxin and IscS was also found to be conserved between *G. intestinalis* mitochondria and *T. vaginalis* hydrogenosomes by overexpression of *G. intestinalis* IscU and ferredoxin in *T. vaginalis*, which showed that efficient targeting to the hydrogenosome is dependent on their presequences (Dolezal et al., 2005). A functional conservation of targeting presequences between *G. intestinalis* mitochondria and mammalian mitochondria could be demonstrated using a (1–18 amino acid) Ferredoxin-green fluorescent protein (GFP) fusion construct, suggesting a common import pathway in these organelles (Regoes et al., 2005).

Lastly, the processing of presequences in *G. intestinalis* has been inferred by the presence of bands corresponding in predicted molecular masses to precursor and mature forms of *G. intestinalis* IscU and ferredoxin in Western blots, and the presence of a β -MPP sequence in the *G. intes-*

tinalis genome (Dolezal et al., 2005; Regoes et al., 2005). The likelihood that this β -MPP is responsible for cleavage is further bolstered by the observation that cleavage of the IscU presequence is EDTA-sensitive with purified rat MPP and hydrogenosomal extracts, which are both consistent with a presequence recognised by β -MPP (Dolezal et al., 2005).

Targeting in microsporidian mitochondria represents another variation derived from mitochondrial targeting, but different from that of either hydrogenosomes or other mitochondria. The first protein shown to be targeted was Hsp70 from *T. hominis*, a gene without a recognizable presequence (Williams et al., 2002), and a number of putatively targeted proteins have been found in other species, in particular *Encephalitozoon cuniculi* and *Antonospora locustae* (Germot et al., 1997; Hirt et al., 1997; Peyretailade et al., 1998; Williams and Keeling, 2005; Burri et al., 2006). Sequence analysis of these genes predicted N-terminal presequences for some of the putative mitochondrial proteins, but in a number of cases presequences are either very reduced or absent altogether (Fast and Keeling, 2001; Katinka et al., 2001; Burri et al., 2006). Whether putative microsporidian targeting signals from two species of microsporidia are recognised by canonical mitochondria was examined by expressing 12 *A. locustae* and four *E. cuniculi* genes as GFP fusion proteins in yeast. Many were not properly targeted but others, including representatives from both species, did target to yeast mitochondria (Burri et al., 2006). Deletion mutants suggested that the N-terminus is often necessary for correct targeting, but in targeted proteins with short presequences such as *A. locustae* ferredoxin and *E. cuniculi* IscU, additional internal signals are required as well.

In contrast to *G. intestinalis* and *T. vaginalis*, where processing of presequences has now been demonstrated to occur in at least some proteins and MPP enzymes are known (Dolezal et al., 2005), no MPP could be identified in the fully sequenced genome of *E. cuniculi* (Katinka et al., 2001). It is possible there is no processing and that minimal targeting information might be tolerated and left attached to mature proteins without influencing its function. The only microsporidian presequence processing that could be found was with *A. locustae* mtG3PDH, which has the longest predicted presequence of around 40 amino acids. Western blots on purified *A. locustae* and *E. cuniculi* spores showed an unprocessed and mature form for *A. locustae* mtG3PDH, whereas *E. cuniculi* mtG3PDH only exists in the unprocessed form. This is an interesting exception because in yeast G3PDH (Gut2) is a substrate of an alternative processing pathway involving the Inner Membrane Peptidase (IMP) (Esser et al., 2004). *Antonospora locustae* has been shown to encode a gene for an IMP-2 enzyme (Williams and Keeling, 2005) and when expressed in yeast, the *A. locustae* mtG3PDH was further shown to be a substrate of the IMP complex (Burri et al., 2006), like its yeast homologue. This suggests that *A. locustae* retains an operating IMP enzyme responsible for processing of

mtG3PDH, while *E. cuniculi* has degenerated even more and can do without either MPP or IMP complex (Burri et al., 2006).

