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Short communication

Trypanosoma brucei has a canonical mitochondrial processing peptidase

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ABSTRACT

Most mitochondrial matrix and inner membrane proteins have N-terminal presequences which serve as import signals. After import these presequences are cleaved by the heterodimeric mitochondrial processing peptidase. In the parasitic protozoa *Trypanosoma brucei* mitochondrial protein import relies on presequences that are much shorter than in other eukaryotes. How they are processed is unknown. The trypansomal genome encodes four open reading frames that are annotated as mitochondrial processing peptidase. Here we show that RNAi-mediated ablation of two of these proteins leads to a growth arrest and a concomitant accumulation of mitochondrial precursor proteins inside mitochondria. Import experiments using isolated mitochondria from RNAi cell lines reveals that both proteins are required for efficient import and processing of the tested precursor protein. Reciprocal immunoprecipitation demonstrates that the proteins interact with each other. In summary these results show that we have identified the two subunits of the trypanosomal mitochondrial processing peptidase.

Most mitochondrial proteins are encoded in the nucleus, synthesized in the cytosol and finally imported into mitochondria. Mitochondrial protein import of matrix and of many inner membrane proteins is mediated by short N-terminal presequences that show little sequence homology but are enriched for positive amino acids and have the capability to form amphiphilic helices. Upon import into the organelle these presequences get removed by several presequence peptidases present in the matrix, the inner membrane or the intermembrane space of mitochondria [1]. The most important of these proteases that processes hundreds of mitochondrial proteins is the mitochondrial processing peptidase (MPP). MPP is essential for eukaryotic life under all conditions. It is active as a heterodimer consisting of the zinc-dependent catalytic β-MPP subunit and a regulatory α-MPP subunit that is involved in substrate recognition [2]. The two subunits are paralogs and conserved in most eukaryotes. In yeast and mammals the two MPP subunits are soluble matrix proteins whereas in plants and some fungi, MPP is integrated into the cytochrome bc_1 complex and functions as both a core protein of the complex and as a presequence protease [1].

The parasitic protozoa *Trypansoma brucei* and its relatives are considered to represent one of the most early diverged branches in the eukaryotic evolutionary tree [3]. In line with this its mitochondrial protein import machinery shows some unique features [4]. The normally conserved mitochondrial outer membrane protein translocase Tom40 could initially not be identified in *T. brucei* [5]. Instead, protein import across the outer membrane is mediated by ATOM, a protein that shows affinities to both the bacterial Omp85-like protein family, involved in bacterial protein export, as well as to the conventional outer membrane protein import channel Tom40 [6,7]. Moreover, only a single member of the Tim17/22/23 protein family occurs in *T. brucei* suggesting that unlike other eukaryotes *T. brucei* may have a single inner membrane protein translocase [8,9]. Finally, trypanosomal proteins that are imported into the mitochondrion, similar to proteins imported into the mitochondria-derived hydrogenosomes or mitosomes, have unusual short presequences [10]. How the trypanosomal presequences are processed is unknown.

MPP belongs to the pitrilysin family of metalloproteases. To identify the trypanosomal orthologs we searched the TriTryp database (version 4.1) for the InterPro entry IPR011249 "Metalloenzyme, LuxS/M16 peptidase-like, metal-binding" and retrieved 7 entries. One of them is predicted to be a pseudogene. Four of the remaining 6 entries are annotated as MPPs. Phylogenetic analysis and the sequence alignments in Fig. 1A show that Tb927.2.4110 groups with α-MPPs of other species and consistent with this exhibits the typical glycine-rich motif. Tb09.160.3110 on the other hand groups with β-MPPs and contains the characteristic inverted $HxxEHx_{76}E$ zinc-binding motif. Tb927.5.1060 and Tb11.02.1480 are components of the recently characterized *T. brucei* respiratome [11]. They encode subunits of the cytochrome bc_1 complex as they have been shown to interact with cytochrome c_1 (Tb927.8.1890) and the Rieske iron-sulfur protein ISP (Tb09.211.4700) but not with Tb927.2.4110 or Tb09.160.3110 [11]. This suggests that in trypanosomes, unlike in plants and some fungi [1], no subunits are shared between the cytochrome bc_1 complex and MPP.

