Biochimica et Biophysica Acta 1833 (2013) 3076–3092

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Ubiquitination of the glycosomal matrix protein receptor PEX5 in *Trypanosoma brucei* by PEX4 displays novel features

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ARTICLE INFO

Article history:
Received 17 May 2013
Received in revised form 8 August 2013
Accepted 12 August 2013
Available online 27 August 2013

Keywords:
Trypanosoma
Glycosome biogenesis
Ubiquitination
Peroxin
PEX5
PEX4

ABSTRACT

Trypanosomatids contain peroxisome-like organelles called glycosomes. Peroxisomal biogenesis involves a cytosolic receptor, PEX5, which, after its insertion into the organellar membrane, delivers proteins to the matrix. In yeasts and mammalian cells, transient PEX5 monoubiquitination at the membrane serves as the signal for its retrieval from the organelle for re-use. When its recycling is impaired, PEX5 is polyubiquitinated for proteasomal degradation. Stably monoubiquitinated TbPEX5 was detected in cytosolic fractions of *Trypanosoma brucei*, indicative for its role as physiological intermediate in receptor recycling. This modification's resistance to dithiothreitol suggests ubiquitin conjugation of a lysine residue. *T. brucei* PEX4, the functional homologue of the ubiquitin-conjugating (UBC) enzyme responsible for PEX5 monoubiquitination in yeast, was identified. It is associated with the cytosolic face of the glycosomal membrane, probably anchored by an identified putative TbPEX22. The involvement of TbPEX4 in TbPEX5 ubiquitination was demonstrated using procyclic Δ*PEX4* trypanosomes. Surprisingly, glycosomal matrix protein import was only mildly affected in this mutant. Since other UBC homologues were upregulated, it might be possible that these have partially rescued PEX4's function in PEX5 ubiquitination. In addition, the altered expression of UBCs, notably of candidates involved in cell-cycle control, could be responsible for observed morphological and motility defects of the Δ*PEX4* mutant.

1. Introduction

Human African trypanosomiasis is an infectious disease caused by the parasitic protist *Trypanosoma brucei* and transmitted by bites of tsetse flies. *T. brucei* belongs to the Kinetoplastea clade and the Trypanosomatidae family. This group also includes *Trypanosoma cruzi*, the causative agent of Chagas disease in Latin America, and *Leishmania* species which are responsible for several forms of leishmaniasis in tropical and subtropical areas of the world [1–3].

The *T. brucei* life cycle involves several successive developmental changes which occur when the parasite alternates between living extracellularly in the mammalian host, where its main form is the proliferative so-called long-slender bloodstream form and in the insect, where procyclic forms are the replicating stage in the fly's midgut. This differentiation, which involves physiological, morphological and metabolic remodelling, allows the parasite to adapt to the different environments [4,5].

One of the unique features that distinguish members of the kinetoplastids from other eukaryotes is the metabolic compartmentalisation of the majority of glycolytic enzymes and some other pathways of core metabolism within organelles called glycosomes [6–8]. Remarkably, correct compartmentalisation of glycolysis in glycosomes of the bloodstream form of *T. brucei* is absolutely essential for survival of the parasites [9,10].

Glycosomes are microbodies that belong to the organelle family of peroxisomes, together with the peroxisomes found in representatives of all eukaryotic lineages. These organelles are bounded by a single phospholipid bilayer membrane and do not contain any detectable DNA. Peroxisomal and glycosomal matrix and membrane proteins are synthesised in the cytosol and post-translationally imported into the organelles. The formation of these organelles involves different proteins (so far a non-redundant set of 33 proteins have been identified in different organisms) commonly named 'peroxins' (abbreviated PEX), which accomplish the different steps of the process: peroxisome

Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; DTT, dithiothreitol; DUB, deubiquitinating; FBPase, fructose-1,6-bisphosphatase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green-fluorescent protein; HXK, hexokinase; NEM, N-ethylmaleimide; PEX, peroxin; PFK, phosphofructokinase; PTS, peroxisomal-targeting signal; ORF, open-reading frame; Tet, tetracycline; TPI, triosephosphate isomerase; TPR, tetratricopeptide repeat; UBC, ubiquitin-conjugating

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http://dx.doi.org/10.1016/j.bbamcr.2013.08.008

membrane formation, import of peroxisomal matrix proteins and peroxisome proliferation [11–13].

Peroxisomal matrix protein import is a multistep process that involves successive interactions between several peroxins in the cytosol and at the peroxisomal membrane. The matrix proteins are targeted to the organelles through their peroxisomal-targeting signals: PTS1, consisting of the motif SKL or physicochemically conserved variants of this tripeptide, with also contributions by adjacent residues, more precisely a dodecamer sequence, located at the extreme C-terminus of the great majority of matrix proteins [14]; PTS2 is a nonapeptide (R/K)(L/V/I/Q/)xx(L/V/I/H/Q)(L/S/G/A/K)X(H/Q)(L/A/F) near the N-terminus of a smaller subset of matrix proteins [13,15,16]. The matrix proteins are recognised in the cytosol through their targeting signals PTS1 or PTS2 by different receptors, PEX5 and PEX7, respectively. PEX5 is a predominantly cytosolic protein that possesses two domains: a C-terminal domain composed of 6–7 tetratricopeptide repeat (TPR) motifs which interact with the PTS1 of the cargo, and a structurally disordered N-terminal domain that functions in the receptor's docking, at the organelle's membrane, through its conserved pentapeptide (WxxxF/Y) repeat motifs, and in the receptor's recycling (see below). PEX7 is also a cytosolic protein that becomes transiently internalised inside the peroxisomes [17,18] belonging to the WD protein family. For the PTS2-protein import, it requires species-specific auxiliary proteins also known as co-receptors: PEX18 and PEX21 in *Saccharomyces cerevisiae*, PEX20 in *Yarrowia lipolytica*, *Pichia pastoris*, *Hansenula polymorpha* and *Neurospora crassa* or a longer splice variant of the PTS1 receptor PEX5 in plants and mammals. The co-receptors form a ternary complex with the cargo-loaded PEX7 in the cytosol and direct the complex to the peroxisomal membrane [19]. A small fraction of proteins that lack canonical PTS1 and 2 motifs may be imported through either their association with PTS-containing proteins ("piggy backing") or via the binding to the N-terminal region of PEX5 (non-PTS import) [20].

The next step in matrix protein import is the association of the receptor–cargo complex, formed in the cytosol, with the peroxisomal membrane. A docking subcomplex minimally composed of PEX14, PEX13 and in *S. cerevisiae* also PEX17, is responsible for this step [21]. The docking subcomplex has been shown to physically associate with a subcomplex of three RING-finger proteins, PEX2-PEX10-PEX12, in order to form an import-competent complex, called the importomer [13,15]. The docking and RING-finger subcomplexes are associated in a PEX8 and PEX3-dependent manner in *S. cerevisiae* and *P. pastoris*, respectively [22].

Peroxisomes are able to import folded proteins and also large oligomeric proteins into the peroxisomal matrix [23,24]. Recently, Meinecke et al. [25] demonstrated that the membrane-associated import receptor PEX5 from *S. cerevisiae* together with the docking-complex protein PEX14 forms a dynamic channel that is opened to a diameter up to 9 nM upon docking of the cytosolic receptor–cargo complex. This discovery explains how peroxisomes are able to import completely folded and even oligomeric proteins of different sizes and illustrates the extremely important, multiple roles of PEX5 in the peroxisomal matrix protein import process.

The next step in matrix protein import is the cargo release in the peroxisomal matrix, the mechanism of which is poorly understood. Several lines of evidence indicate a possible function of PEX8 in the dissociation of cargo proteins from the PEX5–cargo complex; however, this issue remains to be solved [16]. After the cargo release, the two receptors, PEX5 and PEX7, must be retrieved from the peroxisomes. Whereas PEX7 is fully internalised into the matrix [18], PEX5 does not enter the lumen with its cargo, but seems to release it while remaining inserted in the membrane. PEX5 is thus a cycling protein that translocates to the peroxisomal membrane from where it is recycled to the cytosol [26]. The molecular machinery that is involved in this process was defined as the exportomer [16]. It comprises two cytosolic AAA^+-ATPases, PEX1 and PEX6, which are anchored to the peroxisomal membrane through PEX15 in yeasts or PEX26 in mammalian cells, and which have been identified as the mechanoenzymes responsible for the removal of PEX5 from the peroxisomal membrane [27]. Also part of the exportomer is a member of the E2 ubiquitin-conjugating enzyme family, a function performed in yeasts and plants by PEX4 together with its membrane anchor protein PEX22 [28–30]. In mammalian cells, this function is accomplished by the cytosolic ubiquitin-conjugating enzymes UbcH5a/b/c [31]. Additionally involved in the receptor recycling are the RING-finger peroxins which have E3-ubiquitin ligase activity [32].

Unlike the docking event, which is ATP independent, the receptor recycling requires the free energy from the hydrolysis of ATP, both by the AAA^+-ATPases and for the activation of ubiquitin. Recently, it has been proved that the ATP-dependence of the ubiquitination-mediated export of *S. cerevisiae* PEX18, via the mechanistic linkage, drives the translocation of the PEX7 receptor-bound cargo into the matrix [33], supporting the export driven protein import model proposed by Schliebs et al. [34].

In yeast PEX5 is mono- or polyubiquitinated at the peroxisomal membrane. The monoubiquitination of the receptor serves as the signal for its recycling and depends on the concerted action of the peroxisomal membrane-bound PEX4 and PEX12, which ubiquitinate PEX5 at a conserved cysteine residue (Cys6) located in its N-terminal part. The polyubiquitination has been demonstrated to occur as a quality control mechanism that serves to signal the removal of not properly recycled receptor molecules. This type of ubiquitination depends on the redundant activities of the cytosolic ubiquitin-conjugating enzymes UBC4/5/1 and membrane-bound PEX2 and occurs at different lysine residues located also in the N-terminal part of the protein (Lys18 and Lys24 in *S. cerevisiae* and Lys 21 in *H. polymorpha*) [28,29,32,35–37]. Importantly, in *S. cerevisiae* the co-receptor PEX18 has been proved to be also monoubiquitinated at a conserved cysteine residue (Cys6) as a signal for its recycling and polyubiquitinated at lysine residues (Lys13 and Lys20) for its proteasome-dependent degradation [33]. Very recently, it has been shown that the *P. pastoris* co-receptor PEX20 is also mono- or diubiquitinated (Cys8) and polyubiquitinated (Lys19) in a PEX4 and PEX4–PEX7 dependent manner, respectively, providing new roles for PEX4 in the ubiquitination cascade of the PTS2 import pathway [38].

PEX5 in mammalian cells, as with its counterpart in yeast, is also monoubiquitinated at the peroxisomal membrane at a well conserved cysteine residue (Cys11) [39]. However, the ubiquitin-conjugating enzyme involved in this modification is the cytosolic broad range UbcH5a/b/c [31]. It was recently proved that mammalian PEX10 is necessary for PEX5 ubiquitination as well [40]. Interestingly, in mammalian cells two different monoubiquitinated PEX5 species were detected in distinct cellular locations. While the cysteine-monoubiquitinated [dithiothreitol (DTT) sensitive] form of PEX5 was exclusively detected in the organellar fraction of mammalian cells, another monoubiquitinated form (DTT resistant), for which no function has yet been ascribed, was located in the cytosol [40,41].

In trypanosomes, several of the peroxins involved in the glycosomal matrix protein import have been identified and characterised. These include TbPEX2, TbPEX5, TbPEX6, TbPEX7, TbPEX10, TbPEX12, TbPEX13 (two different isoforms, TbPEX13.1 and TbPEX13.2) and TbPEX14. All these peroxins are essential for glycosome biogenesis and therefore for the survival of the parasites (reviewed in [9,10,42]). Nevertheless, few data are available yet about the mechanism of the import process and how some of these peroxins operate in TbPEX5 recycling. In this paper we provide the first experimental evidence of TbPEX5 ubiquitination and present the identification and functional characterisation of the *T. brucei* orthologue of the peroxisomal ubiquitin-conjugating enzyme TbPEX4.

2. Materials and methods

2.1. Parasite cultures, transfections and cell growth measurements

Monomorphic bloodstream and procyclic-form *T. brucei* strain Lister 427 (hereafter called *T. brucei* 427), cell lines 449 [43] that were used in

this study constitutively express the *Escherichia coli* tetracycline (Tet) repressor gene from the chromosomally integrated plasmid pHD449, also endowing phleomycin resistance. This cell line is metabolically indistinguishable from the wild type. Bloodstream forms were cultured in HMI-9 medium containing 20% heat-inactivated foetal calf serum (Gibco) and 0.18 μg/ml phleomycin (Cayla) at 37 °C under water-saturated air with 5% CO_2. Procyclic trypanosomes were grown in SDM79 medium [44] supplemented with 12% foetal calf serum and 0.5 μg/ml phleomycin at 28 °C under water-saturated air with 5% CO_2.