5. Discussion

In yeast, the mitochondrial protein import machinery consists of a complex network of many proteins including hetero-oligomeric membrane translocases, chaperones and processing peptidases (Wiedemann et al., 2004). There is no complete functional picture of the import system for any cryptic mitochondrion yet, however bioinformatic studies on the fully sequenced genomes of *G. intestinalis* and *E. cuniculi* suggest that the mitochondrial import machinery may be greatly reduced, such that only nine different components have been identified collectively in these two systems so far (Fig. 2). In the *E. cuniculi* genome, only two putative components of the TOM complex (Tom70 and Tom40) and one putative component of the TIM complex (Tim22) have been identified (Katinka et al., 2001; Burri et al., 2006), while in the *G. intestinalis* genome only one outer membrane component (Tom40) and only one inner membrane component (Tim14) have been found (Dolezal et al., 2005, 2006). The mitochondrial chaperone Hsp70 is found in *T. vaginalis*, *G. intestinalis*, *E. histolytica*, and several microsporidia, and Cpn60 in *T. vaginalis*, *G. intestinalis* and *E. histolytica*, but not *E. cuniculi*. Lastly, processing peptidases identified to date are the microsporidian IMP-2 (Williams and Keeling, 2005), and the *T. vaginalis* and *G. intestinalis* matrix-located MPP (Dolezal et al., 2005). There are certainly more components to these systems than have been described, even from

complete genomes, because many are small and probably not highly conserved, and distantly related genes can be difficult to annotate or identify, even using sensitive comparative tools. Direct methods of protein identification such as mass spectrometry will likely be needed to give a complete picture of the minimal set of proteins required for the mitochondrial import machinery.

With a conserved but reduced matrix-targeting pathway, the main difference between presequences in many parasites and those of yeast and mammalian cells is their length. The majority of yeast and mammalian presequences are between 20 and 80 amino acids (Hendrick et al., 1989), but can be as short as seven to 12 amino acids when used in artificial precursor proteins (Hurt et al., 1984; Verner and Lemire, 1989). Hydrogenosomal presequences are present in all genes identified so far. Fungal hydrogenosomal presequences range between 20 and 30 amino acids (Van Der Giezen et al., 1997, 1998), while *T. vaginalis* presequences are as short as 5–14 amino acids (Bradley et al., 1997). They are similar in length to the mitochondrial presequences of kinetoplastids, which are sometimes exceptionally short, only between eight or nine amino acids, but can be as long as 20 amino acids (Priest and Hajduk, 1992, 1995, 1996). Presequences responsible for targeting to mitosomes are more variable. Some proteins retain recognizable presequences (e.g., the 21 amino acid *E. histolytica* Hsp60 presequence or the ~40 amino acid presequence of the microsporidian mtG3PDH), but many other proteins seem to lack them (e.g., Hsp70 in *T. hominis* or Cpn60, IscS and Hsp70 in *G. intestinalis*), perhaps relying instead on internal signals for correct localisation.

A second emerging characteristic that is unusual is processing. In canonical mitochondria only a small number of mitochondrial matrix proteins are known to keep their targeting signal after import: rhodanese, 3-oxoacyl-CoA thiolase, the β -subunit of the human electron transfer flavoprotein, the mitochondrial ribosomal protein YmL8, and chaperonin 10 (Amaya et al., 1988; Miller et al., 1991; Finocchiaro et al., 1993; Matsushita and Isono, 1993; Rospert et al., 1993; Ryan et al., 1994). Only in the case of 3-oxoacyl-CoA thiolase has it been experimentally demonstrated that the targeting information lies within the first 14–16 amino acids (Arakawa et al., 1990). If it is true that processing-independent import is common in some mitosomes (e.g., those of microsporidia), it may relate to the same pressures that are reducing the complexity of the targeting signals in general, namely that reductive evolution is minimizing the system to only the information essential for targeting.