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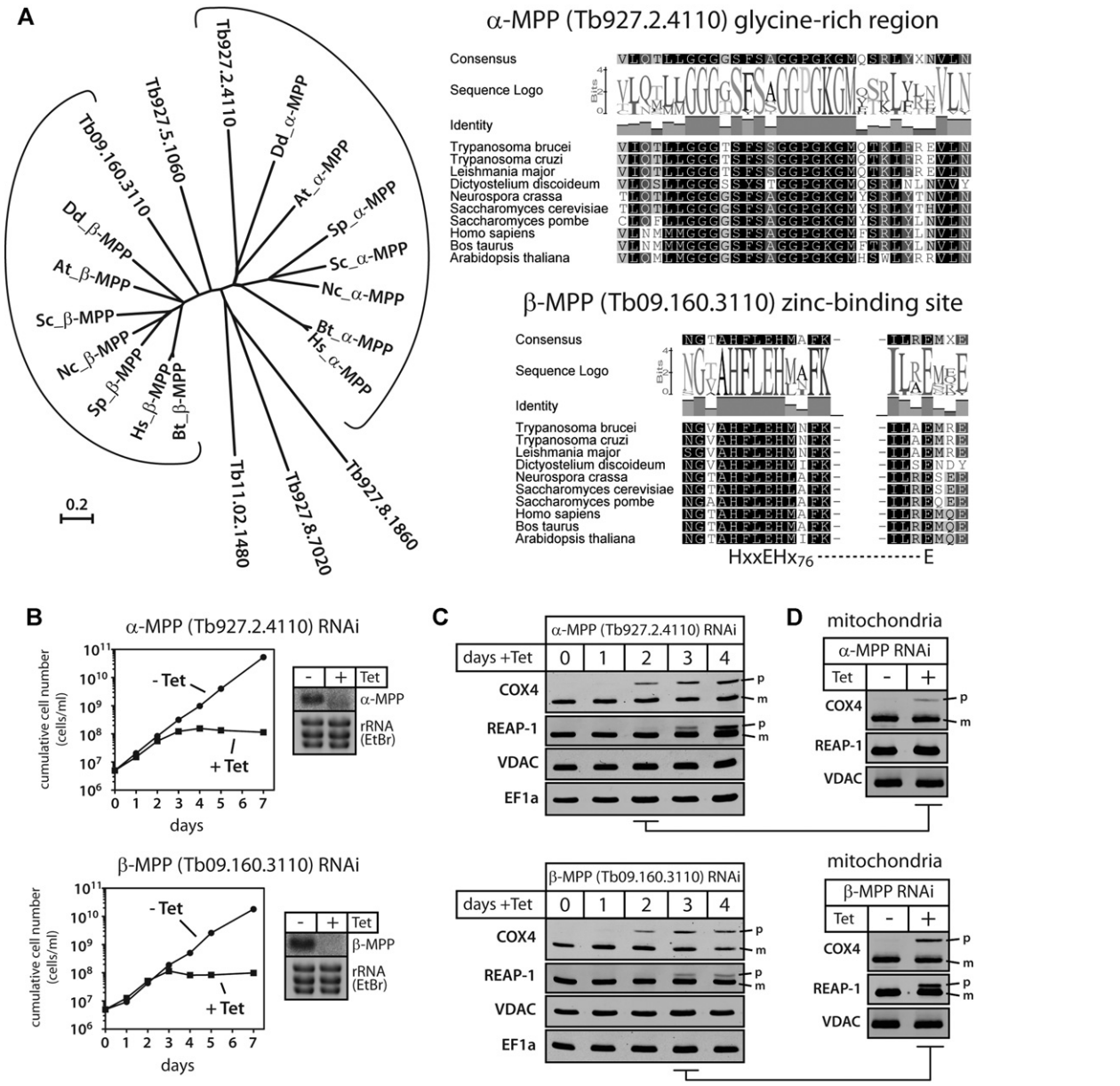