Cultures were always harvested in the exponential growth phase, i.e., at densities lower than 1×10^6 cells/ml for bloodstream forms and 1×10^7 cells/ml for procyclic cells, by centrifugation at 700 ×*g* for 10 min. The method used for transfection of bloodstream and procyclic-form trypanosomes has previously been described [45]. For cell growth measurements cultures of 1 ml were started at 2×10^5 cells/ml (bloodstream form) and 2×10^6 cells/ml (procyclic form). Motile cells were daily counted in a Bürker–Türk (Merck) cell counting grid (0.1 mm in depth). After dilution down to the initial cell density, cells were recounted similarly. Accumulative growth curves were constructed by plotting the $\log_{10}$ of the product of cell density (cells/ml) and total dilution as a function of time.

2.2. Preparation of DNA constructs and cloning procedures

Standard methodologies in molecular biology were carried out essentially according to Sambrook et al. [46]. Chemicals and enzymes were obtained from Fermentas, Roche Diagnostics and Promega Corporation. The sequences of primers used are available in Table S1 of the Supplementary data file. All constructs were checked by sequencing.

For myc-ubiquitin cloning the full-length gene of one monoubiquitin (derived from the putative polyubiquitin gene; TriTrypDB (http://tritrypdb.org) accession code Tb11.01.1680) was amplified by PCR from *T. brucei* 427 genomic DNA using the set of primers MycUbi forward and reverse. The PCR-amplified fragment digested with *Hind*III–*Bam*HI was cloned using the trypanosome-specific tetracycline-inducible expression vector pHD1701 (a gift from Dr Frank Voncken), which directs the synthesis of an N-terminal 2×-myc-tagged fusion protein.

All TbPEX4 constructs described here were derived from the *T. brucei* gene with accession code Tb927.8.920. For bacterial expression, the full open-reading frame (ORF) of *TbPEX4* (FL) was amplified by PCR from genomic DNA of *T. brucei* 427 using the primers TbPEX4FL forward and reverse. The amplified TbPEX4FL gene was first inserted in the cloning vector pJET1.2 (Thermo Fisher Scientific) and from there transferred to the bacterial expression vector pET28a (Novagen) between the restriction sites *Nde*I and *Bam*HI. This vector directs the expression of an N-terminally $(His)_6$-tagged recombinant protein under the control of the Lac promoter.

To express in trypanosomes an N-terminally green-fluorescent protein (GFP) tagged-TbPEX4 protein, the *TbPEX4* full-length gene was amplified by PCR from *T. brucei* 427 genomic DNA using the primers GFP-TbPEX4FL forward and reverse. PCR amplified fragments were digested with *Hind*III and *Bam*HI and ligated in the trypanosome-specific vector pGC1 containing already the GFP gene (previously prepared by the insertion of the *EGFP* gene into the trypanosome-specific tetracycline-inducible expression vector pHD1336 [47]).

A TbPEX4 knock-out construct was prepared as follows: a segment of the 5′UTR region of the *TbPEX4* locus from position −581 to −16 and a segment of the 3′UTR region comprising the last 22 bp of the ORF of *TbPEX4* until position +592 were amplified by PCR from *T. brucei* 427 genomic DNA using the following sets of primers: 5′UTRKOTbPEX4 forward and reverse, 3′UTRKOTbPEX4 forward and reverse. Each fragment was individually cloned using the pGEMT Easy Vector (Promega Corporation) to create the plasmids pGEMT-5′UTRTbPEX4 and pGEMT-3′UTRTbPEX4, respectively. Subsequently, the 3′UTRTbPEX4 fragment was liberated from the vector and subcloned after ligating it in the pGEMT-5′UTRKOTbPEX4 vector between the restriction enzymes *Eco*RI and *Xba*I to generate pGEMT5′-3′UTRKOTbPEX4. The puromycin resistance gene (*Streptomyces alboniger* puromycin N-acetyltransferase gene) and blasticidin resistance gene (*Aspergillus terreus* blasticidin S deaminase gene) were excised from plasmid pGEMT-puro and pGEMT-bla (gifts from Dr Frédéric Bringaud) using the restriction enzymes *Xba*I and *Xho*I and each was individually ligated in pGEMT5′-3′UTRKOTbPEX4 to generate pGEMT5′-PURO-3′UTRKOTbPEX4 and pGEMT5′-BLA-3′UTRKOtbPEX4. These two plasmids were then digested with the restriction enzyme *Hpa*I to liberate the fragments TbPEX4-5′-PURO-3′UTR and TbPEX4-5′-BLA-3′UTR containing the corresponding selection markers flanked by 500 bp of the UTR region of the *TbPEX4* locus. These DNA fragments were sequentially used to transfect *T. brucei* procyclic and bloodstream forms and replace each allele of the *PEX4* gene by homologous recombination by the respective antibiotic resistance markers.

A GFP-PTS1 trypanosome expression plasmid was constructed by PCR amplification of the gene coding for GFP with at its C-terminus fused a PTS1 (SKL) from the yeast expression vector pEW88 (a gift from Dr Ben Distel) using primers GFP-SKL forward and reverse. The amplified PCR fragment was digested with *Hind*III and *Bam*HI and ligated to the trypanosome-specific tetracycline-inducible expression vectors pHD1336 and pHD677 (gifts from Prof. Christine Clayton).

2.3. Genome integration analysis

Total genomic DNA was extracted from 5×10^7 cells using the Magnesyl KF genomic system (Promega Corporation) and the Kingfisher Labsystem (Thermo Fisher Scientific). Briefly, after selection of positive clones (resistant to the respective antibiotic markers) of procyclic- and bloodstream-form *T. brucei* cells transfected with the relevant plasmids containing knock-out constructs, they were analysed to confirm the correct integration of the corresponding gene(s) by PCR from genomic DNA. The criteria to assess the integration of *TbPEX4* knock-out constructs were based on a PCR reaction performed with the following sets of primers: TbPEX4-RTPCR forward and reverse, Blast forward and reverse, Puro forward and reverse, Tubulin forward and reverse. The negative result of a non-amplification of the *TbPEX4* gene and the simultaneous positive results in amplification of the blasticidin and puromycin resistance marker genes were used as evidence for successful double gene knock-out. This was only achieved for procyclic trypanosomes; despite repeated attempts, no bloodstream-form null mutant was obtained, suggesting that it would have a lethal phenotype.

2.4. RT-PCR and qPCR

Total RNA was isolated from 5×10^7 cells using the SV total RNA isolation system (Promega Corporation). After quantification, 2 μg of total RNA was used to synthesise cDNA using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) following the manufacturer's instructions. Total cDNA was diluted 4-fold and used as a template for PCR amplification using the same primer set for the genomic integration analysis. Fixed volumes were loaded into slots of 2% agarose gels and stained with ethidium bromide. For qPCR analysis of the Δ*PEX4* cell line, cDNA was not diluted after synthesis and used for a qPCR reaction with 10 μl of KAPA SYBR® FAST Bio-Rad iCycler™ qPCR Master Mix (Sopachem, Kapa Biosystems, Eke, Belgium) and 10 pmol of each primer. A single denaturing step at 94 °C for 300 s was followed by 40 cycles: 94 °C for 30 s and 60 °C for 3 s. For quantification, the copy number of each mRNA was normalised to the tubulin mRNA copy number. Sets of forward and reverse primers were used for the following genes: TbPEX4-RTPCR, Tb927.5.1000, Tb11.01.5510, Tb927.7.2540, Tb927.8.6090, Tb927.2.3720, Tb09.211.0050, Tb927.4.3460, Tb11.01.5790, Tb927.4.2710, Tb927.4.3190 and Tubulin, as specified in Table S1.

2.5. Western blot analysis

Immunoblotting was performed according to a standard procedure [48] with slight modifications. Briefly, proteins were size separated by SDS-PAGE and then transferred to a nitrocellulose membrane (Amersham Biosciences, Inc.) using a wet transfer tank (Hoefer Instruments). Non-specific binding of antibodies to the membrane was prevented by blocking in PBS supplemented with 5% dried low-fat milk for 1 h. Incubation with primary antibodies was done in PBS supplemented with 1% dried low-fat milk overnight at room temperature. Membranes were subsequently washed by consecutive 10 min incubations in PBS (2×), PBS supplemented with 0.05% NP-40 (1×) and PBS (2×). Incubation with the secondary antibody was done in PBS supplemented with 1% dried low-fat milk for 1 h at room temperature. For detection of the following proteins, polyclonal antisera raised in rabbits were used at the indicated dilutions: TbPEX5 (1:20,000), TbPEX4 (1:4000), TbPEX4 peptide based (affinity purified) (1:300), human ubiquitin (Santa Cruz Biotechnology, Inc.) (1:300), TbPEX11 (1:2500), TbEnolase (1:150,000), TbAldolase (1:200,000), cytosolic glyceraldehyde-3-phosphate dehydrogenase (TbcGAPDH) (1:10,000), hexokinase (TbHXK) (1:100,000). Furthermore, a mouse monoclonal antibody against heat-shock protein 60 (TbHSP60) (1:100) was used (courtesy of Dr Frédéric Bringaud). The secondary antibodies, anti-rabbit IgG and anti-mouse IgG conjugated to horseradish peroxidase (Rockland Immunochemicals, Inc.) were diluted 1:10,000 and visualised with the ECL Western Blotting System, a luminol-based system (Amersham Biosciences, Inc.).

2.6. Immunofluorescence

Immunofluorescence analysis of trypanosomes was performed as described by Galland et al. [49], with the following modifications. Procyclic- and bloodstream-form trypanosomes (1×10^7 cells) were centrifuged at 918 ×*g* in a T15A61 VWR rotor for 5 min at 4 °C and washed twice in ice-cold PBS. Cell fixation was done with 4% formaldehyde in PBS for 10 min at room temperature in a rotating device followed by a permeabilisation step involving treatment with 1% Triton X-100 for 15 min. Finally cells were incubated for 1 h with PBS containing 0.5 M glycine and then washed once in ice-cold PBS. After applying a suspension of trypanosomes on poly-L-lysine coated microscope slides and drying during 1 h at room temperature, unspecific antibody binding was blocked by incubation in PBS containing 5% BSA for 45 min at 37 °C, followed by incubation with primary antiserum in PBS containing 2% BSA at the following dilutions: mouse monoclonal anti-*T. brucei* triosephosphate isomerase (TbTPI) (1:5000), rabbit polyclonal TbAldolase (1:1500) and anti-*T. brucei* glycosomal GAPDH (TbgGAPDH) (1:5000). After extensive washes with PBS, the slides were incubated for 45 min at 37 °C with fluorescein-conjugated secondary antibodies (Alexa Fluor 488 anti-mouse IgG, Alexa Fluor 488 anti-rabbit, Alexa 568 anti-mouse IgG and Alexa 568 anti-rabbit IgG) (Molecular Probes, Invitrogen Corporation) in PBS containing 2% of BSA at a 1:1000 dilution. After washes with PBS, cells were incubated with 4′,6-diamidino-2-phenylindole (DAPI) (1:500) in PBS for 30 min at room temperature followed by washes in PBS. The preparations were finally mounted in Dako (Dako North America, Inc.) and visualised using a Zeiss Axiovert 200 inverted fluorescence microscope coupled to an MRC-1024 confocal scanning laser imaging system (Bio-Rad Laboratories, Inc.). Digital images were captured using the software AxioVision 4.8.2 (Zeiss). Images to be analysed were imported in AxioVision (4.8.2; Zeiss, Germany).

2.7. Immunoprecipitation

Native TbPEX5 was immunoprecipitated under denaturing conditions from *T. brucei* wild-type procyclic cells as described in Leung et al. [50].

2.8. Differential centrifugation

A total of 8×10^9 exponentially growing wild-type bloodstream (5×10^5 cells/ml) and procyclic (5×10^6 cells/ml) trypanosomes were collected, centrifuged at 680 ×*g* in a SLA-3000 Sorvall rotor for 15 min at 4 °C and washed twice in isotonic STE buffer (250 mM sucrose, 25 mM Tris–HCl, pH 7.4, 1 mM EDTA). Cells were homogenised (>95%, as microscopically checked) in a pre-chilled mortar with carborundum powder as described by Opperdoes and Borst [6]. Differential centrifugation was performed as described by Verplaetse et al. [51]. Equal amounts of protein of each fraction were analysed by SDS-PAGE followed by Western blotting.

