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Processing of the glycosomal matrix-protein import receptor PEX5 of *Trypanosoma brucei*

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ABSTRACT

Glycolysis in kinetoplastid protists such as *Trypanosoma brucei* is compartmentalized in peroxisome-like organelles called glycosomes. Glycosomal matrix-protein import involves a cytosolic receptor, PEX5, which recognizes the peroxisomal-targeting signal type 1 (PTS1) present at the C-terminus of the majority of matrix proteins. PEX5 appears generally susceptible to *in vitro* proteolytic processing. On western blots of *T. brucei,* two PEX5 forms are detected with apparent M_r of 100 kDa and 72 kDa. 5′-RACE-PCR showed that TbPEX5 is encoded by a unique transcript that can be translated into a protein of maximally 72 kDa. However, recombinant PEX5 migrates aberrantly in SDS–PAGE with an apparent M_r of 100 kDa, similarly as observed for the native peroxin. *In vitro* protease susceptibility analysis of native and ^{35}S-labelled PEX5 showed truncation of the 100 kDa form at the N-terminal side by unknown parasite proteases, giving rise to the 72 kDa form which remains functional for PTS1 binding. The relevance of these observations is discussed.

1. Introduction

Kinetoplastea protists possess organelles called glycosomes which contain enzymes for the major part of the glycolytic pathway, as well as enzymes of other core metabolic processes [1]. Glycosomes belong to the organelle family of peroxisomes; they share with the other family members some typical peroxisomal enzymes and morphological features. Moreover, the biogenesis of glycosomes and peroxisomes occurs via similar routes, mediated by homologous proteins called peroxins (PEX) [2].

The kinetoplastid *Trypanosoma brucei* is responsible for African sleeping sickness in humans and ‘nagana’ in cattle. Trypanosomes live in the bloodstream of mammals and are transmitted between mammalian hosts by tsetse flies. Glycosome biogenesis has been extensively studied in mammalian bloodstream and procyclic insect forms.

Peroxisomal and glycosomal matrix proteins are synthesized in the cytosol and posttranslationally imported into the organelles. Proteins to be imported are recognized by their peroxisome-targeting signal (PTS). Two main types of PTS exist: PTS1 comprising the C-terminal tripeptide SKL or variations having similar amino acids and PTS2 with a more complex nonapeptide motif near the N-terminus. PTS-containing proteins are recognized by cytosolic receptors, PTS1 by PEX5 and PTS2 by PEX7. The cargo-loaded receptors associate with a docking complex comprising minimally PEX13 and PEX14 [3]. This association leads to integration of multiple PEX5 molecules into the membrane, to form an oligomeric transient pore also involving PEX14 that allows the delivery of PEX5’s cargo into the matrix [4]. PEX5 is subsequently retrieved from the organelles by an ubiquitination-dependent process. Monoubiquitination of PEX5 mediates retrieval of the receptor for new rounds of import, whereas polyubiquitination targets it for degradation by proteasomes in case of recycling impairment [5].

In yeasts and fungi, PTS2-loaded PEX7 interacts also directly with the docking complex, in association with cytosolic co-receptors, PEX18 and PEX21 in *Saccharomyces cerevisiae*, or PEX20 in *Yarrowia lipolytica*, *Pichia pastoris*, *Hansenula polymorpha* and *Neurospora crassa*. However, in mammals, plants and possibly also in trypanosomatids, the PTS2-PEX7 complex binds to PEX5 and together the receptors bind to the docking complex [6].

In plants and trypanosomatids, PEX5 is present as only one isoform that is directly involved in PTS1 import and, together with PEX7, also in PTS2 protein import [6]. Also in yeast, only one

Abbreviations: PEX, peroxin; PGK, phosphoglycerate kinase; PMS, post-mitochondrial supernatant; PTS, peroxisome-targeting signal; Tet, tetracycline; TCA, trichloroacetic acid; TPR, tetratricopeptide repeat.