This raises the larger question, what are the criteria that dictate what information is essential for targeting? The minimal mitochondrial targeting information can be found randomly by chance in up to 5% of *Escherichia coli* proteins (Lucattini et al., 2004), suggesting the information is not particularly complex. However, this disregards the fact that *E. coli* does not have a mitochondrion itself, so its cytosolic proteins are not under selection to lack

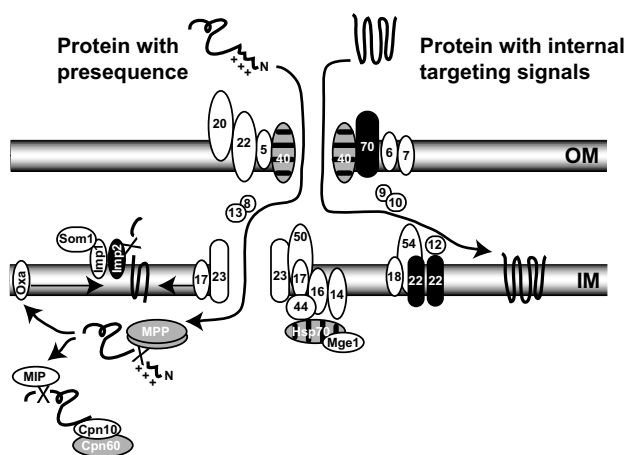


Fig. 2. Components of the mitochondrial targeting system known from mitosomes. Proteins in the yeast mitochondrial import system are shown and where homologues have been identified in *Giardia intestinalis* they are shown in grey, where they have been found in microsporidia they are shown in black, where they have been found in both *G. intestinalis* and microsporidian genome databases they are striped in black-grey, and where they have been found in neither they are in white. Proteins are depicted with their full or numerical description (40 indicates Tom40). Abbreviations: TOM, translocase of the outer membrane; TIM, translocase of the inner membrane; OM, outer membrane; IM, inner membrane.

mitochondrial targeting information. Indeed, it is possible that the stringency of mitochondrial targeting information may be relaxed in parasites like microsporidia simply due to the fact that their proteome has undergone an overall reduction in complexity. There are only about 2,000 proteins in *E. cuniculi*, so there are not only fewer proteins targeted to the mitochondrion, but there are also far fewer proteins that must be excluded from the mitochondrial targeting pathway. It stands to reason, therefore, that adequate targeting specificity could be achieved with less information. By this principle, a targeting system with fewer constraints may have evolved in some of these parasites both because the decreased biochemical and functional complexity of mitochondria-derived organelles themselves imposed fewer pressures on the system and also the decreased informational complexity of the proteins in the cells as a whole, affecting the targeting information and the number of import components needed for organellar protein translocation.

Acknowledgements

Research in the Keeling lab on microsporidian mitochondria was supported by a Grant (MOP-42517) from the Canadian Institutes for Health Research (CIHR) to P.J.K. P.J.K. is a Fellow of the Canadian Institute for Advanced Research and a senior investigator of Michael Smith Foundation for Health Research (MSFHR). L.B. is supported by fellowships from MSFHR and CIHR.