Fig. 1. Identification and in vivo analysis of the putative trypanosomal MPP. (A) Left panel: phylogenetic tree of α- and β-MPPs of various eukaryotes and the six trypanosomal open reading frames annotated as "metallo-peptidase, Clan ME, Family M16". Sequences were aligned using MUSCLE and the tree was built using the BioNJ algorithm (distance: poisson; all gap sites ignored; 100 bootstrap replicates) in SeaView (version 4). Right panel: multiple sequence alignments of the α-MPP glycine-rich regions and the β-MPP zinc-binding sites, the signature sequences of the MPP subunits. Sequences are of the same species as shown on the left including the putative α- and β-MPPs of trypanosomatids. The highly conserved consensus sequences are indicated at the top and the β-MPP zinc-binding motif is highlighted at the bottom. (B) Growth curves of uninduced (−Tet) and induced (+Tet) RNAi cell lines directed against the putative α- and β-MPP. Northern blots confirming the ablation of the corresponding α- and β-MPP mRNAs are shown (−Tet, 48 h +Tet). RNAi cell lines were produced using pLew100-based stem loop constructs [18,19] targeting nucleotides 729–1263 of Tb927.2.4110 for α-MPP and nucleotides 221–715 of Tb09.160.3110 for the β-MPP and procyclic *T. brucei* 29-13 as a host strain. (C) Total cellular extracts of the two RNAi cell lines were prepared at the indicated time points after RNAi induction and analyzed by immunoblots (1.5×10^6 cells per lane) using the indicated antisera. The positions of the mature forms (m) of COX4 and REAP-1 as well as their putative precursor forms (p) are indicated. (D) Isotonically isolated mitochondria (5 μg) [20] from uninduced RNAi cell lines (−Tet) and from cells that had been induced (+Tet) for two days (α-MPP) and three days (β-MPP) were analyzed on immunoblots using the indicated antisera. Positions of precursor and mature forms are indicated.

To analyze the function of the putative trypanosomal MPP we produced RNAi cell lines of procyclic *T. brucei* that allow tetracycline inducible ablation of either of the two proteins. Fig. 1B shows that ablation of the putative α- and β-subunits results in a growth arrest 2 and 3 days after induction of RNAi. This indicates that both proteins are essential for normal growth, as expected should they be subunits of the trypanosomal MPP. The inner membrane protein cytochrome oxidase subunit 4 (COX4) and the matrix-localized kinetoplastid RNA-editing-associated protein 1 (REAP-1) [12] are nuclear encoded, synthesized in the cytosol and imported into mitochondria. Immunoblot analysis of total cellular extracts from uninduced and induced RNAi cell lines revealed a time-dependent accumulation of slower migrating bands, which likely correspond to the uncleaved precursor forms of the proteins (Fig. 1C). Interestingly, the accumulation of the precursor form of both proteins is more pronounced in the putative α-MPP RNAi cell line. The mitochondrial outer membrane protein voltage-dependent anion channel (VDAC) [5] which lacks a presequence is not affected by the knockdown. The cytosolic protein translation elongation factor 1a (EF1a) serves as a loading control. Analysis of gradient-purified

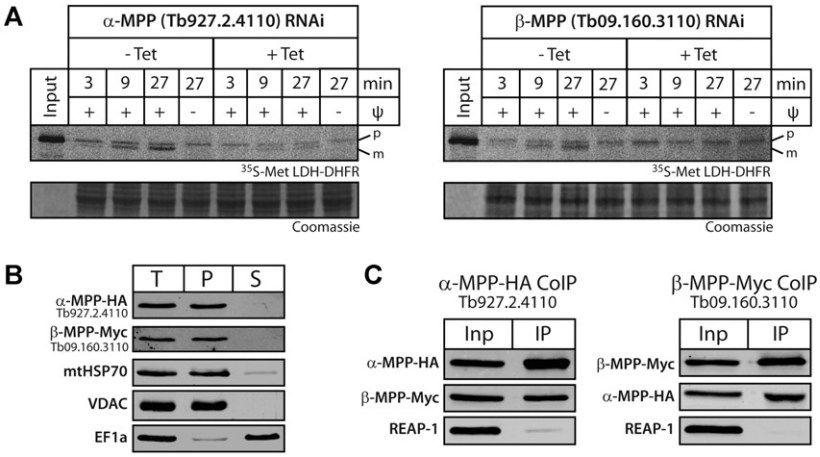

Fig. 2. In organello and biochemical analysis of the putative trypanosomal MPP. (A) Isotonically isolated mitochondria [20] from uninduced RNAi cell lines (−Tet) and from cells that had been induced (+Tet) for two days (α-MPP) and three days (β-MPP) were tested for in vitro import of ^{35}S-Met-labeled in vitro translated LDH-DHFR and analyzed by SDS-PAGE. Positions of precursor (p) and mature forms (m) of LDH-DHFR are indicated. The coomassie stained gel is shown as a loading control. All import reactions were proteinase K treated. Input: 10% of added substrate. ψ, membrane potential. In vitro translation of LDH-DHFR and in vitro import assays were done as described [6,13]. (B) C-terminally HA- and Myc-tagged versions of α- and β-MPP were expressed in the same *T. brucei* 29-13 cell line. α-MPP was tagged in situ with HA as described [21]. Myc-tagging of β-MPP was done using a pLew100-derived vector [18]. The resulting cells (T) were fractionated after 48 h of Tet-induction by digitonin yielding a supernatant (S) and a crude mitochondrial fraction (P) [15] which were analyzed on immunoblots using the indicated antisera. (C) A digitonin lysate of the α-MPP-HA and β-MPP-Myc expressing cells (48 h induced) was immunoprecipitated using either anti-HA antibodies (left panel) or anti-Myc antibodies (right panel), respectively. Five percent of the total extract (Inp) and 100% of the bound fraction (IP) were analyzed by immunoblots using anti-HA, anti-Myc and anti-REAP-1 antisera.

