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Studies on the organization of the docking complex involved in matrix protein import into glycosomes of *Trypanosoma brucei*

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ABSTRACT

Trypanosoma brucei contains peroxisome-like organelles designated glycosomes because they sequester the major part of the glycolytic pathway. Import of proteins into the peroxisomal matrix involves a protein complex associated with the peroxisomal membrane of which PEX13 is a component. Two very different PEX13 isoforms have recently been identified in *T. brucei*. A striking feature of one of the isoforms, TbPEX13.1, is the presence of a C-terminal type 1 peroxisomal-targeting signal (PTS1), the tripeptide TKL, conserved in its orthologues in all members of the Trypanosomatidae family so far studied, but absent from TbPEX13.2 and the PEX13s in all other organisms. Despite their differences, both TbPEX13s function as part of a docking complex for cytosolic receptors with bound matrix proteins to be imported. We further characterized TbPEX13.1's function in glycosomal matrix-protein import. It provides a frame to anchor another docking complex component, PEX14, to the glycosomal membrane or information to correctly position it within the membrane. To investigate the possible function of the C-terminal TKL, we determined the topology of the C-terminal half of TbPEX13.1 in the membrane and show that its SH3 domain, located immediately adjacent to the PTS1, is at the cytosolic face.

1. Introduction

Protists grouped in the order Kinetoplastea possess organelles called glycosomes because they have the peculiarity to harbor the major part of the glycolytic pathway [1,2]. Based on morphological data and the fact that additional enzymes found in glycosomes are typical peroxisomal, these organelles are considered as members of the organelle family of peroxisomes. Moreover, for kinetoplastids such as *Trypanosoma brucei*, the parasite responsible for African sleeping sickness in humans and similar diseases in other mammals, it has been shown that glycosome biogenesis requires the function of proteins homologous to the peroxins (indicated with the acronym PEX) involved in peroxisome biogenesis in other organisms [3,4]. Trypanosomes, transmitted by tsetse flies, live in the blood of their mammalian hosts. Glycolysis is essential for the parasites when living in the bloodstream because glucose degradation is their sole source of free energy at this stage of their life cycle [5]. The proper compartmentalization of glycolytic enzymes inside glycosomes is also crucial for parasite survival [6–8].

Like for peroxisomes, glycosomal matrix and membrane proteins are encoded by nuclear genes, synthesized in the cytosol and post-translationally imported into the organelles. Peroxisomal or glycosomal matrix proteins contain a peroxisomal-targeting signal (PTS). Two main targeting signals have been described and are relatively well conserved between species; the type 1 PTS (PTS1) is a C-terminal tripeptide with the general consensus sequence (S/C/A)-(K/R/H)-(L/M) [9]. It is recognized by the cytosolic receptor protein PEX5. The PTS2 is a nonapeptide located near the N-terminus of proteins to be imported and corresponds to the sequence [(R/K)-(L/V/I/Q)-xx-(L/V/I/H)-(L/S/G/A)-x-(H/Q)-(L/A)] [10]. PTS2 motifs are recognized by PEX7, also cytosolically located. After recognition and binding their cargo in the cytosol, the loaded PEX5 and PEX7 steer for the peroxisomes and interact with a docking complex present in the membrane and that in yeasts has been shown to comprise minimally two peroxins, PEX13 and PEX14. Peroxisome targeting of membrane proteins follows a different pathway and requires a routing signal that consists of a stretch of basic amino acids and a transmembrane segment [11,12].

Despite a low sequence identity compared with mammalian or yeasts homologues, *T. brucei* PEX5, PEX7 and PEX14 have been identified in databases by sequence homology or by PCR with degenerate primers [13–15]. However, such analyses were unsuccessful to identify PEX13. Recently, two very different PEX13

Abbreviations: PBS, phosphate-buffered saline; PEX, peroxin; PFK, phosphofructokinase; PPDK, pyruvate phosphate dikinase; PTS, peroxisomal-targeting signal; tet, tetracycline.

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isoforms have been identified, TbPEX13.1 and TbPEX13.2, by using different bespoke bioinformatics approaches involving pattern searches plus domain analysis and jackhammer database searches, respectively [16,17]. The proteins display a very low sequence identity with PEX13s from other organisms, but are, like these other PEX13s, predicted to be integral membrane proteins with two transmembrane segments. Indeed, their localization in the glycosomal membrane has been experimentally confirmed. TbPEX13.1, but not TbPEX13.2, shares with other PEX13s a SH3 domain in its C-terminal half. A most remarkable, unique feature of TbPEX13.1 is the presence of a PTS1 motif, a C-terminal TKL, directly adjacent to the SH3 domain of this membrane protein [16]. Strikingly, this TKL sequence is fully conserved in PEX13.1s of other trypanosomatid genera, strongly suggesting an important function.

In *Saccharomyces cerevisiae* and fibroblasts, PEX13 has been shown to function not only in receptor docking, but also as a membrane anchor or peroxisomal membrane association factor to PEX14 [18–20]. Previously, we reported that *T. brucei* PEX14 is peripherally associated with the cytosolic face of the glycosomal membrane [15]. For TbPEX14 and both TbPEX13 isoforms, the involvement in the glycosomal biogenesis process as a membrane docking factors for the cargo-loaded receptors TbPEX5 or TbPEX7 was confirmed by RNAi: glycosomal PTS-containing matrix proteins became mislocalized to the cytosol when the levels of TbPEX14, TbPEX13.1 or TbPEX13.2 were decreased [15–17].