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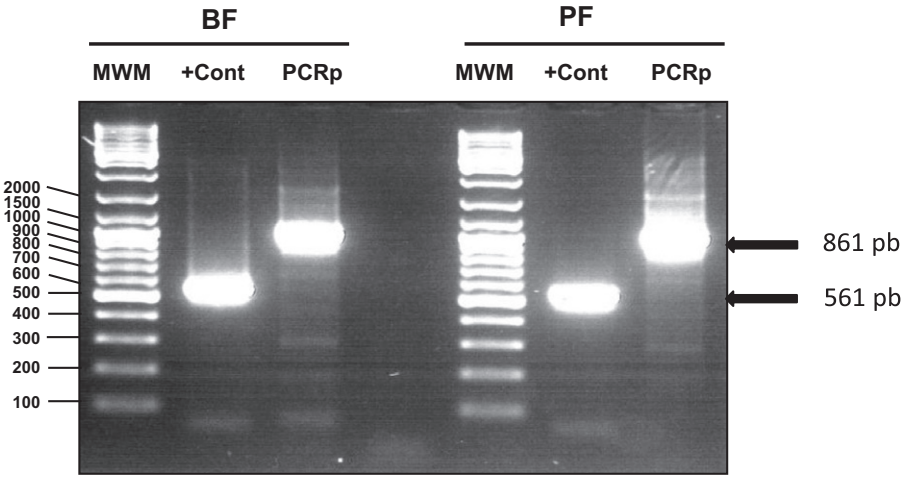

Fig. 1. *T. brucei* contains a single unique transcript coding for the TbPEX5 protein, in both the bloodstream and procyclic form. 5′-RACE-PCR was performed using cDNAs of *T. brucei* as templates for amplification using as a forward primer the sequence corresponding to the conserved 5′ miniexon sequence present at *T. brucei* mRNAs, and an internal reverse primer (Supplementary Fig. S2). The molecular size of the singly amplified product (PCRp) (861 bp) corresponds to a transcript not allowing translation of a protein that starts upstream of the predicted start codon of TbPEX5 thus confirming that it must have a molecular weight as predicted from the gene sequence. The positive control (+Cont) involving a PCR reaction with internal primers shows the integrity of PEX5 cDNA. Molecular weight marker (MWM).

isoform of PEX5 exists, which is exclusively devoted to PTS1-protein import. By contrast, mammalian cells contain two isoforms resulting from alternative splicing: the shorter PEX5S and a longer PEX5L, differing in an internal segment of 37 amino acids which contributes to the so-called PEX7-binding box. PEX5S only binds PTS1-containing proteins, whereas PEX5L interacts with PEX7. A consensus sequence is found in the PEX7-binding box of PEX5L and the yeast co-receptors PEX18, PEX20 and PEX21 [5] and is also present in plant and trypanosomatid PEX5 [6].

PEX5 is composed of two separate domains (Supplementary data, Fig. S1): A structurally disordered N-terminal half and a C-terminal domain consisting of tetratricopeptide repeats (TPR). The poorly conserved N-terminal half functions in the docking of PEX5 to the peroxisomal membrane and its recycling [7,8]. A species-dependent number of a pentapeptide motif, WxxxF/Y, is distributed in this domain and some of these motifs are involved in the binding of PEX13 and PEX14 [2]. The C-terminal domain is almost entirely composed of seven TPRs, organized in two clusters, TPR1–3 and TPR5–7, connected by the 4th TPR. The cavity between the clusters forms the PTS1-binding site.

Kiel et al. [9] reported that *H. polymorpha* PEX5 is a highly unstable protein *in vitro* and that especially the cytosolic pool of PEX5 is subject to proteolysis. Proteolytic cleavage of the human, *S. cerevisiae* and *P. pastoris* PTS1 receptor was also mentioned by Gould et al. [10], who observed that the pronounced protease sensitivity of *P. pastoris* PEX5 could only be inhibited by the presence of NaF during cell lysis. Kiel et al. [9] described that protein precipitation by treatment of whole cells with trichloroacetic acid (TCA) prior to cell breakage prevented PEX5's proteolysis.