References

- Abe, Y., Shodai, T., Muto, T., Mihara, K., Torii, H., Nishikawa, S., Endo, T., Kohda, D., 2000. Structural basis of presequence recognition by the mitochondrial protein import receptor Tom20. *Cell* 100, 551–560.
- Abrahamsen, M.S., Templeton, T.J., Enomoto, S., Abrahante, J.E., Zhu, G., Lancto, C.A., Deng, M., Liu, C., Widmer, G., Tzipori, S., Buck, G.A., Xu, P., Bankier, A.T., Dear, P.H., Konfortov, B.A., Spriggs, H.F., Iyer, L., Anantharaman, V., Aravind, L., Kapur, V., 2004. Complete genome sequence of the apicomplexan, *Cryptosporidium parvum*. *Science* 304, 441–445.
- Akhmanova, A., Voncken, F., Van Alen, T., Van Hoek, A., Boxma, B., Vogels, G., Veenhuis, M., Hackstein, J.H., 1998. A hydrogenosome with a genome. *Nature* 396, 527–528.
- Ali, V., Shigeta, Y., Tokumoto, U., Takahashi, Y., Nozaki, T., 2004. An intestinal parasitic protist, *Entamoeba histolytica*, possesses a non-redundant nitrogen fixation-like system for iron–sulfur cluster assembly under anaerobic conditions. *J. Biol. Chem.* 279, 16863–16874.
- Amaya, Y., Arakawa, H., Takiguchi, M., Ebina, Y., Yokota, S., Mori, M., 1988. A noncleavable signal for mitochondrial import of 3-oxoacyl-CoA thiolase. *J. Biol. Chem.* 263, 14463–14470.
- Arakawa, H., Amaya, Y., Mori, M., 1990. The NH₂-terminal 14–16 amino acids of mitochondrial and bacterial thiolases can direct mature ornithine carbamoyltransferase into mitochondria. *J. Biochem. (Tokyo)* 107, 160–164.
- Arretz, M., Schneider, H., Guiard, B., Brunner, M., Neupert, W., 1994. Characterization of the mitochondrial processing peptidase of *Neurospora crassa*. *J. Biol. Chem.* 269, 4959–4967.
- Bakatselou, C., Kidgell, C., Graham Clark, C., 2000. A mitochondrial-type hsp70 gene of *Entamoeba histolytica*. *Mol. Biochem. Parasitol.* 110, 177–182.
- Borst, P., 1991. Why kinetoplast DNA networks? *Trends Genet.* 7, 139–141.
- Boxma, B., De Graaf, R.M., Van Der Staay, G.W., Van Alen, T.A., Ricard, G., Gabaldon, T., Van Hoek, A.H., Moon-Van Der Staay, S.Y., Koopman, W.J., Van Hellemond, J.J., Tielens, A.G., Friedrich, T., Veenhuis, M., Huynen, M.A., Hackstein, J.H., 2005. An anaerobic mitochondrion that produces hydrogen. *Nature* 434, 74–79.
- Bradley, P.J., Lahti, C.J., Plumper, E., Johnson, P.J., 1997. Targeting and translocation of proteins into the hydrogenosome of the protist *Trichomonas*: similarities with mitochondrial protein import. *EMBO J.* 16, 3484–3493.
- Brix, J., Rudiger, S., Bukau, B., Schneider-Mergener, J., Pfanner, N., 1999. Distribution of binding sequences for the mitochondrial import receptors Tom20, Tom22, and Tom70 in a presequence-carrying preprotein and a non-cleavable preprotein. *J. Biol. Chem.* 274, 16522–16530.
- Bui, E.T., Bradley, P.J., Johnson, P.J., 1996. A common evolutionary origin for mitochondria and hydrogenosomes. *Proc. Natl. Acad. Sci. USA* 93, 9651–9656.