2.9. Isopycnic centrifugation

Exponentially growing procyclic-form trypanosomes (1.5 l; 5×10^6 cells/ml) were centrifuged at 680 ×*g* in a SLA-3000 Sorvall rotor for 15 min at 4 °C, washed once with 150 ml of TEDS buffer (25 mM Tris–HCl, pH 7.8, 1 mM EDTA, 1 mM DTT, 250 mM sucrose) followed by a second wash with 50 ml of the same buffer. Cells were homogenised (microscopically checked) in a pre-chilled mortar with carborundum powder as described previously [6]. Differential centrifugation was done as described by Verplaetse et al. [51] to prepare a large granular fraction enriched in mitochondria and glycosomes. 2.5 ml of this large granular fraction was loaded on the top of a preformed linear 20–40% (v/v) Optiprep density gradient (33 ml) mounted on top of a 3.5 ml 50% (v/v) Optiprep cushion according to the manufacturer's instruction (Optiprep application sheet S09, Axis-shield). The gradients were centrifuged at 4 °C in a VTi-50 vertical rotor (Beckman Coulter, Inc.) at 100,000 ×*g* overnight, using the slow acceleration and deceleration modes. Fractions of 1 ml were collected from the bottom of the tube using an automatic fraction collector. Protein concentrations were determined by Bradford method. Proteins present in 300 μl of each fraction were precipitated with trichloroacetic acid and cold acetone according to standard procedures. Western blot analysis of each fraction was performed as previously explained. The diffraction index was measured for each fraction and the respective densities were then calculated from the indices

2.10. Protease protection assay

Protein protection assays were performed using a method previously described by Igoillo-Esteve et al. [52].

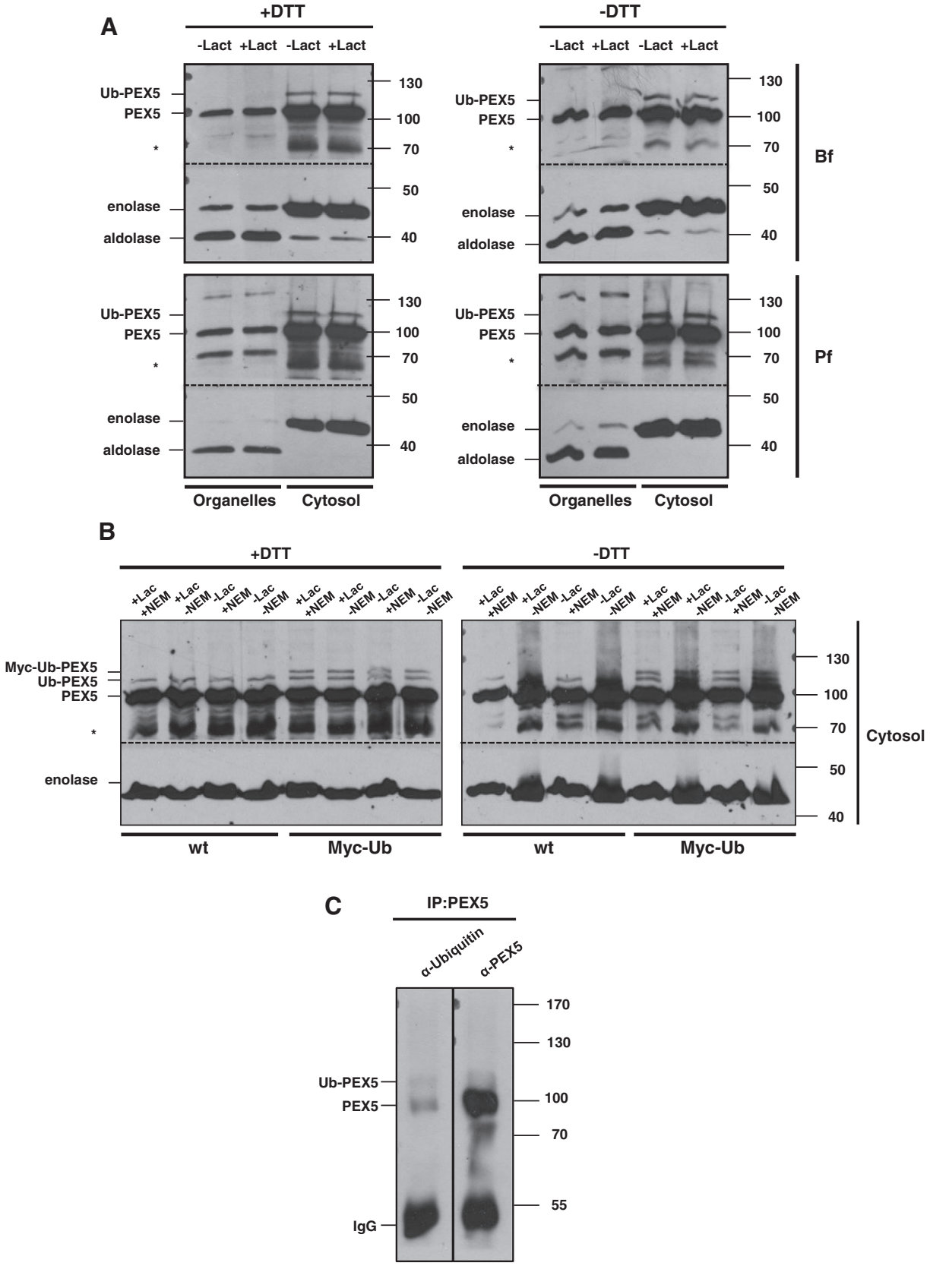
2.11. Proteasome inhibition and cellular fractionation

The following exponentially growing *T. brucei* cells were treated for 6 h with the proteasome inhibitor lactacystin (Sigma-Aldrich Corporation) at a concentration of 1 μM, that has proved to be effective in trypanosomes by Böhme and Cross [53]: wild-type bloodstream (5×10^5 cells/ml) (100 ml) and procyclic (5×10^6 cells/ml) forms, the procyclic Myc-Ub cell line as well as the procyclic Δ*PEX4* cell line (each 50 ml). Subsequently, the cells were centrifuged at 700 ×*g* in an HS-4 Sorvall rotor and washed once in STE buffer. Cell pellets were resuspended in a minimal volume of STE buffer (300 μl) and the protein content in each cell suspension was quantified by the Bradford method. Aliquots of cells containing 300 μg of protein were mixed with HBSS-IP-NEM [Hank's buffered salt solution supplemented with a cocktail of protease inhibitors (Fermentas, Inc.) and 20 mM of N-ethylmaleimide (NEM)] to adjust each volume to 300 μl. Cells were treated with 0.1 mg digitonin/mg of protein for 4 min at room temperature and immediately centrifuged at 20,000 ×*g* in a T15A61 VWR rotor for 2 min at 4 °C. 280 μl of the supernatant (cytosolic fraction) was taken. The organellar pellet was washed once in HSSB-IP-NEM buffer, centrifuged at 20,000 ×*g* for 2 min at 4 °C and finally resuspended in 150 μl of HSSB-IP-NEM buffer. Supernatant (135 μl) and pellet samples (67.5 μl) were diluted for SDS-PAGE in 4× Laemmli buffer containing

or not DTT (25 mM). Samples containing DTT were boiled at 100 °C for 5 min, while samples without DTT were heated at 56 °C for 10 min. The proteins in 60 μl of the preparations were resolved by SDS-PAGE and analysed by Western blotting.

2.12. Scanning electron microscopy

Trypanosomes were allowed to sediment on poly-L-lysine coated coverslips for 30 min. The samples were subsequently rinsed three



times in PBS-Ca/Mg (PBS, pH 7.0, with 3.6 mM $CaCl_2$ and 3.0 mM $MgCl_2$) at room temperature and fixed in successively 0.1 M cacodylate buffer with 0.1% glutaraldehyde for 30 min, 1% glutaraldehyde for 30 min and finally 2% glutaraldehyde overnight. After three wash steps in 0.1 M cacodylate buffer, post-fixation was performed with 1% OsO_4 in cacodylate buffer for 4 h. Subsequently, the samples were washed extensively in this buffer for 30 min, then 6 × 10 min in water and finally dehydrated by overnight incubation in 70% ethanol. The next day samples were further dehydrated in a graded series of ethanol, critical point dried, mounted and sputter coated with a 10 nm gold layer. Samples were then examined in a CM12 electron microscope (Philips) at 80 kV by use of the secondary electron detector (scanning mode). The cell size was estimated by the calculation of the cell body projected area of 30 cells from scanned images of the wild-type and Δ*PEX4* cells using the AxioVision software (4.8.1; Zeiss, Germany). Values obtained were analysed by a "Kolmogorov–Smirnov two sample statistical test".

3. Results

3.1. PEX5 is ubiquitinated in wild-type bloodstream and procyclic-form T. brucei

To explore the possibility that PEX5 in *T. brucei* is ubiquitinated, like its counterparts in yeast and mammalian cells, Western blot analysis of wild-type bloodstream- and procyclic-form cells was performed. In addition, some of the cells were, prior to this analysis, treated with the proteasome inhibitor lactacystin. The purpose of this treatment was to investigate if inhibition of proteasome activity would lead to accumulation of polyubiquitinated forms of TbPEX5, as a response to failure of the quality-control system involved in the disposal of non-recycled molecules of PEX5, as occurs in yeast cells. Western blot analysis showed that the 100 kDa native form of TbPEX5 was highly abundant in the cytosolic fraction (defined by the cytosolic marker enolase), while a minor part was associated with the organellar fraction containing the glycosomal marker aldolase (Fig. 1A). Note the presence of a 70 kDa TbPEX5 species, that has previously been identified in our laboratory as a proteolytically processed form [54]. Importantly, in the conditions tested, the TbPEX5 antiserum also detected a slower migrating form of about 110 kDa exclusively in the cytosolic fraction of both bloodstream and procyclic cells. Based on its molecular weight, we hypothesise that this newly detected TbPEX5 species could represent a monoubiquitinated form of the receptor. Surprisingly, this additional band was absent from the organellar fraction. It should be mentioned that a 130 kDa band was also detected by TbPEX5 antiserum in the organellar fraction, which at face value might be interpreted as a polyubiquitinated form of TbPEX5. However, later experiments (discussed below) showed that the detection of this band should most likely be attributed to cross-reactivity of the antiserum with a unidentified, unrelated protein. The treatment with lactacystin did not affect the abundance of the additional cytosolic 110 kDa TbPEX5 species, indicating no role of proteasome inhibition in the stabilisation of this modified TbPEX5 form. The intensity of this new band was not affected either by the presence of DTT, at concentrations up to 25 mM, suggesting that the ubiquitin is attached to a lysine residue. The sequences of the N-terminal half of PEX5s are highly variable (Supplementary data, Fig. S1), but importantly in all mammalian and yeast species they contain a cysteine residue that, under physiological conditions, is a target of monoubiquitination. TbPEX5 has also a cysteine (Cys3) in a similar region as its counterparts in other species. Thus, one might expect that, if TbPEX5 monoubiquitination occurs, it would involve this cysteine residue. Future investigation should provide a definitive assignment of the TbPEX5 monoubiquitination at Cys3 or a Lys residue.

As a strategy to prove the ubiquitinated nature of the newly detected form of TbPEX5, a procyclic *T. brucei* cell line was created that expresses an N-terminally 2×-myc-tagged-ubiquitin protein (with a predicted additional molecular weight of 12.5 kDa) under the control of a tetracycline-inducible promoter. The wild-type and myc-tagged ubiquitin-expressing procyclic cell lines were treated with lactacystin for 6 h, followed by a cell fractionation performed similarly as described above, but in the presence or absence of the nonspecific sulfhydryl alkylating agent NEM that is known to inhibit many Cys-containing enzymes. Since NEM also affects ubiquitin-dependent isopeptidases, we aimed to see in this manner if inhibition of deubiquitinating (DUB) enzymes affects the presence of the additionally detected TbPEX5 forms. Inhibition of DUB enzymes by high concentrations of NEM is a standard method previously used to study monoubiquitination of PEX5 in cells from different organisms [39,55] and the PEX7 co-receptors PEX18 and PEX20 in yeasts [33,38]. Western blot analysis showed in the myc-ubiquitin expressing procyclic cells a new, slower migrating form of about 115 kDa, detectable by the TbPEX5 antiserum, present in the cytosolic fractions, in addition to the 110 kDa form also detected in the wild-type cells (Fig. 1B). By including the proteasome inhibitor lactacystin, we confirmed once more that addition of this drug did not lead to stabilisation of the slower migrating forms of TbPEX5 in the wild-type and myc-ubiquitin expressing cells, suggesting that these forms correspond to stable species of TbPEX5 present in the cell. Importantly, this figure shows that the presence or absence of NEM during fractionation did not lead to a change in the abundance of the slower migrating forms of TbPEX5, proving that inhibition of deubiquitinating enzymes is not required for the detection of these modified PEX5 forms in trypanosomes, different from the situation in other organisms.