Literature reviewing revealed that PEX5 proteolysis is commonly observed in western blot analyses performed with lysates from yeasts ([8]: Fig. 1C; [11]: Figs. 1B, 2A and 7A; [12]: Figs. 1A and 2A; [4]: Fig. S3) as well as from mammalian cells ([13]: Figs. 1D, 3B and 4A). Although faster migrating forms of PEX5 are usually detectable, they are often not mentioned and discussed, probably because of the assumption that they result from nonspecific *in vitro* degradation. The proteolysis seems to occur only with PEX5, not with any other cytosolic or peroxisomal proteins studied under similar conditions. It occurs even in the presence of several protease inhibitors and, in some cases, also in TCA lysates (as in [8]: Fig. 1C), but can be inhibited under certain conditions, as mentioned above. Furthermore, it involves PEX5 truncation rather than

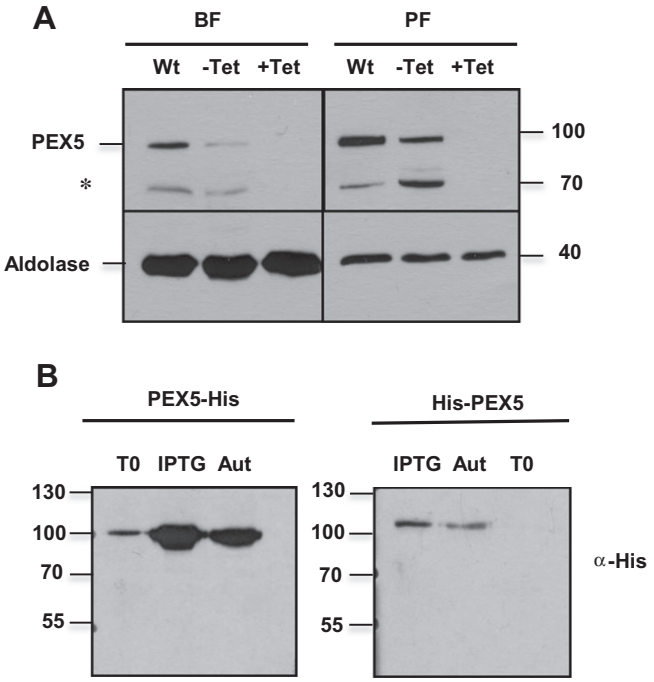

Fig. 2. Migration of *T. brucei* PEX5 in SDS–PAGE. (A) Two forms of TbPEX5 are detected by western blot analysis using affinity-purified antibodies. TbPEX5 RNAi cells were grown in the presence (+Tet) and absence of tetracycline (−Tet). Wild-type (Wt), induced and non-induced cells, taken 24 h after induction, were lysed in the presence of protease inhibitors. Proteins were separated by SDS–PAGE, blotted and analysed with TbPEX5 antibodies (*72 kDa PEX5). (B) N- and C-terminally His-tagged forms (PEX5-His and His-PEX5, respectively) of TbPEX5 were expressed in *E. coli* at 37 °C. Ten micrograms of total protein were analysed by SDS–PAGE followed by western blotting. The electrophoretic migration of the proteins suggests a molecular weight of 100 kDa. T0, time 0, IPTG, induced with 1 mM IPTG for 3.5 h, AUT, grown in autoinduction medium overnight.

general fragmentation. Strikingly, different bands were also observed for PEX5 in *T. brucei* lysates [6]. It is intriguing that this truncation is observed in widely different organisms, although PEX5 is poorly conserved, notably in its N-terminal domain [2,14]. To explore a possible physiological processing of PEX5, we performed a more detailed analysis of the phenomenon in *T. brucei*.