- Burri, L., Williams, B.A.P., Bursac, D., Lithgow, T., Keeling, P.J., 2006. Microsporidian mitochondria retain elements of the general mitochondrial targeting system. *Proc. Natl. Acad. Sci. USA*, 103, 15916–15920.
- Cavalier-Smith, T., 1987. The simultaneous symbiotic origin of mitochondria, chloroplasts, and microbodies. *Ann. N. Y. Acad. Sci.* 503, 55–71.
- Clark, C.G., Roger, A.J., 1995. Direct evidence for secondary loss of mitochondria in *Entamoeba histolytica*. *Proc. Natl. Acad. Sci. USA* 92, 6518–6521.
- Clayton, C., Hausler, T., Blattner, J., 1995. Protein trafficking in kinetoplastid protozoa. *Microbiol. Rev.* 59, 325–344.
- Dolezal, P., Smid, O., Rada, P., Zubacova, Z., Bursac, D., Sutak, R., Nebesarova, J., Lithgow, T., Tachezy, J., 2005. Giardia mitochondria and trichomonad hydrogenosomes share a common mode of protein targeting. *Proc. Natl. Acad. Sci. USA* 102, 10924–10929.
- Dolezal, P., Likic, V., Tachezy, J., Lithgow, T., 2006. Evolution of the molecular machines for protein import into mitochondria. *Science* 313, 314–318.
- Dyall, S.D., Koehler, C.M., Delgado-Correa, M.G., Bradley, P.J., Plumper, E., Leuenberger, D., Turck, C.W., Johnson, P.J., 2000. Presence of a member of the mitochondrial carrier family in hydrogenosomes: conservation of membrane-targeting pathways between hydrogenosomes and mitochondria. *Mol. Cell. Biol.* 20, 2488–2497.
- Embley, T.M., Van Der Giezen, M., Horner, D.S., Dyal, P.L., Bell, S., Foster, P.G., 2003. Hydrogenosomes, mitochondria and early eukaryotic evolution. *IUBMB Life* 55, 387–395.
- Esser, K., Jan, P.S., Pratje, E., Michaelis, G., 2004. The mitochondrial IMP peptidase of yeast: functional analysis of domains and identification of Gut2 as a new natural substrate. *Mol. Genet. Genomics* 271, 616–626.
- Fast, N.M., Keeling, P.J., 2001. Alpha and beta subunits of pyruvate dehydrogenase E1 from the microsporidian *Nosema locustae*: mitochondrion-derived carbon metabolism in microsporidia. *Mol. Biochem. Parasitol.* 117, 201–209.
- Finocchiaro, G., Colombo, I., Garavaglia, B., Gellera, C., Valdameri, G., Garbuglio, N., Didonato, S., 1993. cDNA cloning and mitochondrial import of the beta-subunit of the human electron-transfer flavoprotein. *Eur. J. Biochem.* 213, 1003–1008.
- Germot, A., Philippe, H., Le Guyader, H., 1997. Evidence for loss of mitochondria in Microsporidia from a mitochondrial-type HSP70 in *Nosema locustae*. *Mol. Biochem. Parasitol.* 87, 159–168.
- Gray, M.W., Burger, G., Lang, B.F., 1999. Mitochondrial evolution. *Science* 283, 1476–1481.
- Hackstein, J.H., Tjaden, J., Huynen, M., 2006. Mitochondria, hydrogenosomes and mitosomes: products of evolutionary tinkering! *Curr. Genet.* 50, 225–245.
- Hancock, K., Hajduk, S.L., 1990. The mitochondrial tRNAs of *Trypanosoma brucei* are nuclear encoded. *J. Biol. Chem.* 265, 19208–19215.
- Hauser, R., Pypaert, M., Hausler, T., Horn, E.K., Schneider, A., 1996. In vitro import of proteins into mitochondria of *Trypanosoma brucei* and *Leishmania tarentolae*. *J. Cell Sci.* 109 (Pt 2), 517–523.