Additionally, it should be mentioned that the 130 kDa band, exclusively observed in the organellar fraction (Fig. 1), was not affected by the expression of myc-ubiquitin (not shown), allowing us to discard this band as a polyubiquitinated form of PEX5 and rather attribute it to non-specific binding of the TbPEX5 antiserum to another protein.

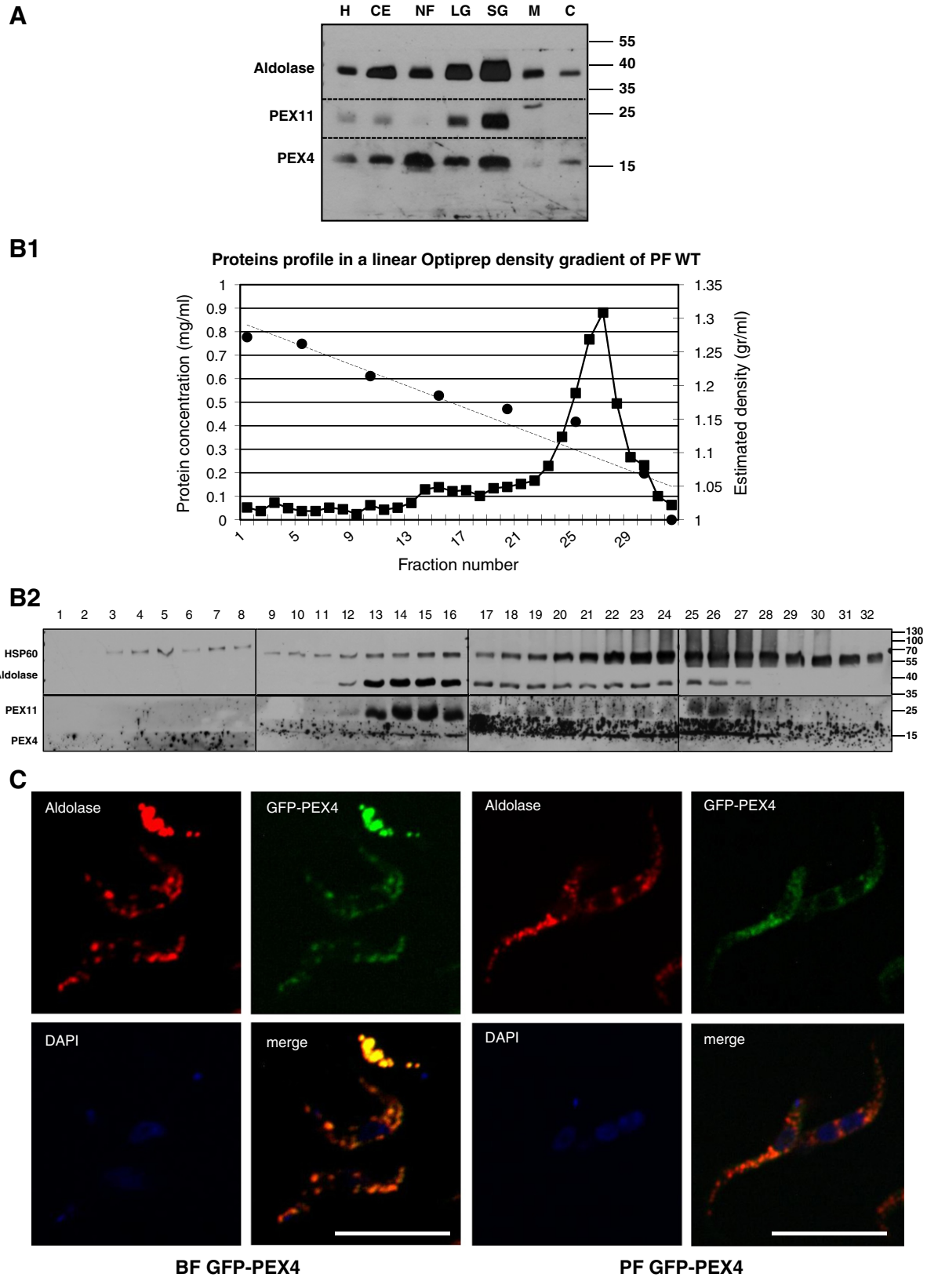
As further proof that TbPEX5 is ubiquitinated we have immunoprecipitated this receptor from wild-type procyclic cells and performed Western blot analysis using commercially available antibodies against human ubiquitin. Fig. 1C shows that the eluted fraction after immunoprecipitation of TbPEX5 contains the native form (100 kDa) as well as the slower migrating form of 110 kDa detectable with anti-PEX5, although the latter was only detected as a very faint band. When this sample was analysed with human ubiquitin antibodies, the TbPEX5 band of 110 kDa was detected. It should be noted that

Fig. 1. (A). A slower migrating TbPEX5 form is present in *T. brucei* wild-type cells. Wild-type bloodstream-form (Bf) and procyclic-form (Pf) cells were cultured in the presence of the proteasome inhibitor lactacystin for 6 h. Cytosolic and organellar fractions were prepared after treatment of cells with 0.1 mg of digitonin/mg of protein in the presence of 20 mM NEM followed by centrifugation. Protein samples were prepared in Laemmli buffer containing DTT or not and SDS-PAGE followed by Western blot was performed. A slower migrating form of TbPEX5 (Ub-PEX5) was exclusively detected in cytosolic fractions, irrespective of the presence of DTT. The * indicates an in vitro proteolytically processed form of TbPEX5. Enolase and aldolase were used as markers for cytosolic and organellar fractions respectively. (B). A second slower migrating TbPEX5 form is detected upon expression of myc-ubiquitin. Trypanosomes of wild-type procyclic-form (PF) and a PF Myc-Ub cell line expressing 2×-myc-ubiquitin were treated as in panel A, but in the presence or absence of NEM. A slower migrating band was detected by the TbPEX5 antiserum; it corresponds to Myc-Ub-TbPEX5 and is exclusively found in cytosolic fractions. Both slower migrating forms of TbPEX5 were lactacystin/NEM independent. The * indicates an in vitro proteolytically processed form of TbPEX5. Note that sample migration was somewhat disturbed in lines 2, 4, 6 and 8 of the −DTT panel probably due to incomplete denaturation of proteins. (C). The slower migrating form of TbPEX5 detected in wild-type cells corresponds to ubiquitin-TbPEX5. TbPEX5 was immunoprecipitated from *T. brucei* wild-type procyclic-form cells. Western blot analysis was performed with the elution fraction using antisera against human ubiquitin and TbPEX5. The slower migrating form of TbPEX5 was detected by the anti-ubiquitin antiserum proving that TbPEX5 is ubiquitinated in wild-type cells. The horizontal dashed lines in panels A and B indicate where the blots were cut to incubate the different parts separately with the respective antisera.

a band at approximately the same molecular weight as the native TbPEX5 was also detected with the human ubiquitin antiserum. However, it is likely that this should be attributed to a non-specific reaction of the antiserum due to the very large amount of TbPEX5 protein present in the elution fraction.

3.2. Identification of TbPEX4 and TbPEX22

To study in more detail the mechanism of TbPEX5 ubiquitination during glycosomal matrix protein import in *T. brucei*, we looked for an orthologue of the ubiquitin-conjugating enzyme PEX4 and its possible



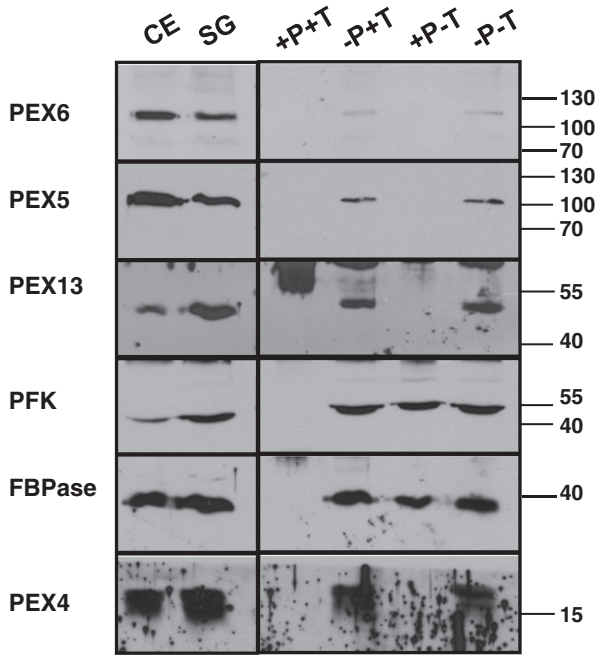


Fig. 3. TbPEX4 is associated with glycosomes at their cytosolic face. Intact glycosomes (latency of 95%) present in a small granular fraction obtained by differential centrifugation were used to perform a protease protection assay. CE: cellular extract, SG: small granular, +P/+T: proteinase K/Triton X-100, −P/+T: no-proteinase K/Triton X-100, +P/−T: proteinase K/no-Triton X-100, −P/−T: no-proteinase K/no-Triton X-100. TbPEX4 was shown to be susceptible to proteinase K proteolysis when glycosomes were intact, similarly as the AAA^{+}-ATPase TbPEX6, TbPEX13 (SH3 domain), but in contrast to the glycosomal matrix proteins FBPase and PFK which were protected under that condition. Notice that the blot was cut to incubate the different parts separately with the respective antisera.

membrane-anchoring protein, PEX22. By homology searches we have identified a likely candidate for each of these two peroxins in the *T. brucei* genome database. The corresponding genes were cloned, sequenced and expressed as recombinant proteins in *E. coli*. This work and detailed in silico analyses of the sequences, as well as structure modelling and yeast two-hybrid studies supporting the identity of these proteins as the respective peroxins and their interaction, are presented in the Supplementary data file, with Figs. S2 to S6.

3.3. TbPEX4 is an ubiquitin-conjugating enzyme present at the cytosolic face of the glycosomal membrane

The localisation of the likely TbPEX4 candidate was initially analysed in subcellular fractions of wild-type bloodstream and procyclic-form cells prepared by differential centrifugation. The matrix protein aldolase and membrane protein TbPEX11 were used as glycosomal markers. A representative fractionation experiment performed with bloodstream-form cells is shown in Fig. 2A. Aldolase and TbPEX11 are, as characteristic for glycosomal proteins, mostly enriched in the small granular fraction and to a lesser extent in the large granular fraction. The putative TbPEX4 protein was found, as expected, in the homogenate and post-nuclear fraction, while after fractionation its distribution profile corresponds, for a major part, with a glycosomal localisation. The presence of a relatively large amount of PEX4, and to a lesser extent aldolase, in the nuclear fraction compared to PEX11 is considered an artefact (see the Discussion section). Additional evidence for an association of the putative TbPEX4 with glycosomes was obtained by equilibrium centrifugation of procyclic (Fig. 2B; fractions 13–16) and bloodstream-form trypanosomes (data not shown) using a linear Optiprep density gradient. It is important to note that such isopycnic centrifugation of peroxisomes and glycosomes leads unavoidably to the formation of so-called "peroxisomal/glycosomal ghosts" which are formed as a result of osmotic effects causing breakage of the organelles followed by resealing or leakage of matrix content during the cell fractionation, generating less dense vesicles which co-migrate with lighter fractions in the gradient [56,57]. This phenomenon was evidenced by the presence of a minor amount of aldolase and TbPEX11, together with TbPEX4, in other fractions of the gradient, notably 23–28 (Fig. 2B).

As an alternative approach, the localisation of a GFP–TbPEX4 fusion protein in bloodstream and procyclic wild-type cells was studied by confocal immunofluorescence microscopy. Stable cell lines were created for the tetracycline-inducible expression of an N-terminally GFP-tagged fusion protein as described in the Materials and methods section. After induction of GFP-TbPEX4 expression, the subcellular localisation of the fluorescence signal was determined. Fig. 2C shows that the GFP-TbPEX4 protein colocalised with the glycosomal matrix marker aldolase, confirming once more its glycosomal localisation.