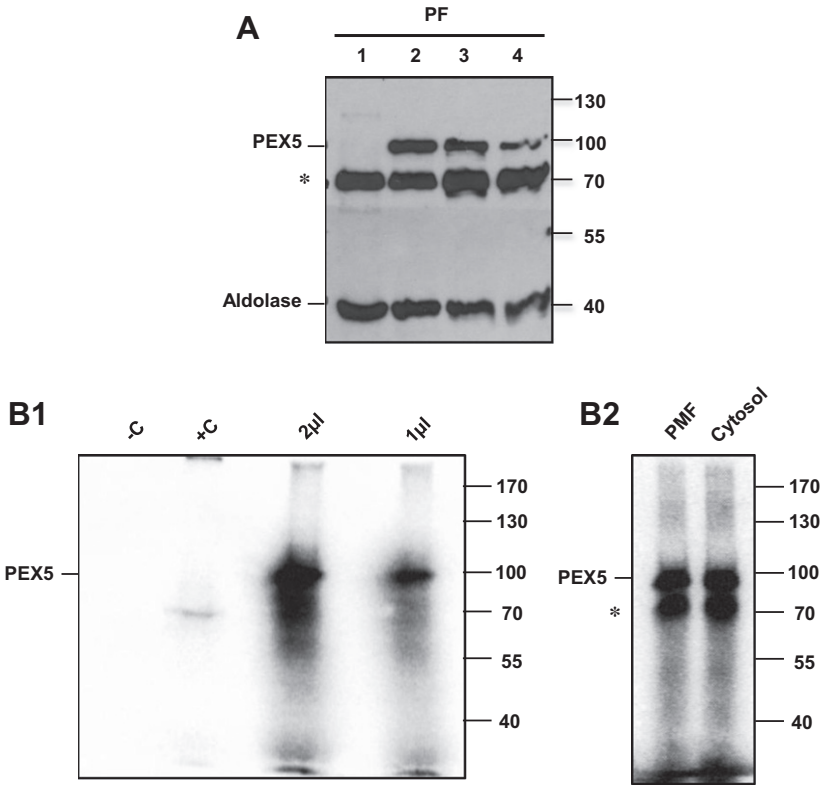

Fig. 3. TbPEX5 is susceptible to endogenous proteases. (A) Total lysates of procyclic-form *T. brucei* were incubated overnight in the presence or absence of different cocktails of protease inhibitors. Samples were analysed by western blotting using anti-PEX5 purified antibodies. 1: No protease inhibitors, 2: Home-made protease inhibitors cocktail, 3: Fermentas® Proteoblock, 4: Roche®. (*72 kDa PEX5). (B1) ^{35}S-TbPEX5 *in vitro* transcription/translation was performed in a cell-free commercial system. Different amounts of the DNA template (plasmid pET24-TbPEX5-His-tagged) were used (1 µl and 2 µl). −C: no DNA in the preparation; +C: ^{35}S-Luciferase control from the kit. (B2) Twenty percentage of the *in vitro* transcribed/translated ^{35}S-TbPEX5 was incubated in a post-mitochondrial fraction of procyclic wild-type cells, followed by ultracentrifugation of the suspension to separate cytosol from organelles. Ten percentage of the PMS and cytosolic fractions were analysed by SDS–PAGE followed by autoscanning of the gels. (*72 kDa PEX5).

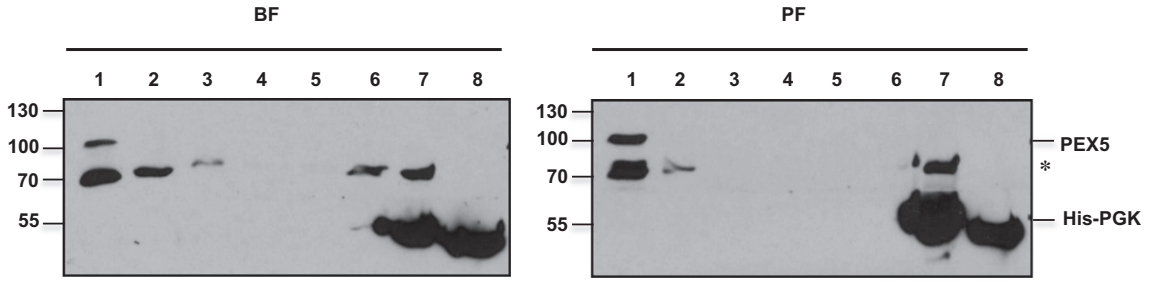

Fig. 4. The short form of TbPEX5 interacts with the PTS1-motif containing TbPGKc. Total lysates of bloodstream- and procyclic-form *T. brucei* were analysed by affinity chromatography under native conditions using a Talon resin with N-terminally His-tagged TbPGKc bound. The TbPGKc interacting proteins were eluted with imidazole and further analysed by western blotting using affinity purified anti-PEX5 and anti-His antibodies for PGKc detection. 1: total lysate, 2: total lysate after free-Talon incubation, 3: elution from free-Talon resin (nonspecifically interacting proteins), 4: flow-through from PGK resin, 5: wash 1, 6: wash 2, 7: elution with 50 mM imidazole, 8: elution with 100 mM imidazole. (*72 kDa PEX5).