mitochondria from uninduced RNAi cell lines and from RNAi cell lines that had been induced for 2 days in the case of the putative α-MPP and for 3 days in the case of the putative β-MPP showed that precursor proteins detectable at these times points likely accumulate inside mitochondria (Fig. 1D). This is expected if the two proteins are required for presequence processing.

Isotonically isolated mitochondria of *T. brucei* retain the capability to import proteins. In vitro import of proteins is monitored by the time- and membrane potential-dependent appearance of the protease-protected mature form of the added import substrate [13]. Fig. 2A shows that an in vitro translated fusion protein consisting of the N-terminal 150 amino acids of mitochondrial dihydrolipoamide dehydrogenase of *T. brucei* and mouse dihydrofolate reductase (LDH-DHFR) [6] is efficiently imported into mitochondria isolated from uninduced RNAi cell lines. In mitochondria from induced α-MPP RNAi cells, however, we observe a great reduction and in the induced β-MPP RNAi cell line even a disappearence of the mature form of LDH-DHFR. This suggests that ablation of α- and β-MPP not only affects processing of mitochondrial matrix proteins as shown above but also inhibits the import process itself. Similar observations have been made previously for the yeast MPP [14].

The α- and the β-subunit of the MPP form a heterodimer. We investigated whether the two subunits of the putative trypanosomal MPP interact with each other in vivo. To that end we expressed a C-terminally hemagglutinin (HA)-tagged version of α-MPP and a C-terminally Myc-tagged version of β-MPP within the same cell line. Separation of cytosolic and mitochondrial compartments by digitonin extraction [15] shows that both tagged MPP subunits are expressed and that they exclusively co-fractionate with mitochondrial marker proteins. Reciprocal immunoprecipitations using anti-HA and anti-Myc antibodies, respectively, demonstrate that the proteins interact with each other (Fig. 2C).

In summary our results show that the two proteins encoded by the open reading frames Tb927.2.4110 and Tb09.160.3110, (i) have sequence similarity to the α- and β-subunits of MPP from other species, (ii) exclusively localize to mitochondria, (iii) interact with each other and (iv) are required for normal growth as well as (v) for processing of imported mitochondrial proteins in vivo and in organello. Based on these results we conclude that we have identified the bona fide trypanosomal orthologs of the MPP.

MPPs have recently been characterized in the mitosome of *Giardia* and in the hydrogenosomes of *Trichomonas* [16]. *T. brucei*, *Giardia* and *Trichomonas* all belong to the eukaryotic supergroup of the *Excavata* [3] and their organellar proteins share the propensity for very short presequences [17]. Interestingly, it was shown that the *Giardia* mitosomes, which have a very small proteome, have an MPP that is active as a single β-subunit, whereas in the more protein-rich hydrogenosomes of *Trichomonas* the MPP consists of two subunits, as in other eukaryotes [16]. The trypanosomal MPP in this respect resembles the *Trichomonas* enzyme suggesting that cleavage of a larger number of substrates proteins requires a heterodimeric MPP. Thus, despite the many unique features of mitochondrial protein import in *T. brucei*, when it comes to presequence processing trypanosomes rely on a canonical mitochondrial MPP.

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References

[1] Mossmann D, Meisinger C, Vögtle FN. Processing of mitochondrial presequences. Biochimica et Biophysica Acta (BBA)–Gene Regulatory Mechanisms 2012;1819:1098–106.
[2] Hawlitschek G, Schneider H, Schmidt B, Tropschug M, Hartl F-U, Neupert W. Mitochondrial protein import: identification of processing peptidase and of PEP, a processing enhancing protein. Cell 1988;53:795–806.
[3] Cavalier-Smith T. Kingdoms Protozoa and Chromista and the eozoan root of the eukaryotic tree. Biology Letters 2010;6:342–5.
[4] Schneider A, Bursać D, Lithgow T. The direct route: a simplified pathway for protein import into the mitochondrion of trypanosomes. Trends in Cell Biology 2008;18:12–8.
[5] Pusnik M, Charrière F, Mäser P, Waller RF, Dagley MJ, Lithgow T, et al. The single mitochondrial porin of *Trypanosoma brucei* is the main metabolite transporter in the outer mitochondrial membrane. Molecular Biology and Evolution 2009;26:671–80.