Subsequently, we determined whether the protein is located in the matrix or associated with the glycosomal membrane at its cytosolic face. To that end, protease protection assays were performed on purified glycosomes obtained by differential centrifugation. Fig. 3 shows the results obtained with glycosomes from bloodstream-form *T. brucei*; the same analysis was done with glycosomes from procyclic cells with similar results. TbPEX4 was efficiently degraded by proteinase K irrespective of whether the glycosomal membrane was permeabilised with Triton X-100 or not. This susceptibility indicates that TbPEX4 is associated with the side of the glycosomal membrane facing the cytosol, as was also observed for TbPEX5, TbPEX6 and the C-terminal part (containing the SH3 domain) of TbPEX13 that are known to be exposed to the cytosol. The analysis of glycosomal matrix proteins such as fructose-1,6-bisphosphatase (FBPase) and phosphofructokinase (PFK) shows that glycosomes remained intact during the proteolytic treatment. Other matrix markers such as aldolase and hexokinase showed a more intriguing behaviour. Both enzymes seemed to be slightly sensitive to proteinase K, resulting in a specific, short truncation of only a fraction of these proteins. In addition, it was observed that such minor proteolysis occurred also in the absence of the detergent, when the glycosomal membrane was supposedly intact (data not shown). Since the glycosome-rich fraction used in this experiment had a high latency (based on hexokinase activity measurement) and both FBPase and PFK were efficiently protected against the proteinase K degradation in the absence of Triton X-100, we feel confident about the integrity of the organelles during the assay. Taking all these data together, we conclude that TbPEX4 is a glycosomal protein associated to the cytosolic face of the glycosomal membrane. Since this protein does not possess any predicted transmembrane region, we speculate that it is attached to the membrane via its interaction with the PEX22 orthologue identified in *T. brucei*, as described in the Supplementary data file.

Fig. 2. *T. brucei* PEX4 is associated with glycosomes. (A). TbPEX4 is present in a small granular fraction enriched in glycosomal proteins. *T. brucei* bloodstream-form cells were fractionated by differential centrifugation and the fractions analysed by Western blotting. H: homogenate, CE: cellular extract, NF: nuclear fraction, LG: large granular fraction, SG: small granular fraction, M: microsomes, C: cytosol. TbPEX4 was identified in the small granular fraction enriched in the glycosomal matrix and membrane markers aldolase and TbPEX11. Notice that differential centrifugation is an enrichment procedure that still leaves some glycosomal protein aldolase and a considerable amount of TbPEX4 in the NF. The horizontal dashed lines indicate where the blots were cut to incubate the different parts separately with the respective antisera. (B). TbPEX4 is present in glycosome-rich fractions of an isopycnic density gradient. Glycosomes from *T. brucei* procyclic forms (PF) were purified by isopycnic centrifugation using the commercial Optiprep medium. B1. Protein profile in the linear density gradient. B2. Western blot analysis was performed on the collected fractions. Antisera directed against the glycosomal matrix marker aldolase, glycosomal membrane marker TbPEX11, as well as TbPEX4 were used. Additional organelles like the mitochondria were examined with antibodies against TbHSP60. Notice that the blot was cut to incubate the different parts separately with the respective antisera. (C). GFP-TbPEX4 is present in *T. brucei* glycosomes. An N-terminal GFP-TbPEX4 fusion protein was expressed in *T. brucei* bloodstream- (BF) and procyclic-form (PF) cells. Confocal immunomicroscopy was performed using aldolase as glycosomal marker. GFP-TbPEX4 was shown to colocalise with aldolase suggesting its association with glycosomes. Scale bar: 10 μm.

3.4. TbPEX4 double knockout

To address the molecular function of TbPEX4 we have tried as an initial approach an RNAi-based strategy. However, several attempts each yielded only clones exhibiting a short, transient reduction of TbPEX4 levels and no growth phenotype. It should be mentioned that in an RNAi-based high-throughput screening of *T. brucei* bloodstream and procyclic forms, it was also found that targeting the mRNA corresponding to TbPEX4 gene (Tb927.8.920) did not cause an effect on growth [58]. After our unsuccessful RNAi approach, we embarked on a knockout strategy. In the case of bloodstream-form cells, we never succeeded in obtaining clones in which both *TbPEX4* alleles were replaced since no cell growth was observed after the second round of selection with antibiotics. We also tried to create *TbPEX4* conditional knockout bloodstream-form cells. Unfortunately, clones positive for the three marker antibiotics, when analysed by Western blot in the absence of tetracycline, appeared to still express the TbPEX4 protein (data not shown). Apparently, the second allele had not been efficiently replaced, because gene duplication must have occurred. We infer from this fact that TbPEX4 might be an important enzyme in the bloodstream form, causing the cell to adjust the gene copy number when losing the *PEX4* gene alleles, similarly as has been observed previously for other genes in trypanosomes (e.g. [59]). In the case of the procyclic form, several clones were shown to have lost the *TbPEX4* gene and to have acquired the respective antibiotic resistance genes. This was proved by genomic PCR, RT-PCR and Western blot. Fig. 4 shows the analysis performed with the clone used for the phenotypic characterisation described below.

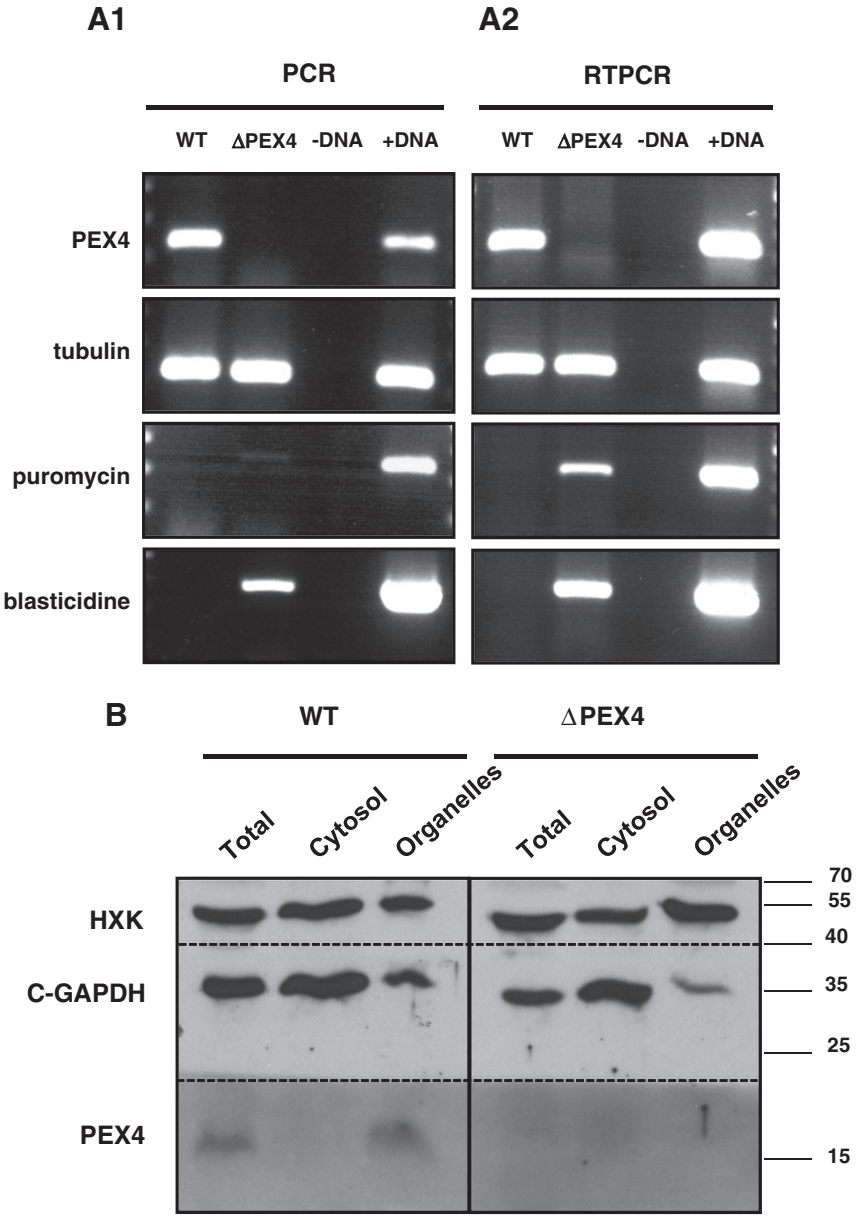


Fig. 4. Validation of the Δ*PEX4* procyclic *T. brucei* cell line. Both alleles of the TbPEX4 gene were successfully replaced by the blasticidin and puromycin resistance genes. (A1). PCR amplification of TbPEX4 and antibiotic resistance genes (Puro and Bla) from genomic DNA of the wild-type (WT) and the *ΔPEX4* cell line. (For further explanations, see the Materials and methods section). (A2). RT-PCR analysis of cDNA prepared from transcripts of TbPEX4 and antibiotic resistance genes of the wild-type and the *ΔPEX4* cell line. (For further explanations, see the Materials and methods section). (B). Western blot analysis of the TbPEX4 protein in cytosolic and organellar fractions of wild-type cells and the *ΔPEX4* cell line. Cytosolic marker: C-GAPDH, dual cytosolic and glycosomal marker: HXK. Note that the antiserum used for TbPEX4 is an affinity purified-peptide based antiserum that causes the high background observed. The horizontal dashed lines indicate where the blots were cut to incubate the different parts separately with the respective antisera. The three approaches confirm the effective knockout of both alleles of the TbPEX4 gene and that the encoded protein is absent in the *ΔPEX4* cell line.

3.5. Absence of TbPEX4 leads to a partial mislocalisation of glycosomal PTS1-containing matrix proteins and to a mild cell growth defect

A phenotypic characterisation of the *TbPEX4* double gene knockout procyclic cells was performed involving analyses of growth behaviour, glycosomal matrix protein import and the ubiquitination status of TbPEX5. The Δ*PEX4* cells presented a decreased growth rate when compared with wild-type cells (Fig. 5A). The 2-fold decrease, which was consistently found in independent experiments, seems not to be very pronounced, taking into account the essentiality of glycosome biogenesis for the viability of the cells as shown in many previous studies (reviewed by [9,10]). Very interestingly, we observed by phase contrast microscopy unexpected features with regard to the morphology and motility of the Δ*PEX4* cells. These features concerned a reduced cell size and a slower displacement of the cells in culture. In order to gain insight into this morphological defect, scanning electron microscopy of the Δ*PEX4* cells was performed. A large part of the cell population presented an aberrant cell shape with a shorter and wider cell body compared to the characteristic elongated-thin shape of normal procyclic trypanosomes (Fig. 5B). To quantify this morphology defect, the projected area of the cell body was determined for 30 cells of each sample. The results are presented in Table S2. The mean area of projections of Δ*PEX4* cells was only 69% of that of wild-type cells. This cell size difference was shown to be extremely significant ($p < 0.001$) upon applying a "Kolmogorov–Smirnov Two Sample Statistical Test". In an attempt to obtain, at least by rough approximation, evidence for the defect in the motility of the Δ*PEX4* cells, their displacement in culture was measured. We performed time-lapse image capturing of procyclic wild-type and Δ*PEX4* cells in culture and analysed the changes in the position of individual random cells. Table S3 presents the measured distance (μm) and velocity (μm/s). It was interesting to see a great variability in the speed between cells of the wild-type culture, indicating that, in general, procyclic trypanosomes are able to swim in liquid cultures both slow and fast. Nevertheless, we found that a higher proportion of cells in the Δ*PEX4* culture had a lower motility, making effective displacement less frequent than observed for wild-type cells. Note that transmission electron microscopy has been used previously to study mutant *T. brucei* cell lines in which other peroxins involved in glycosomal matrix protein