- Hausler, T., Stierhof, Y.D., Blattner, J., Clayton, C., 1997. Conservation of mitochondrial targeting sequence function in mitochondrial and hydrogenosomal proteins from the early-branching eukaryotes *Crithidia*, *Trypanosoma* and *Trichomonas*. *Eur. J. Cell Biol.* 73, 240–251.
- Hawlichek, G., Schneider, H., Schmidt, B., Tropschug, M., Hartl, F.U., Neupert, W., 1988. Mitochondrial protein import: identification of processing peptidase and of PEP, a processing enhancing protein. *Cell* 53, 795–806.
- Hendrick, J.P., Hodges, P.E., Rosenberg, L.E., 1989. Survey of amino-terminal proteolytic cleavage sites in mitochondrial precursor proteins: leader peptides cleaved by two matrix proteases share a three-amino acid motif. *Proc. Natl. Acad. Sci USA* 86, 4056–4060.
- Hirt, R.P., Healy, B., Vossbrink, C.R., Canning, E.U., Embley, T.M., 1997. A mitochondrial Hsp70 orthologue in *Vairimorpha necatrix*: molecular evidence that microsporidia once contained mitochondria. *Curr. Biol.* 7, 995–998.
- Hrdy, I., Muller, M., 1995a. Primary structure and eubacterial relationships of the pyruvate:ferredoxin oxidoreductase of the amitochondriate eukaryote *Trichomonas vaginalis*. *J. Mol. Evol.* 41, 388–396.
- Hrdy, I., Muller, M., 1995b. Primary structure of the hydrogenosomal malic enzyme of *Trichomonas vaginalis* and its relationship to homologous enzymes. *J. Eukaryot. Microbiol.* 42, 593–603.
- Hurt, E.C., Pesold-Hurt, B., Schatz, G., 1984. The amino-terminal region of an imported mitochondrial precursor polypeptide can direct cytoplasmic dihydrofolate reductase into the mitochondrial matrix. *EMBO J.* 3, 3149–3156.
- Johnson, P.J., D'oliveira, C.E., Gorrell, T.E., Muller, M., 1990. Molecular analysis of the hydrogenosomal ferredoxin of the anaerobic protist *Trichomonas vaginalis*. *Proc. Natl. Acad. Sci USA* 87, 6097–6101.
- Johnson, P.J., Lahti, C.J., Bradley, P.J., 1993. Biogenesis of the hydrogenosome in the anaerobic protist *Trichomonas vaginalis*. *J. Parasitol.* 79, 664–670.
- Katinka, M.D., Duprat, S., Cornillot, E., Metenier, G., Thomarat, F., Prensier, G., Barbe, V., Peyretailade, E., Brottier, P., Wincker, P., Delbac, F., El Alaoui, H., Peyret, P., Saurin, W., Gouy, M., Weissenbach, J., Vivares, C.P., 2001. Genome sequence and gene compaction of the eukaryote parasite *Encephalitozoon cuniculi*. *Nature* 414, 450–453.
- Lahti, C.J., D'oliveira, C.E., Johnson, P.J., 1992. Beta-succinyl-coenzyme A synthetase from *Trichomonas vaginalis* is a soluble hydrogenosomal protein with an amino-terminal sequence that resembles mitochondrial presequences. *J. Bacteriol.* 174, 6822–6830.
- Lange, S., Rozario, C., Muller, M., 1994. Primary structure of the hydrogenosomal adenylate kinase of *Trichomonas vaginalis* and its phylogenetic relationships. *Mol. Biochem. Parasitol.* 66, 297–308.
- Lill, R., Kispal, G., 2000. Maturation of cellular Fe-S proteins: an essential function of mitochondria. *Trends Biochem. Sci.* 25, 352–356.
- Lucattini, R., Likic, V.A., Lithgow, T., 2004. Bacterial proteins predisposed for targeting to mitochondria. *Mol. Biol. Evol.* 21, 652–658.
- Mai, Z., Ghosh, S., Frisardi, M., Rosenthal, B., Rogers, R., Samuelson, J., 1999. Hsp60 is targeted to a cryptic mitochondrion-derived organelle (crypton) in the microaerophilic protozoan parasite *Entamoeba histolytica*. *Mol. Cell Biol.* 19, 2198–2205.
- Marvin-Sikkema, F.D., Kraak, M.N., Veenhuis, M., Gottschal, J.C., Prins, R.A., 1993. The hydrogenosomal enzyme hydrogenase from the anaerobic fungus *Neocallimastix* sp. L2 is recognized by antibodies, directed against the C-terminal microbody protein targeting signal SKL. *Eur. J. Cell Biol.* 61, 86–91.
- Matsushita, Y., Isono, K., 1993. Mitochondrial transport of mitoribosomal proteins, YmL8 and YmL20, in *Saccharomyces cerevisiae*. *Eur. J. Biochem.* 214, 577–585.
- Miller, D.M., Delgado, R., Chirgwin, J.M., Hardies, S.C., Horowitz, P.M., 1991. Expression of cloned bovine adrenal rhodanese. *J. Biol. Chem.* 266, 4686–4691.