2. Materials and methods

2.1. Trypanosomes

Wild-type bloodstream and procyclic-form *T. brucei* strain Lister 427, cell lines 449, and TbPEX5 RNAi cell lines derived from 449 cells have been used. Their properties, culturing and RNAi induction have been described previously [6]. Cultures were always harvested in the exponential growth phase, *i.e.*, at densities lower than 2×10^6 cells mL^{-1} for bloodstream forms and 2×10^7 cells mL^{-1} for procyclic cells, by centrifugation at 700*g* for 10 min.

2.2. 5′-RACE-PCR

Total RNA from wild-type bloodstream and procyclic trypanosomes was isolated from 5×10^7 cells using the SV Total RNA Isolation System (Promega). Two micrograms of total RNA were used to synthesize TbPEX5 cDNA using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Thermo Scientific) with a 3′ specific reverse primer (RT3′: 5′-TGGGGTCGTACACATCATCA-3′) starting 934 bp downstream of the predicted start codon of the PEX5 ORF. The TbPEX5 cDNA product was then treated with 10 U of RNase A for 15 min at room temperature followed by its purification

(Fermentas PCR purification kit). PCR was performed using the TbPEX5 cDNA as a template, the forward primer 5′-AACTAACGCTATTATTAGAACAGTTTCTG-3′ corresponding to the miniexon sequence and the reverse primer 5′-CTGCATGTGGTCCATGACACT-3′ starting 861 bp downstream of the start codon. As a control for the integrity of the cDNA, PCR was performed with the forward primer 5′-CCACAGCAAGCCAACGCTGGA-3′, positioned 301 bp downstream of the open-reading frame's start and the already described reverse primer (Fig. S2). Ten microliters per reaction were electrophoresed in agarose gels for size determination of the amplified fragments.

2.3. RNAi-dependent PEX5 depletion

Hundred millilitres cultures of bloodstream and procyclic TbPEX5 RNAi cell lines were induced during 24 h as described [6]. Parasites were lysed in PBS containing 0.6% CHAPS and a cocktail of protease inhibitors during 20 min on ice, followed by three times sonication, and DNase treatment for 15 min at 37 °C. The lysate was then centrifuged at 13,800*g* for 15 min at 4 °C and the supernatants recovered for protein quantification by the Bradford method. Western blot analyses were done as previously described [15] using purified anti-PEX5 antiserum.

2.4. Expression of recombinant T. brucei PEX5

Hundred millilitres cultures of *E. coli* BL21 cells containing constructs [pET24a_PEX5FL and pProEX_PEX5FL (N. Galland and P. Michels, unpublished)], for the production of full-length TbPEX5 with respectively a C- and N-terminal His-tag, were grown in LB medium supplemented with 30 microgram mL^{-1} kanamycin and 100 μg mL^{-1} ampicillin, to an OD_{600nm} = 0.4. 1 mM of β-D-1-thiogalactopyranoside was added to the cultures followed by incubation at 37 °C for 3.5 h. The same strains were simultaneously grown in ZYM-5052 autoinduction medium (0.5% yeast extract, 25 mM Na_2HPO_4, 25 mM KH_2PO_4, 50 mM NH_4Cl, 5 mM Na_2SO_4, 2 mM $MgSO_4$, 0.5% glycerol, 0.05% glucose and 0.2% lactose), supplemented with 30 μg mL^{-1} kanamycin and 100 μg mL^{-1} ampicillin. Cells were harvested by centrifugation at 2000*g* for 15 min at 4 °C and the pelleted cells resuspended in 20 mL of buffer A (20 mM Tris–HCl, pH 8, 200 mM NaCl) containing a cocktail of protease inhibitors (Roche) and broken by two passages through a French pressure cell at 6.9 MPa. After nucleic acids degradation with 1 U of Benzonase (Merck) at 37 °C for 15 min, protein concentrations were determined followed by SDS–PAGE and western blot analysis using anti-His antisera.