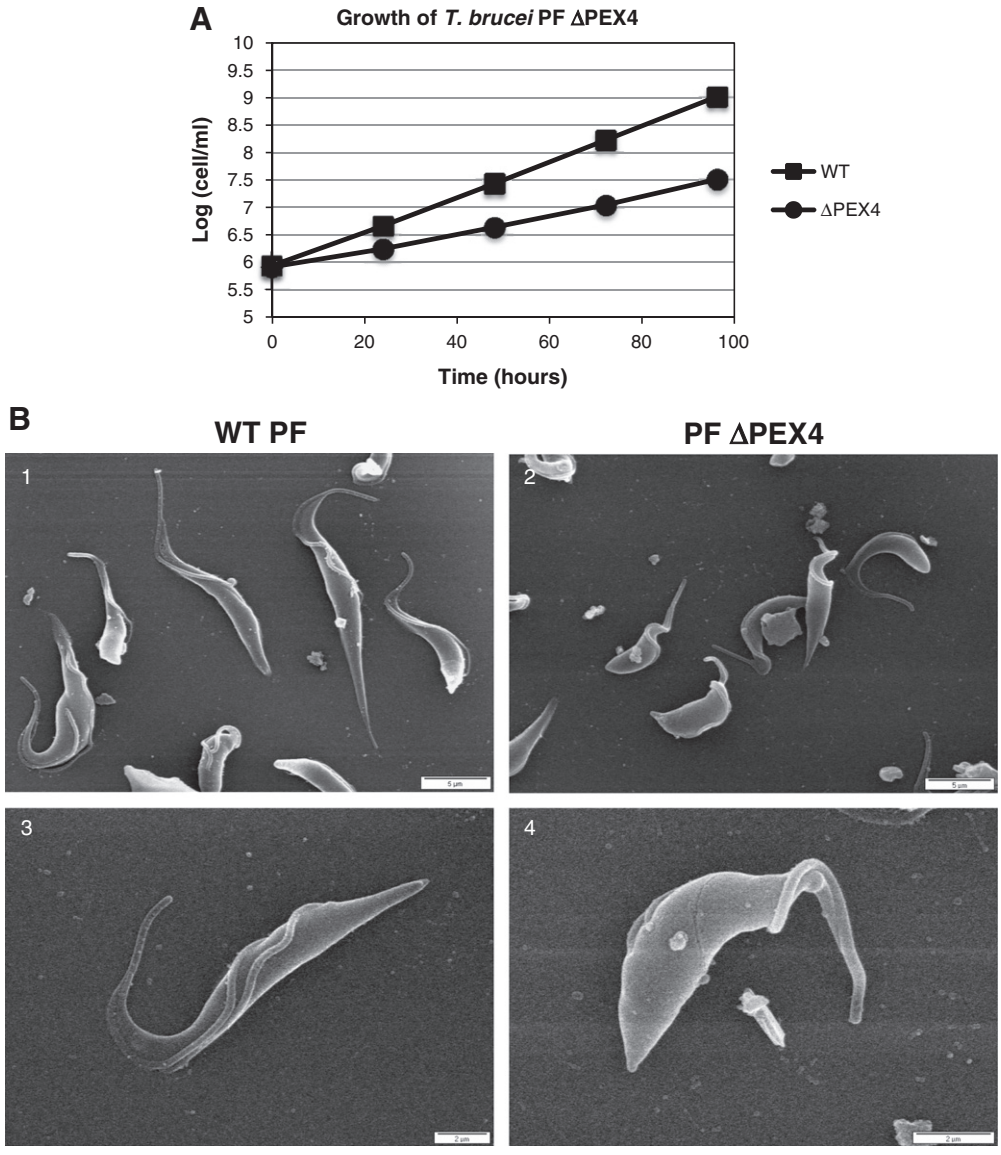


Fig. 5. Growth behaviour and cellular morphology is affected in the Δ*PEX4* cell line. (A). Growth curve of wild-type and *ΔPEX4* procyclic cells. Cell growth was followed during 5 days in SDM79 medium. *ΔPEX4* cells were shown to have a lower growth rate (by a factor 2) compared to wild-type cells. (B). Scanning electron-microscopy of *T. brucei* wild-type and *ΔPEX4* procyclic-form cells. 100% of the wild-type cells presented a typical elongated thin shape (panels 1 and 3). Procyclic *ΔPEX4* trypanosomes formed a heterogeneous population in which approximately 85% of the cells (panels 2 and 4) presented an aberrant cell-shape (shorter cell body and reduced cellular size). About 15% of the trypanosomes displayed an apparent normal morphology (not shown). Cells in panels 3 and 4 show two flagella indicating cell division.

import were depleted, notably PEX5 and PEX7 [49]. Morphological changes were also observed in these RNAi mutants: an altered shape, apoptotic bodies and lysed cells. However, no specific comparison of this phenotype with that observed for the Δ*PEX4* cells can be made with the currently available data due to the different microscopy techniques used.

To determine if glycosomal protein import was affected in the Δ*PEX4* cells wild-type and Δ*PEX4* cell lines were created that express GFP with the C-terminal PTS1 motif SKL (GFP-PTS1) under the control of a tetracycline-inducible promoter. The correct glycosomal localisation of the expressed GFP-PTS1 protein was then compared with the endogenous glycosomal enzyme gGAPDH (a PTS1 containing protein) by confocal fluorescence immunomicroscopy. Fig. 6 shows a representative image of the results obtained. In wild-type as well as in Δ*PEX4* cells, the colocalisation of GFP positive signals with the immunostained gGAPDH in puncta is evident, confirming its glycosomal localisation and validating our system for studying glycosomal protein import using GFP-PTS1. In addition to the fluorescent glycosomes containing the GFP-PTS1 protein, a fluorescent shadow-like signal throughout the cell body was also observed in the Δ*PEX4* cells, but which was absent in wild-type trypanosomes. Although this cytosolic staining was not prominent, it might represent a partial mislocalisation, indicative of a minor defect

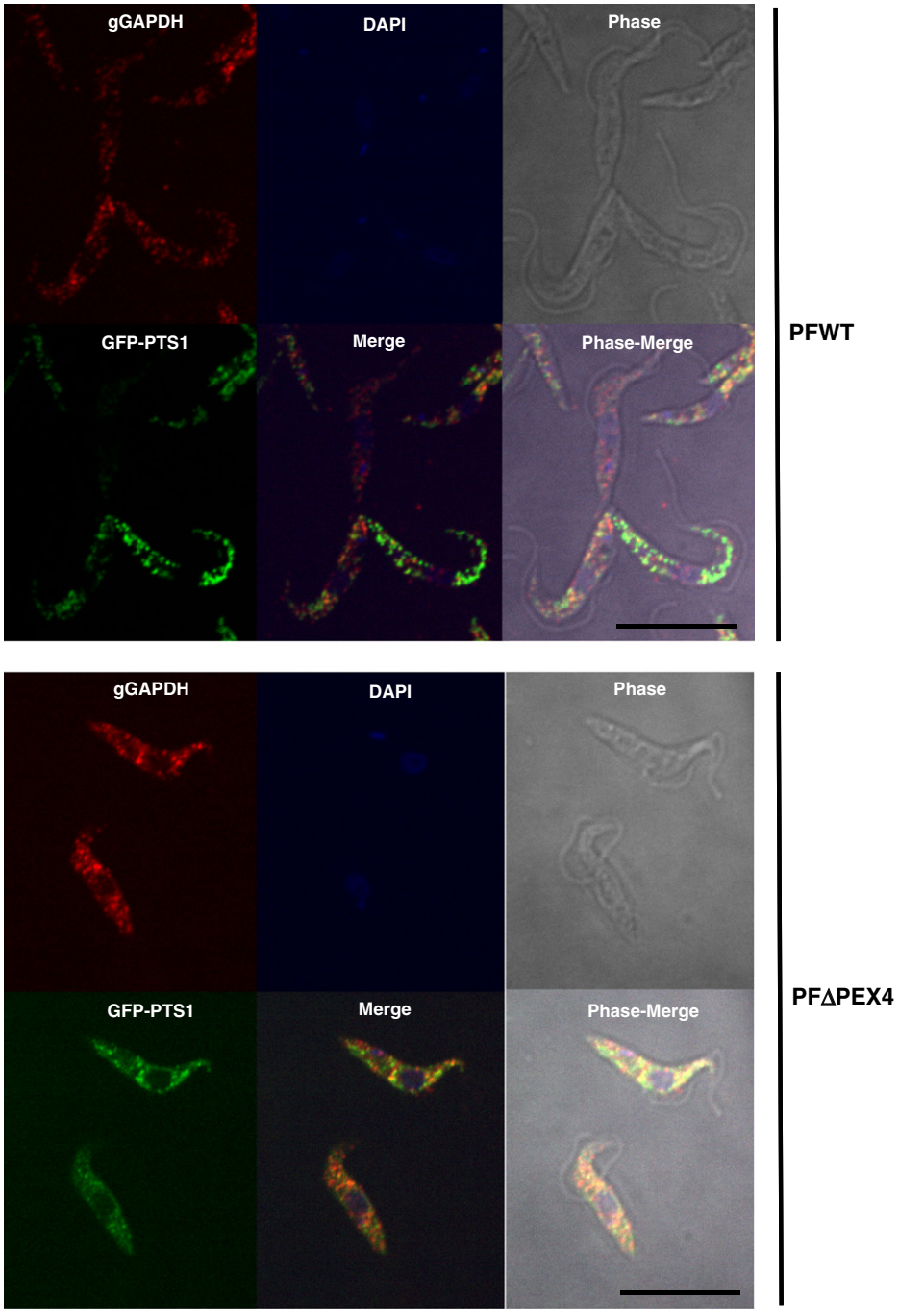


Fig. 6. PTS1 proteins are partially mislocalised in the Δ*PEX4* cell line. Wild-type and Δ*PEX4* GFP-PTS1 expressing cells were fixed, permeabilised and immunolabelled with an antiserum for gGAPDH (a PTS1 containing protein). The colocalisation of the GFP-PTS1 protein with the glycosomal matrix marker was studied by confocal fluorescence microscopy. It was found that, GFP-PTS1 colocalised perfectly with the glycosomal marker gGAPDH in wild-type cells, validating the use of GFP-PTS1 expression as a new means for studying glycosomal protein import in *T. brucei*. Although in the Δ*PEX4* cells all gGAPDH-stained glycosomes also contain GFP-PTS1, a partial mislocalisation to the cytosol was observed as well. Scale bar 10 μm.

of glycosomal protein import. Similar results were found when comparing the GFP-PTS1 localisation with the glycolytic enzyme TPI (not shown).

In spite of the good colocalisation of the two PTS1 containing proteins, a very intriguing observation came from images of the wild-type cells; some cells presented only the punctate red pattern coming from the immunostaining of gGAPDH but not the green fluorescence by the expressed GFP-PTS1 protein. More striking was the fact that some cells presented, besides glycosomes in which both signals merged, also puncta in which either one or the other fluorescent label was present (Fig. 6). This observation might suggest that the population of glycosomes within a single trypanosome is heterogeneous with regard to their protein content, a notion that deserves future study.

To determine, at the molecular level, if the Δ*PEX4* cell line was affected in its TbPEX5 recycling, the receptor's ubiquitination was analysed using the same strategy as established for the study of this modification in wild-type cells (see above and Fig. 1). Fig. 7 shows that, in the Δ*PEX4* cell line, TbPEX5 is an equally abundant cytosolic protein as in wild-type cells and in cells expressing myc-ubiquitin. No significant changes were observed in the steady-state levels of the protein in the cytosol or in the organellar fraction of the mutant cells when compared to wild-type and myc-ubiquitin cells, indicating no important accumulation in the glycosomal membrane. Importantly, the intensity of the slower migrating form of TbPEX5 in wild-type cells, which corresponds to the ubiquitinated form as demonstrated above, was considerably decreased in the cytosol of the Δ*PEX4* cell line to barely detectable levels and did not show accumulation in the organellar fraction. This result clearly shows that the protein identified as TbPEX4 plays a role in this form of TbPEX5 ubiquitination. In several yeast species, the absence of PEX4 leads to the polyubiquitination of PEX5 at the organellar fractions, an event that occurs in a UBC4-dependent manner [35–37,60]. Our results show that in *T. brucei* the deletion of PEX4 did not cause the accumulation of such higher molecular weight forms that could correspond to polyubiquitinated PEX5 (Fig. 7). The abundance of the ubiquitinated form of TbPEX5 was not affected by the presence of DTT in the protein preparation. However, as mentioned before, further investigation will be required to obtain unambiguous proof for a lysine-linked ubiquitination.