- Mottram, J.C., Bell, S.D., Nelson, R.G., Barry, J.D., 1991. tRNAs of *Trypanosoma brucei*. Unusual gene organization and mitochondrial importation. *J. Biol. Chem.* 266, 18313–18317.
- Müller, M., 1993. The hydrogenosome. *J. Gen. Microbiol.* 139, 2879–2889.
- Neupert, W., 1997. Protein import into mitochondria. *Annu. Rev. Biochem.* 66, 863–917.
- Peyretailade, E., Broussolle, V., Peyret, P., Méténier, G., Gouy, M., Vivares, C.P., 1998. Microsporidia, amitochondrial protists, possess a 70-kDa heat shock protein gene of mitochondrial evolutionary origin. *Mol. Biol. Evol.* 15, 683–689.
- Pfanner, N., Geissler, A., 2001. Versatility of the mitochondrial protein import machinery. *Nat. Rev. Mol. Cell Biol.* 2, 339–349.
- Pfanner, N., Hoeben, P., Tropschug, M., Neupert, W., 1987. The carboxyl-terminal two-thirds of the ADP/ATP carrier polypeptide contains sufficient information to direct translocation into mitochondria. *J. Biol. Chem.* 262, 14851–14854.
- Priest, J.W., Hajduk, S.L., 1992. Cytochrome c reductase purified from *Crithidia fasciculata* contains an atypical cytochrome c1. *J. Biol. Chem.* 267, 20188–20195.
- Priest, J.W., Hajduk, S.L., 1995. The trypanosomatid Rieske iron-sulfur proteins have a cleaved presequence that may direct mitochondrial import. *Biochim. Biophys. Acta* 1269, 201–204.
- Priest, J.W., Hajduk, S.L., 1996. In vitro import of the Rieske iron-sulfur protein by trypanosome mitochondria. *J. Biol. Chem.* 271, 20060–20069.
- Priest, J.W., Hajduk, S.L., 2003. *Trypanosoma brucei* cytochrome c1 is imported into mitochondria along an unusual pathway. *J. Biol. Chem.* 278, 15084–15094.
- Priest, J.W., Wood, Z.A., Hajduk, S.L., 1993. Cytochromes c1 of kinetoplastid protozoa lack mitochondrial targeting presequences. *Biochim. Biophys. Acta* 1144, 229–231.
- Rapaport, D., 2003. Finding the right organelle. Targeting signals in mitochondrial outer-membrane proteins. *EMBO Rep.* 4, 948–952.
- Regoes, A., Zourmanou, D., Leon-Avila, G., Van Der Giezen, M., Tovar, J., Hehl, A.B., 2005. Protein import, replication, and inheritance of a vestigial mitochondrion. *J. Biol. Chem.* 280, 30557–30563.
- Reichert, A.S., Neupert, W., 2004. Mitochondriomics or what makes us breathe. *Trends Genet.* 20, 555–562.
- Roger, A.J., 1999. Reconstructing early events in eukaryotic evolution. *Am. Nat.* 154, S146–S163.
- Roger, A.J., Svard, S.G., Tovar, J., Clark, C.G., Smith, M.W., Gillin, F.D., Sogin, M.L., 1998. A mitochondrial-like chaperonin 60 gene in *Giardia lamblia*: evidence that diplomonads once harbored an endosymbiont related to the progenitor of mitochondria. *Proc. Natl. Acad. Sci USA* 95, 229–234.
- Rospert, S., Junne, T., Glick, B.S., Schatz, G., 1993. Cloning and disruption of the gene encoding yeast mitochondrial chaperonin 10, the homolog of *E. coli* groES. *FEBS Lett.* 335, 358–360.
- Ryan, M.T., Hoogenraad, N.J., Hoj, P.B., 1994. Isolation of a cDNA clone specifying rat chaperonin 10, a stress-inducible mitochondrial matrix protein synthesised without a cleavable presequence. *FEBS Lett.* 337, 152–156.
- Schatz, G., Dobberstein, B., 1996. Common principles of protein translocation across membranes. *Science* 271, 1519–1526.
- Simpson, A.M., Suyama, Y., Dewes, H., Campbell, D.A., Simpson, L., 1989. Kinetoplastid mitochondria contain functional tRNAs which are encoded in nuclear DNA and also contain small minicircle and maxicircle transcripts of unknown function. *Nucleic Acids Res.* 17, 5427–5445.
- Simpson, L., 1990. RNA editing – a novel genetic phenomenon? *Science* 250, 512–513.
- Smagula, C.S., Douglas, M.G., 1988. ADP-ATP carrier of *Saccharomyces cerevisiae* contains a mitochondrial import signal between amino acids 72 and 111. *J. Cell. Biochem.* 36, 323–327.
- Tachezy, J., Sanchez, L.B., Muller, M., 2001. Mitochondrial type iron-sulfur cluster assembly in the amitochondriate eukaryotes *Trichomonas vaginalis* and *Giardia intestinalis*, as indicated by the phylogeny of IscS. *Mol. Biol. Evol.* 18, 1919–1928.