2.5. Protease susceptibility assay

T. brucei procyclic (200 mL) cells were harvested, washed once in PBS, resuspended in 1 mL of lysis buffer (0.1 M triethanolamine–HCl, pH 7.6, 1 mM $KHPO_4$, 200 mM KCl, 0.1% Triton X-100) and immediately distributed over four eppendorf tubes, each then supplemented with protease inhibitors from different sources: ProteoBlock Protease Inhibitor Cocktail from Fermentas; Complete Mini, EDTA-free Protease Inhibitor Cocktail tablets from Roche; home-made cocktail (final concentrations used: 1 μM pepstatin, 1 μM leupeptin, 10 μM AEBSF, 0.25 μM E-64, 0.75 mM PMSF). Cells were incubated on ice for 20 min and treated with 1 U of Benzonase for 15 min at 37 °C. Lysates were centrifuged at 13,800*g* for 15 min at 4 °C and the supernatants recovered. Proteins were quantified and suspensions kept at 4 °C overnight before analysis by SDS–PAGE followed by western blotting.

2.6. In vitro transcription and translation of TbPEX5

^{35}S-labelled TbPEX5 was synthesized *in vitro* using the expression plasmid pET24_TbPEX5FL as a template in the TNT Quick Coupled transcription/translation system (Promega) with ^{35}S-methionine (Perkin-Elmer). Proper *in vitro* synthesis was confirmed by SDS–PAGE followed by autoscanning of the gels. Differential centrifugation of procyclic wild-type *T. brucei* cells (800 mL trypanosomes at 8×10^6 cells mL^{-1}) was performed using buffer I (0.25 M sucrose, 5 mM Hepes-KOH, pH 7.4, 0.1% ethanol, 5 mM methionine and 50 mM KCl) to obtain a post-mitochondrial supernatant (PMS) adjusted to 1 mg protein mL^{-1}. 400 μg of PMS was incubated with 10 μL of ^{35}S-TbPEX5 in the absence of protease inhibitors at 26 °C for 30 min. The PMS suspension was centrifuged at 100,000*g* for 1 h at 4 °C to obtain a cytosolic fraction enriched in PEX5. 40 μL of the PMS and cytosolic fractions were analysed by SDS–PAGE followed by autoscanning of the gels using a Typhus scanner (GE Healthcare).

2.7. In vitro binding assay

T. brucei bloodstream (400 mL) and procyclic cells (200 mL) were lysed in Binding buffer (0.1 M triethanolamine–HCl, pH 7.6, 1 mM $KHPO_4$, 200 mM KCl, 0.1% Triton-X-100) in the presence of a cocktail of protease inhibitors (Fermentas) for 1 h on ice. DNA was degraded using 1 U of Benzonase for 15 min at 37 °C. Two 15-s cycles of sonication at 4 °C were performed prior to centrifugation at 13,800*g* for 10 min at 4 °C. Protein concentration was determined and lysates normalized to 1 mg mL^{-1}. Lysates (2 mg protein) were incubated overnight at 4 °C with Talon resin (Clontech) pre-equilibrated with Binding buffer to eliminate proteins that bind non-specifically to the resin. The flow-through (cleared lysate) was collected, its protein content quantified and kept for the *in vitro* binding experiment with *T. brucei* glycosomal phosphoglycerate kinase (PGKc), which contains a PTS1. N-terminal His-tagged TbPGKc was expressed in *E. coli* BL21 (pET28a_TbPGKc) and affinity purified by standard procedures over a Talon resin, using 100 mM imidazole for its elution. The imidazole in the purified TbPGKc sample was diluted to 10 mM by several cycles of concentration and dilution in Centricon devices (30 kDa) (Millipore). Subsequently, one milligram of the protein was bound to pre-equilibrated Talon resin overnight at 4 °C. After extensive washes (with 10 bed volumes), the TbPGKc-Talon was incubated with the cleared trypanosome lysate (0.5 mg protein) overnight at 4 °C. The flow-through was collected and washes were done with 10 bed volumes of binding buffer. Elution of TbPGKc with interacting trypanosome proteins was done in two fractions of 500 μL with 50 mM and 100 mM imidazole, respectively. Total proteins in the elution fractions were precipitated with chloroform/methanol and dissolved in 1× Laemmli buffer. Interacting proteins were analysed by SDS–PAGE followed by western blotting.