The fact that in the complete absence of TbPEX4, TbPEX5 ubiquitination was strongly impaired but not totally abolished and that the glycosomal protein import process and cell growth were only mildly affected, raised the question whether there could be a redundancy in the cellular ubiquitination machinery that still allowed TbPEX5 recycling to occur in Δ*PEX4* cells. To investigate this possibility, the mRNA levels of other putative ubiquitin-conjugating (UBC) proteins were analysed by qPCR in wild-type and Δ*PEX4* procyclic cells. From the 15 UBC genes identified in the *T. brucei* genome database, 10 were selected for these qPCR studies, based on their having the highest degree of sequence similarity and most similar predicted molecular weight to TbPEX4 (Table S4). The results obtained showed consistently that four different putative UBC mRNAs were overexpressed, in a statistically significant manner, in the Δ*PEX4* cell line (Fig. 8). Interestingly, two of them have been predicted to possess a PTS1 motif [61], implying that a glycosomal association is likely, although within the matrix. These UBCs might be candidates for at least partially functionally replacing

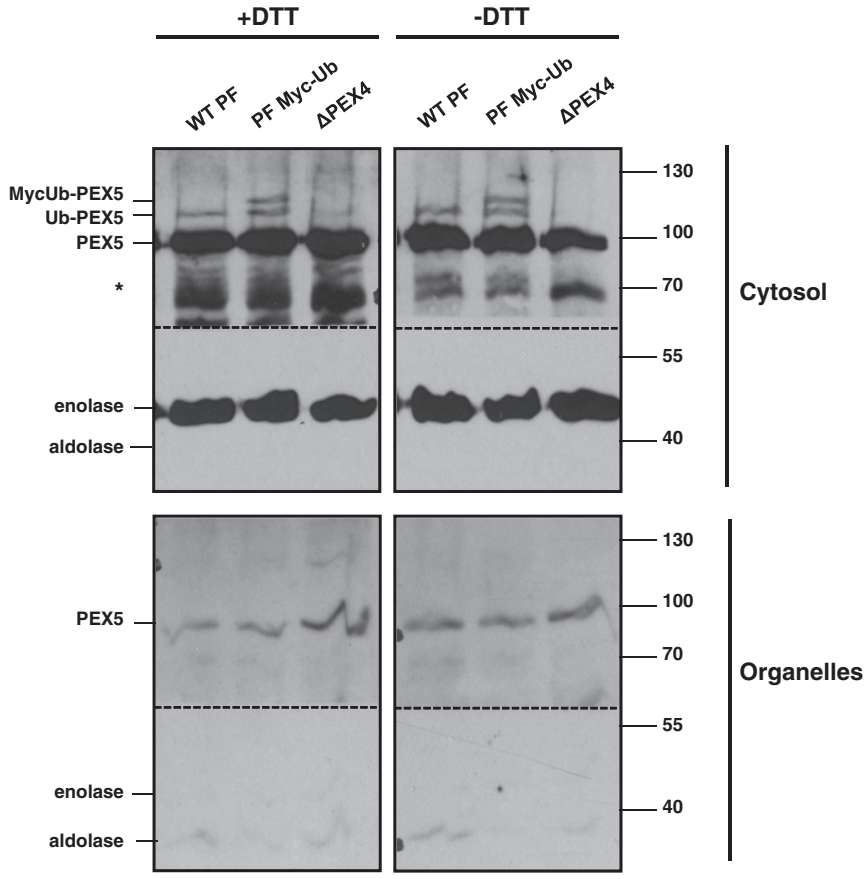


Fig. 7. TbPEX5 ubiquitination is impaired in the Δ*PEX4* cell line. Cytosolic and organellar fractions were obtained by selective permeabilisation of the plasma membrane with a low concentration of digitonin in the presence of 20 mM NEM. Western blot analysis shows an important decrease in the level of the Ub-TbPEX5 form in the Δ*PEX4* cell line, indicating that TbPEX4 functions as a ubiquitin-conjugating enzyme involved in TbPEX5 ubiquitination in wild-type cells. No important changes in the steady-state levels of TbPEX5 were observed in the cytosolic neither in the organellar fraction. The * indicates an in vitro proteolytically processed form of TbPEX5. The horizontal dashed lines indicate where the blots were cut to incubate the different parts separately with the respective antisera.

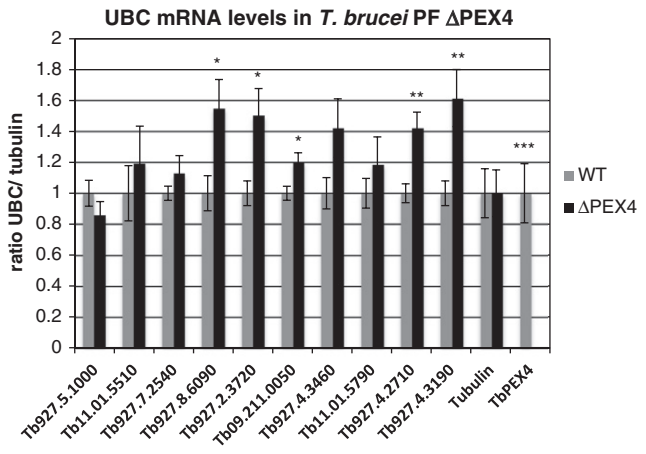


Fig. 8. Expression levels of genes coding for putative *T. brucei* ubiquitin-conjugating (UBC) enzymes in the Δ*PEX4* cell line. Total RNA from three independent biological samples was extracted and used to synthesise cDNA. qPCR analysis of wild-type cells and the *ΔPEX4* cell line was performed on three technical replicates and mRNA expression levels of 10 putative UBC proteins were compared. mRNA abundance was normalised to tubulin and data were analysed for statistical relevance via a Student's *t*-test (homoscedastic with 2 tail distribution): $^{*}p < 0.1$, $^{**}p < 0.01$, $^{***}p < 0.001$. Error bars represent the relative standard error.

PEX4 in the knockout cells. In order to gain an insight into their possible authentic function, a preliminary reciprocal BLAST analysis was performed for those UBC enzymes that are overexpressed in the *T. brucei* Δ*PEX4* cell line, querying them against the *S. cerevisiae* and human genomes. The results of this analysis are presented in Table S4. The yeast and/or human orthologues of some of these overexpressed UBC enzymes have a function in cell-cycle regulation. As will be argued in the Discussion section, it is conceivable that increased expression of one or several of these proteins is responsible for partially rescuing the phenotype of the *PEX4* knockout cells, while also causing the additional phenotypic features. Although this issue is beyond the scope of the TbPEX5 ubiquitination analysis presented in this paper, the intriguing, suggestive results warrant further investigation of the rescue possibility in the future.

All these data together confirm that both alleles of the TbPEX4 gene were successfully knocked out in procyclic cells. We hypothesise that the conditions established for preparing the knockout in this cell line have triggered compensatory/adaptive mechanisms in which the expression of other UBC enzymes was significantly upregulated above wild-type levels. This has allowed, at least partially, the rescue of glycosomal protein import and the survival of the cells, but could also have been responsible for the aberrant phenotype by affecting other cellular processes, thus causing the defects in shape, size and cellular motility.

4. Discussion

In this paper, we have addressed an essential aspect of the glycosomal matrix protein import in *T. brucei*: the recycling of the matrix protein receptor TbPEX5. We have proved that TbPEX5 is a cytosolic protein that also interacts with organellar fractions containing glycosomes. The receptor becomes ubiquitinated, like its fungal and mammalian homologues, indicating that the principle of the mechanism by which it is recycled has been conserved throughout eukaryotic evolution, because trypanosomatids belong to the phylogenetic supergroup of the Excavata, whereas yeasts and mammals are part of the supergroup Opisthokonta. The common origin of these and other supergroups has been traced back to the so-called LECA or last eukaryotic common ancestor which must have possessed already a peroxisome [8].

Despite these common features, the data obtained in our research collectively indicate that the TbPEX5 recycling process exhibits also important differences compared to that of other organisms. Part of TbPEX5 was found to be ubiquitinated in both bloodstream and procyclic wild-type trypanosomes (Fig. 1A). This modification was always detected exclusively in the cytosolic fractions after cellular fractionation. A minor amount of only unmodified native TbPEX5 was detected at steady-state levels in the glycosomal membrane, similar to what has been reported for PEX5 in mammalian cells (only about 10% of total PEX5) [41], implying that in wild-type conditions, the protein is efficiently retrieved to the cytosol after matrix-protein import. The PEX5 ubiquitination machinery of *T. brucei* is, as in other organisms, present at the membrane of the organelle, proving that this modification must occur at this cellular location. However, we did not find conditions allowing for the detection of membrane-bound receptor trapped in the recycling stage with any ubiquitin molecules attached by Western blot analysis of wild-type cells. This might indicate that in *T. brucei* the ubiquitination and subsequent export of TbPEX5 occur as a concerted fast process. For yeast peroxisomes, it has been reported that a minor fraction of the membrane-bound PEX5 is ubiquitinated [27]. The fact that in trypanosomes ubiquitinated PEX5 was only detectable in the cytosol, albeit as a minor fraction of the total TbPEX5, seems to reflect another important difference compared to the *S. cerevisiae* peroxisomal protein import, where an AAA^{+}-ATPase-associated ubiquitin hydrolase is in charge of the de-ubiquitination of the receptor PEX5, immediately after or during its export to the cytosol [55]. In *T. brucei*, we demonstrated that, even in the absence of deubiquitinase inhibitors like NEM, the ubiquitinated forms of TbPEX5 were stable in the cytosol (Fig. 1B). This might indicate that the trypanosomal steady-state process is more similar to that of mammalian cells, where such stable ubiquitinated forms of PEX5 are also found in the cytosol of CHO and Fao cells [40,41]. According to the current model of PEX5 recycling in mammalian cells, the removal of ubiquitin from the receptor occurs in the cytosol and is required to enable new rounds of protein import [62]. Indeed, Grou et al. [63] identified the ubiquitin hydrolase responsible for the deubiquitination of the cysteine-monoubiquitin of mammalian PEX5. We envisage that the TbPEX5 deubiquitination process must be regulated similarly as in mammalian cells.

Another intriguing aspect of the TbPEX5 ubiquitination concerns the residue that is modified by ubiquitin. In yeast and mammalian cells, the signal for PEX5 recycling is the monoubiquitination at a conserved cysteine residue (Cys6 in *S. cerevisiae* and Cys11 in mammals) present in the N-terminal half [29–31]. Our analysis shows that the ubiquitination occurring on TbPEX5 is not sensitive to reducing agents such as DTT at 25 mM (Fig. 1A and B), indicative of a lysine-linked ubiquitin moiety rather than modification at a cysteine. PEX5 in several species of yeast is also a target of lysine-linked ubiquitin chains, but this kind of modification occurs only when recycling is impaired as in PEX1–PEX6 and PEX4–PEX22 deletion mutants and is not considered as a physiological step in the receptor's recycling [35,36]. The lysine-linked PEX5 ubiquitination process in yeast has been associated with polyubiquitination of non-functional receptors acting as a signal for their routing to proteasomal degradation. This occurs as a quality control mechanism in yeast peroxisomal matrix protein import [36]. However, this seems to be unlikely the case for the apparent Lys-linked ubiquitin-PEX5 in *T. brucei*, because i) these molecules, detected in the cytosol, were stable in wild-type cells and did not accumulate after inhibition of the proteasome by the inhibitor lactacystin and ii) the molecular weights of these modified TbPEX5 forms do not correspond to the conjugation of a considerable number of ubiquitin moieties, they rather correspond with mono- or di-ubiquitination (Fig. 1A and B). These two facts suggest that the detected ubiquitinated TbPEX5 is an authentic intermediate in the physiological recycling of the receptor. The cysteine that serves as ubiquitin target in yeast and mammalian PEX5s has an equivalent in the same region of the trypanosomal receptor's primary structure (Fig. S1). Whether it is Cys3 or another residue – Lys or Cys – in TbPEX5 that is modified by ubiquitin requires further investigation by methods like site-directed mutagenesis of the candidate residues.

We have identified the *T. brucei* orthologue of PEX4, the ubiquitin-conjugating enzyme responsible for the monoubiquitination of PEX5 in yeast and plant cells, and proved its function in the ubiquitination of TbPEX5. This protein is associated with glycosomes (Fig. 2), where it is exposed at the cytosolic face of the membrane (Fig. 3), a topology also presumed for its counterpart in yeast. In spite of the low sequence identity of the identified TbPEX4 with other PEX4s (Fig. S2), amino acids of ScPEX4 involved in the interaction with its membrane-anchor protein ScPEX22 are to considerable extent conserved in the *T. brucei* orthologue (Fig. S3), consistent with the probable existence of such an anchor protein in the trypanosomes. Indeed, a candidate TbPEX22 was identified and the modelled TbPEX4–TbPEX22 interface has shown to be favourable. The predicted interface region of TbPEX22 was proved to interact with TbPEX4 in a yeast two-hybrid system, supporting its identity as the anchor protein of TbPEX4 (Fig. S4). It is worth mentioning that TbPEX4, like the PEX4s of yeasts and plants, does not possess a predicted transmembrane region to attach it directly to the glycosomal or peroxisomal membrane. Nonetheless, in all our analyses TbPEX4 was consistently found in the organellar fraction after cell fractionation, further supporting the notion that it is anchored by binding to another protein. Only in the differential centrifugation analysis (Fig. 2A) a relatively large amount of PEX4 was also found in the nuclear fraction. To a lesser extent also the specific glycosomal marker aldolase, a matrix protein, was found in this fraction, but not the glycosomal integral membrane protein PEX11. The presence of PEX4 and aldolase in the nuclear fraction is considered an artefact, due to the release of both this peripheral membrane and the soluble protein from respectively the outer surface and matrix of the glycosomes upon damage of some organelles during lysis of trypanosomes by grinding, and a non-specific affinity of the released proteins for nuclear constituents.