[6] Pusnik M, Schmidt O, Perry AJ, Oeljeklaus S, Niemann M, Warscheid B, et al. Mitochondrial preprotein translocase of trypanosomatids has a bacterial origin. Current Biology 2011;21:1738–43.
[7] Zarsky V, Tachezy J, Dolezal P. Tom40 is likely common to all mitochondria. Current Biology 2012;22:R479–81.
[8] Gentle IE, Perry AJ, Alcock FH, Likić VA, Dolezal P, Ng ET, et al. Conserved motifs reveal details of ancestry and structure in the small TIM chaperones of the mitochondrial intermembrane space. Molecular Biology and Evolution 2007;24:1149–60.
[9] Singha UK, Peprah E, Williams S, Walker R, Saha L, Chaudhuri M. Characterization of the mitochondrial inner membrane protein translocator Tim17 from *Trypanosoma brucei*. Molecular and Biochemical Parasitology 2008;159:30–43.
[10] Häusler T, Stierhof Y-D, Blattner J, Clayton C. Conservation of mitochondrial targeting sequence function in mitochondrial and hydrogenosomal proteins from the early-branching eukaryotes *Crithidia*, *Trypanosoma* and *Trichomonas*. European Journal of Cell Biology 1997;73:240–51.
[11] Acestor N, Zíková A, Dalley RA, Anupama A, Panigrahi AK, Stuart KD. *Trypanosoma brucei* mitochondrial respiratome: composition and organization in procyclic form. Molecular and Cellular Proteomics 2012, http://dx.doi.org/10.1074/mcp.M110.006908.
[12] Madison-Antenucci S, Sabatini RS, Pollard VW, Hajduk SL. Kinetoplastid RNA-editing-associated protein 1 (REAP-1): a novel editing complex protein with repetitive domains. EMBO Journal 1998;17:6368–76.
[13] Hauser R, Pypaert M, Häusler T, Horn EK, Schneider A. In vitro import of proteins into mitochondria of *Trypanosoma brucei* and *Leishmania tarentolae*. Journal of Cell Science 1996;109:517–23.
[14] Witte C, Jensen RE, Yaffe MP, Schatz G. MAS1, a gene essential for yeast mitochondrial assembly, encodes a subunit of the mitochondrial processing protease. EMBO Journal 1988;7:1439–47.
[15] Schneider A, Bouzaidi-Tiali N, Chanez A-L, Bulliard L. ATP production in isolated mitochondria of procyclic *Trypanosoma brucei*. Methods in Molecular Biology 2007;372:379–87.
[16] Smíd O, Matusková A, Harris SR, Kucera T, Novotný M, Horváthová L, et al. Reductive evolution of the mitochondrial processing peptidases of the unicellular parasites trichomonas vaginalis and giardia intestinalis. PLoS Pathogens 2008:4.
[17] Dolezal P, Smíd O, Rada P, Zubácová Z, Bursać D, Suták R, et al. Giardia mitosomes and trichomonad hydrogenosomes share a common mode of protein targeting. Proceedings of the National Academy of Sciences of the United States of America 2005;102:10924–9.
[18] Wirtz E, Leal S, Ochatt C, Cross GA. A tightly regulated inducible expression system for conditional gene knock-outs and dominant-negative genetics in *Trypanosoma brucei*. Molecular and Biochemical Parasitology 1999;99:89–101.
[19] Bochud-Allemann N, Schneider A. Mitochondrial substrate level phosphorylation is essential for growth of procyclic *Trypanosoma brucei*. Journal of Biological Chemistry 2002;277:32849–54.
[20] Schneider A, Charrière F, Pusnik M, Horn EK. Isolation of mitochondria from procyclic *Trypanosoma brucei*. Methods in Molecular Biology 2007;372:67–80.
[21] Oberholzer M, Morand S, Kunz S, Seebeck T. A vector series for rapid PCR-mediated C-terminal in situ tagging of *Trypanosoma brucei* genes. Molecular and Biochemical Parasitology 2005;145:117–20.