3. Results and discussion

PEX5 in *T. brucei* (and other trypanosomatids) appears encoded by a single gene (http://tritrypdb.org/tritrypdb/, # Tb927.5.1100). The protein's predicted molecular weight is 72 kDa. Transcriptional analysis of this gene by 5′-RACE-PCR resulted in the detection of only a single mRNA species that could be translated into a protein with the predicted molecular weight; no mRNAs with coding potential for larger PEX5 forms were found (Fig. 1). Nonetheless, western blot analysis using a polyclonal antiserum raised against a quasi full-length recombinant protein revealed two different bands, of approximately 72 kDa and 100 kDa (Fig. 2A). Although 72 kDa corresponds to the protein's molecular mass as predicted from the ORF, it is the 100 kDa form that should be considered as

the native form of TbPEX5. This is concluded from the fact that His-tagged recombinant forms of full-length TbPEX5 migrate aberrantly in SDS–PAGE (Fig. 2B and [14]). The notion that the 100 kDa form is the native one is further supported by the observation of a third, faint band with an apparent mass of 110 kDa that was identified as a monoubiquitinated form of TbPEX5, the physiological intermediate of the receptor during its recycling (our unpublished data).

To verify that the 72 kDa band is indeed a TbPEX5 form and not a cross-reacting band, we analysed a cell line in which TbPEX5 can be depleted by RNAi. As shown in Fig. 2A, the two most intense bands (72 kDa and 100 kDa) disappeared completely in bloodstream and procyclic cells after RNAi induction, confirming that PEX5, in addition to its native 100 kDa form, exists in *T. brucei* as an additional species with an apparent M_r of 72 kDa.

Mass spectrometry analysis of immunoprecipitated TbPEX5 from wild-type bloodstream- and procyclic-form cells proved that both proteins correspond to the PTS1 receptor TbPEX5 (Fig. S3 and Table S1).

Importantly, the aberrant migration of native PEX5 in SDS–PAGE as a protein with an apparent higher molecular mass has been previously reported also for a truncated, recombinant form of TbPEX5 [14] and for PEX5 of *P. pastoris*, plants and the human long and short isoforms. It has been attributed to a high density of negatively charged residues within the N-terminal half of the protein [16].

In order to explore the possibility that the 100 kDa native TbPEX5 is shortened to the 72 kDa form, we analysed lysates of procyclic *T. brucei* prepared in the presence of protease inhibitor cocktails from different commercial sources. As shown in Fig. 3, in total lysates prepared in the absence of protease inhibitors, the abundance of the native 100 kDa TbPEX5 was importantly decreased whereas the 72 kDa form remained unaffected. By contrast, a protease inhibitor cocktail in the lysate, irrespective of its commercial source, stabilized the higher molecular weight form, while the shorter one was also abundantly present in each of the preparations, apparently being equally stable. This result suggests that full-length TbPEX5 is susceptible to (an) unknown parasite protease(s) present in the lysate, which is (are) only partially inhibited by common protease inhibitors. It should be mentioned that the experiments performed in this investigation involved disruption of trypanosomes in a standard, detergent-containing lysis buffer, supplemented (unless otherwise stated) with a protease inhibitor cocktail. Under these conditions, we observed consistently the two molecular weight forms of TbPEX5 with the purified antibody.

To test further the hypothesis of an involvement of a trypanosome-specific protease, we *in vitro* synthesized radiolabelled ^{35}S-TbPEX5 (Fig. 3B1) and incubated it in a post-mitochondrial fraction prepared from wild-type procyclic cells by differential centrifugation in the absence of protease inhibitors. Fig. 3B2 shows that under this condition, the ^{35}S-TbPEX5 also undergoes the proteolytic processing as observed for the native TbPEX5, confirming the susceptibility of TbPEX5 to (an) unknown parasite-specific protease(s).