Future studies should confirm the candidate TbPEX22 unambiguously as the membrane-anchor protein of TbPEX4. A very strong support for the confirmation of our candidate proteins as TbPEX4 and TbPEX22 was obtained very recently by their identification in a proteomic analysis of *T. brucei* glycosomes (Dr Lucia Guther and Prof. Michael Ferguson, personal communication).

The function of TbPEX4 was studied by knocking out the two alleles of the TbPEX4 gene (Fig. 4). This strategy allowed us to prove firmly its role in the ubiquitination of TbPEX5 in procyclic forms (Fig. 7). The results of the phenotypic analysis were however intriguing, since only minor mislocalisation of glycosomal proteins was found in this mutant (Fig. 6). In *S. cerevisiae* cells, the impairment of ubiquitination of PEX5 by the absence of PEX4 led to the mislocalisation of PTS1 proteins in the cytosol, as a consequence of the inability of the PEX5 receptor to cycle back to the cytosol, which is evident by its massive accumulation in the peroxisomal membrane. The peroxisomal import defect in this mutant severely affected growth in oleate-based media [35–37]. In other yeast species such as *P. pastoris* and *H. polymorpha*, the absence of the complex PEX4/PEX22 caused a strong reduction in the steady-state levels of PEX5 and consequently a defect in the peroxisomal matrix protein import. Nevertheless, in these latter species the PTS1- and PTS2-protein import defect is milder than in baker's yeast [64–66]. Our finding that even with a large reduction of ubiquitinated PEX5 molecules, as observed in the Δ*PEX4* mutant, glycosomal protein import was still functional, TbPEX5 did not accumulate in the glycosomal membrane and growth was only partially slowed down, was surprising. At first sight, these results would suggest that receptor ubiquitination is not required for proper receptor recycling in trypanosomes, contradicting the current model of peroxisomal matrix protein import in yeast and mammalian cells. However, further investigation of the Δ*PEX4* mutant revealed peculiar aspects that may account for this unexpected result. We observed that upon knockout of both alleles of the TbPEX4 gene in procyclic cells, several other UBC genes were significantly over-expressed (Fig. 8). This could have occurred as a compensatory mechanism to overcome the TbPEX5 ubiquitination defect. One of these UBC proteins could have taken over the function of TbPEX4, albeit with less efficiency than TbPEX4 [since a very faint ubiquitinated PEX5 signal was detected in the Δ*PEX4* mutant by Western blot analysis (Fig. 7)], maintaining the cells' ability to import proteins into their glycosomes. Such a “redundant” protein should have similar features to TbPEX4 in order to be able to interact with the E3 ligase present in the glycosomal membrane. One may wonder if it should also be able to interact with the anchor protein TbPEX22. However, permanent attachment to the membrane may not be mandatory, since in mammalian cells the functional counterpart of PEX4, UBCH5, is a soluble cytosolic protein. We speculate that the putative TbPEX4-compensating protein in the trypanosome probably lacks an orthologue in *S. cerevisiae*, or at least not one that shares a similar function as in the trypanosomes, since this compensatory mechanism seems not to have emerged in the *S. cerevisiae* Δ*PEX4* mutants. In contrast, indications for a similar PEX4 rescue have been described in a very recent paper about a study of the in vivo ubiquitination of the *P. pastoris* PEX20 [38]. By gene knockout, PEX4 was found to be necessary for the mono- and di-ubiquitination of this coreceptor on the conserved Cys8, as well as for the polyubiquitination on Lys19. Importantly, when simultaneously with the absence of PEX4 the proteasome-dependent degradation pathway of PEX20 was inactivated by mutation of Lys19, a single ubiquitin moiety was added to both Cys8 (via a DTT sensitive linkage) and, with much lower efficiency, in a DTT-resistant manner to another site of PEX20. This indicates that another, redundant UBC can substitute for the loss of PEX4 activity. Notably, the authors could exclude UBC4 as a candidate for the PEX4 redundancy [38]. These findings for *P. pastoris* indicate the realistic nature of our hypothesis that a similar event could have been possible in the *T. brucei* Δ*PEX4* mutant.

Making a null mutant by knocking out both alleles of the *PEX4* gene in procyclic-form *T. brucei* generated cells with an approximately two-fold increase in their duplication time. Despite the impaired ubiquitination of the glycosomal matrix-protein receptor TbPEX5, protein import appeared not to be significantly affected, leading only to a minor mislocalisation of PTS1- (Fig. 6) and I-PTS-proteins (data not shown). Why then was proliferation so much affected and why were the cell morphology and motility altered? A possible explanation may be found in the fact that three of the UBC family members that showed significantly increased mRNA levels in the mutant (Fig. 8) – Tb09.211.0050, Tb927.8.6090 and Tb927.2.3720 – are firm orthologues of proteins involved in the cell-cycle regulation in yeast and mammalian cells. The latter two, Tb927.8.6090 and Tb927.2.3720, are the respective orthologues of mammalian UBE2C [67,68] and UBE2S [69,70] which promote mitosis progression through the targeting of cell-cycle regulatory factors, including M- and C-cyclins, for proteasome degradation, thus leading to the final separation of sister chromatids. The first one, Tb09.211.0050, which in *T. brucei* possesses a PTS1, is the orthologue of UBC13 in yeast and UBE2N in mammalian cells which are involved in the targeting of substrates during activation of gene transcription as well as during DNA repair [71,72]. The yeast UBC13 is also linked to mitotic checkpoint control by protein–protein interaction [73]. We can imagine that overexpression of one or more UBC genes coding for such functions in the Δ*PEX4* cell line might disturb the ubiquitination – and thus the functioning – of proteins with a regulatory role in cell cycle control. This may lead to defects in coordination of DNA synthesis, mitosis, cytokinesis or cell-cycle coupled processes such as flagellum development and thus offers an explanation for the retardation of growth and altered motility. Moreover, it can also be envisaged that, instead of the usual formation of equally-sized daughter cells, this loss of cell cycle control in the mutants is responsible for a population of cells that is quite heterogeneous with the majority having a reduced cell body and an abnormal shape, as was observed by scanning electron microscopy (Fig. 5B). Future investigation of this phenomenon should be performed to explore the validity of our hypothesis. Such an investigation may involve combined analyses of the transcriptome, the cell cycle and cytokinesis. This would require a detailed morphometric study to quantify all morphological changes observed between mutant and wild-type cells and the ratio

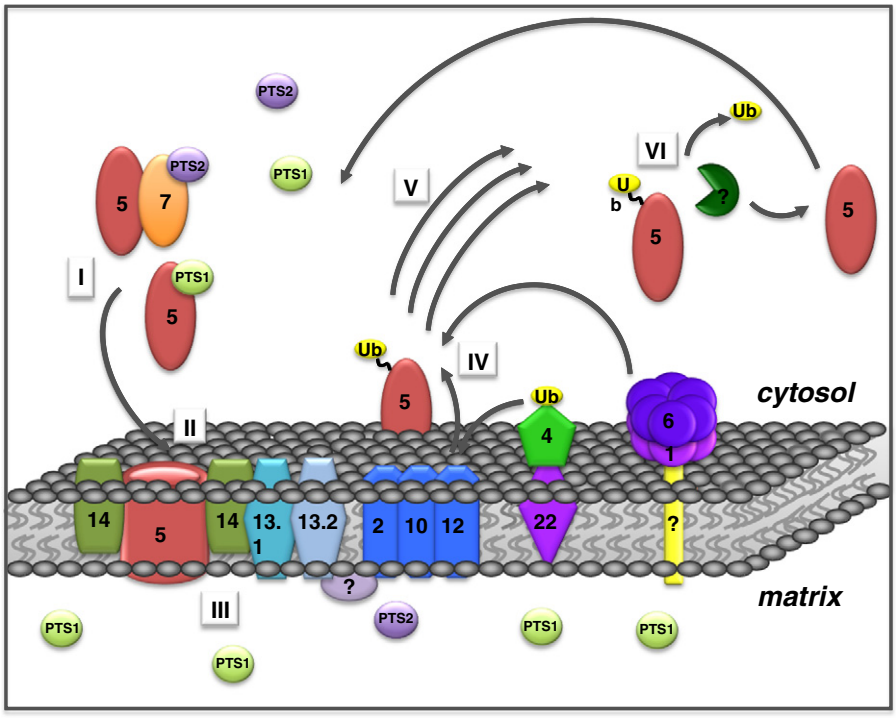


Fig. 9. Model of the import of glycosomal matrix proteins in *Trypanosoma brucei*. Matrix protein import is achieved by a process which involves cyclic receptors, here depicted for TbPEX5, the receptor of matrix proteins with a PTS1 motif. Six distinct steps can conceptually be understood: (I) binding of proteins with a PTS1 to TbPEX5 (and PTS2 to TbPEX7, plus TbPEX7-PTS2-proteins to TbPEX5). (II) Docking of the receptor–cargo complex to the docking subcomplex (TbPEX13.1, TbPEX13.2, TbPEX14) in the glycosomal membrane, followed by the insertion of PEX5 into the membrane, forming in conjunction with TbPEX14 a transient pore. (III) Release of PTS proteins, via the transient pore, into the matrix. (IV) Ubiquitination of the membrane-inserted TbPEX5 by the coordinated action of a cytosolic ubiquitin-conjugating enzyme, TbPEX4, very likely anchored to the membrane by TbPEX22, and an ubiquitin-ligating enzyme of the RING-finger subcomplex, which comprises PEX2, PEX10 and PEX12. (V) Fast retrieval of the ubiquitinated PEX5 from the membrane by the cytosolic AAA^{+}-ATPase complex including PEX1 and PEX6, recruited to the membrane. (VI) Deubiquitination of PEX5, rendering the receptor available to mediate new rounds of PTS-protein import. All peroxins identified in *T. brucei* are indicated in this scheme by the numbers attributed previously to their *S. cerevisiae* homologues.

of the numbers of nuclei and kinetoplasts per cell in the Δ*PEX4* population.

In conclusion, our research on the molecular mechanism involved in the recycling of TbPEX5, together with previous work done by us and others, led us to adjust the model for glycosomal protein import in *T. brucei* (Fig. 9): TbPEX5 is a cytosolic protein that recognises PTS1 proteins and participates in their import, as well as mediates PTS2 protein import through its interaction with the PTS2 receptor TbPEX7. The cargo-loaded PTS1 receptor docks at the peroxisomal membrane by interacting, through its N-terminal domain, with peroxins TbPEX13.1, TbPEX13.2 and TbPEX14. The translocation of the cargo probably occurs similarly as shown in *S. cerevisiae* cells, by the formation of a dynamic and transient pore formed by the receptor TbPEX5 and probably TbPEX14. After the cargo release, the receptor TbPEX5 is ubiquitinated, by the glycosomal E2 enzyme TbPEX4 in concert with the activity of the E3 RING-finger complex (very likely TbPEX12) and then rapidly exported to the cytosol by the action of the TbPEX6/TbPEX1. The ubiquitinated TbPEX5 is stable in the cytosol. The signalling ubiquitin is probably removed by an (enzymatic/or chemical) mechanism homologous to the one in mammalian cells but that operates at a much lower steady-state level.

Acknowledgements

We are grateful to Prof. Ralf Erdmann (Ruhruniversität Bochum) for discussions and advice, during our research, and comments on the manuscript. We would like to acknowledge Drs Chris Williams and Damien Devos (EMBL, Hamburg) for their assistance in the in silico analysis of TbPEX4 and TbPEX22 protein–protein interaction, Dr Lucia Guther and Prof. Michael Ferguson (University of Dundee) for allowing us to cite the identification of the candidate PEX4 and PEX22 in their proteomic analysis of glycosomes, and Prof. Christine Clayton (Universität Heidelberg) and Drs Ben Distel (Universiteit van Amsterdam), Frank Voncken (University of Hull) and Frédéric Bringaud (Université Bordeaux Segalen) for gifts of plasmids and antiserum. MGL gratefully acknowledges her PhD scholarship from the Fonds pour la Formation à la Recherche dans l'Industrie et dans l'Agriculture (FRIA) and the Patrimoine de Faculté Médicine, Université catholique de Louvain. This work received financial support (to PAMM) from the Fonds de la Recherche Scientifique (F.R.S.-FNRS) and the Belgian Interuniversity Attraction Poles-Federal Office for Scientific, Technical and Cultural Affairs.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2013.08.008.

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