- Tovar, J., Fischer, A., Clark, C.G., 1999. The mitosome, a novel organelle related to mitochondria in the amitochondrial parasite *Entamoeba histolytica*. *Mol. Microbiol.* 32, 1013–1021.
- Tovar, J., Leon-Avila, G., Sanchez, L.B., Sutak, R., Tachezy, J., Van Der Giezen, M., Hernandez, M., Muller, M., Lucocq, J.M., 2003. Mitochondrial remnant organelles of *Giardia* function in iron–sulphur protein maturation. *Nature* 426, 172–176.
- Van Der Giezen, M., Birdsey, G.M., Horner, D.S., Lucocq, J., Dyal, P.L., Benchimol, M., Danpure, C.J., Embley, T.M., 2003. Fungal hydrogenosomes contain mitochondrial heat-shock proteins. *Mol. Biol. Evol.* 20, 1051–1061.
- Van Der Giezen, M., Cox, S., Tovar, J., 2004. The iron–sulfur cluster assembly genes *iscS* and *iscU* of *Entamoeba histolytica* were acquired by horizontal gene transfer. *BMC Evol. Biol.* 4, 7.
- Van Der Giezen, M., Kiel, J.A., Sjollem, K.A., Prins, R.A., 1998. The hydrogenosomal malic enzyme from the anaerobic fungus *Neocallimastix frontalis* is targeted to mitochondria of the methylotrophic yeast *Hansenula polymorpha*. *Curr. Genet.* 33, 131–135.
- Van Der Giezen, M., Rechinger, K.B., Svendsen, I., Durand, R., Hirt, R.P., Fevre, M., Embley, T.M., Prins, R.A., 1997. A mitochondrial-like targeting signal on the hydrogenosomal malic enzyme from the anaerobic fungus *Neocallimastix frontalis*: support for the hypothesis that hydrogenosomes are modified mitochondria. *Mol. Microbiol.* 23, 11–21.
- Van Der Giezen, M., Slotboom, D.J., Horner, D.S., Dyal, P.L., Harding, M., Xue, G.P., Embley, T.M., Kunji, E.R., 2002. Conserved properties of hydrogenosomal and mitochondrial ADP/ATP carriers: a common origin for both organelles. *EMBO J.* 21, 572–579.
- Van Der Giezen, M., Tovar, J., 2005. Degenerate mitochondria. *EMBO Rep.* 6, 525–530.
- Van Der Giezen, M., Tovar, J., Clark, C.G., 2005. Mitochondrion-derived organelles in protists and fungi. *Int. Rev. Cytol.* 244, 175–225.
- Verner, K., Lemire, B.D., 1989. Tight folding of a passenger protein can interfere with the targeting function of a mitochondrial presequence. *EMBO J.* 8, 1491–1495.
- Von Heijne, G., Steppuhn, J., Herrmann, R.G., 1989. Domain structure of mitochondrial and chloroplast targeting peptides. *Eur. J. Biochem.* 180, 535–545.
- Wattenberg, B., Lithgow, T., 2001. Targeting of C-terminal (tail)-anchored proteins: understanding how cytoplasmic activities are anchored to intracellular membranes. *Traffic* 2, 66–71.
- Wiedemann, N., Frazier, A.E., Pfanner, N., 2004. The protein import machinery of mitochondria. *J. Biol. Chem.* 279, 14473–14476.
- Wiedemann, N., Pfanner, N., Ryan, M.T., 2001. The three modules of ADP/ATP carrier cooperate in receptor recruitment and translocation into mitochondria. *EMBO J.* 20, 951–960.
- Williams, B.A., Hirt, R.P., Lucocq, J.M., Embley, T.M., 2002. A mitochondrial remnant in the microsporidian *Trachipleistophora hominis*. *Nature* 418, 865–869.
- Williams, B.A., Keeling, P.J., 2005. Microsporidian mitochondrial proteins: expression in *Antonospora locustae* spores and identification of genes coding for two further proteins. *J. Eukaryot. Microbiol.* 52, 271–276.
- Williams, B.A.P., Keeling, P.J., 2003. Cryptic organelles in parasitic protists and fungi. *Adv. Parasitol.* 54, 9–67.
- Xu, P., Widmer, G., Wang, Y., Ozaki, L.S., Alves, J.M., Serrano, M.G., Puiu, D., Manque, P., Akiyoshi, D., Mackey, A.J., Pearson, W.R., Dear, P.H., Bankier, A.T., Peterson, D.L., Abrahamsen, M.S., Kapur, V., Tzipori, S., Buck, G.A., 2004. The genome of *Cryptosporidium hominis*. *Nature* 431, 1107–1112.