In order to determine if the 72 kDa form of TbPEX5 is competent for PTS1 binding, we used an *in vitro* binding assay in which recombinant N-terminally His-tagged PGKc (a PTS1-containing protein) was immobilized to an affinity resin and used as a ligand for binding native TbPEX5. This experiment allows to establish if the 72 kDa form of this receptor is truncated at its C-terminus, since the binding of the PTS1 depends on PEX5's C-terminal TPR-domain. As shown in Fig. 4, in both bloodstream (A) and procyclic (B) cells, we could detect the 100 and 72 kDa proteins in the initial cell lysate. After clearing the lysate overnight at 4 °C from proteins nonspecifically interacting with the resin, only the 72 kDa TbPEX5 form was detected, indicating that the incubation was sufficiently long to allow the unknown protease(s) to process all 100 kDa molecules (as a result of the time-dependent activity loss of the protease inhibitors). The alternative possibility that the slower migrating form of TbPEX5 may not have been present in the cleared lysate as a result of an affinity – lost by the processed form – for the PGK-free resin could be excluded, since it was not detected in that fraction (Fig. 4, lane 3). Concluding that only the 72 kDa TbPEX5 form was left in the cleared lysate, our *in vitro* binding study was thus restricted to this TbPEX5 species. Western blot analysis showed that the 72 kDa TbPEX5 was recovered in the TbPGKc eluted fraction indicating the capability of this TbPEX5 form to interact with the PTS1 motif of TbPGKc. Its TPR domain is thus functional for PTS1 binding and therefore its C-terminus cannot have been shortened. The shortening should thus either be the result of an N-terminal truncation or, less likely, a deletion of an internal segment.

This shorter TbPEX5 form is also capable of interacting with the glycosomal membrane since it is detected in western blots of glycosome-enriched cell fractions obtained by isopycnic centrifugation of lysates (data not shown). We postulate that the proteolytic cleavage occurs in the N-terminal part, upstream of the third pentapeptide motif, a region previously shown to be responsible for TbPEX5's interaction with PEX14 and PEX13 [3,17].

The proteolytic processing occurred in the presence of protease inhibitors, although to a lesser extent (Fig. 3). This partial inhibition renders a non-enzymatic cleavage of PEX5, for example involving its oxidation upon cell lysis, unlikely. We are not aware of similar proteolysis for *Trypanosoma* proteins other than TbPEX5. We argue therefore that, although it seems to be – at least largely – an *in vitro* process, it must be caused by a specific protease that acts preferably on PEX5 and not on other cytosolic proteins. A possible explanation is that the structurally disordered N-terminal domain of PEX5 exposes frequently, during its intramolecular motions, a cleavage site that does not exist or is inaccessible in most well-structured proteins present in cells. It is intriguing that in other organisms such as yeast, mammals and other kinetoplastid parasites such as *Leishmania* species (our unpublished data and A. Jardim, personal communication) this proteolytic processing also occurs, indicating that the putative specific protease must be an enzyme with well-conserved specificity. Moreover, also the cleavage site must have been maintained throughout evolution in the otherwise highly divergent sequence of the N-terminal PEX5 half. We wonder, therefore, if this phenomenon reflects some event, also relevant *in vivo*, in PEX5's function that has been overlooked so far. It might be an important, still unknown key event in peroxisomal matrix-protein import, but artificially stimulated when cells are lysed. Of course, proteolytically truncated PEX5 would not be suitable for recycling, but one may wonder if such proteolytic processing might be another route for disposal of non-functional or redundant PEX5 molecules, additionally to the polyubiquitination-dependent retrieval of non-functional PEX5 proteins from the organelles to be routed to the proteasomes [12]. The identification of the protease involved, its specificity and subcellular localization deserves further analysis in order to understand the puzzling PEX5 processing and to determine if it has indeed a physiological role or is an artefact, surprisingly conserved during evolution.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.12.075.

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