In this paper, we present the results of some further analysis of *T. brucei* PEX13.1. We investigated the topology of this peroxin, important for unraveling the function of the PTS1 sequence and show that PEX13.1 plays a role in correctly positioning PEX14 in the parasite's glycosomal membrane, as was recently also proved for PEX13.2 [17].

2. Materials and methods

2.1. Trypanosomes, growth conditions and transfection

Procyclic (*i.e.* the life-cycle stage living in the tsetse fly's midgut) forms of *T. brucei* strain Lister 427, cell line 449 [21], 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. Procyclic trypanosomes were grown in SDM79 medium [22] supplemented with 15% fetal calf serum and 0.5 μg mL^{-1} 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 2×10^7 cells mL^{-1}, by centrifugation at 700*g* for 10 min. Transfection of trypanosomes and selection of clones were performed as described previously [23]. After transfection and selection, positive clones were stored at −80 °C in appropriate medium containing 12% glycerol.

2.2. Molecular biology methods

For most experiments in molecular biology standard methodologies were used [24] or protocols were followed as provided by suppliers of enzymes used for various forms of DNA manipulation (Fermentas, Roche Applied Science, Promega). *E. coli* strain XL-1-Blue (Stratagene) was used for all plasmid cloning.

2.3. Subcellular fractionation of T. brucei

Procyclic trypanosomes (10^{10} cells) were collected, centrifuged for 20 min at 1,000*g* in a SLA-3000 Sorvall rotor and washed in isotonic SIE buffer (250 mM sucrose, 3 mM imidazole–HCl, pH 7.0, 1 mM EGTA). Cells were broken (microscopically checked) in a mortar with carborundum powder, the suspension then taken up in another 5 mL SIE buffer and centrifuged at 30*g* for 3 min (all centrifugation steps in this procedure were done in a SM-24 Sorvall rotor). The pellet was washed twice with 2.5 mL SIE buffer and then discarded, whereas the pooled supernatants, representing the cell homogenate, were centrifuged at 1500*g* for 10 min giving the nuclear fraction. The post-nuclear supernatant was then centrifuged at 5000*g* for 10 min giving the large-granular (mitochondria-enriched) fraction as pellet. In order to obtain the small-granular (glycosomes-enriched) fraction, the supernatant was then centrifuged at 15000*g* for 30 min. A further centrifugation step (140,000*g* for 1 h using a 50Ti Beckman rotor) gave the microsomal fraction and the cytosol depleted of all organelles.

2.4. Protease protection assay

The integrity of the glycosomes present in the small granular fraction was evaluated by measurement of the hexokinase activity to confirm good latency. 10 mM $CaCl_2$ was added to glycosomes (400 μg of proteins) obtained after subcellular fractionation by differential centrifugation. Aliquots (100 μg of proteins) were incubated with proteinase K (Roche) (120 μg mL^{-1} final concentration) in the presence of 0.3% Triton X-100. Control experiments were performed without protease and without detergent. The volume of each suspension was adjusted to 300 μL with isotonic SIE buffer. Tubes were incubated on ice for 2 h before stopping the reaction by addition of 20 mM phenylmethylsulfonyl fluoride and incubation for 10 min on ice. Proteins were then extracted and precipitated

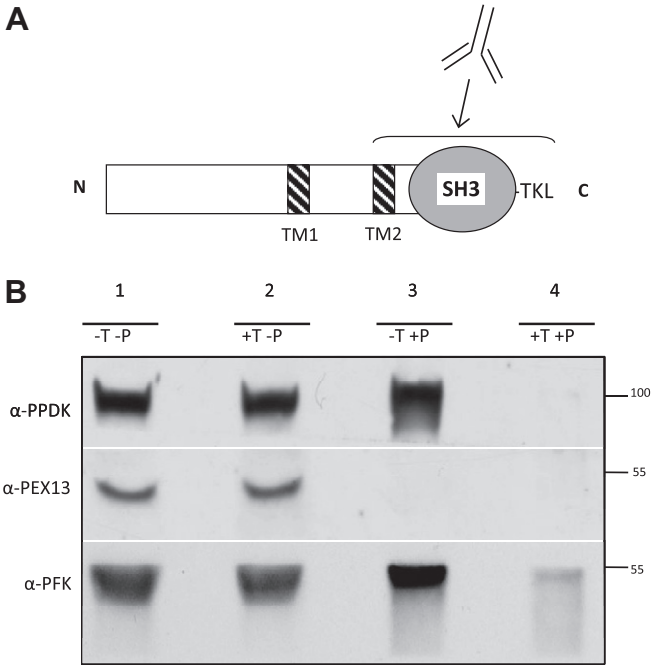

Fig. 1. Determination of the topology of the *T. brucei* PEX13.1 C-terminal half in the glycosomal membrane by a protease protection assay. (A). Schematic representation of TbPEX13.1 features. The antibody symbol indicates the part of PEX13.1 used to raise the antiserum. Abbreviations: SH3, Src homology 3 domain; TKL, the PTS1-like motif; TM, transmembrane segment. (B). 100 μg of glycosomal proteins obtained from a subcellular fractionation by differential centrifugation of procyclic cells were incubated in the presence, or not, of proteinase K (P) and in the presence, or not, of the detergent Triton X-100 (T). Proteins were then precipitated, size-fractionated by SDS–PAGE and transferred onto a nitrocellulose membrane. The same membrane was probed and after stripping re-probed successively with antisera against PEX13.1 (1:20,000) and glycosomal enzymes.

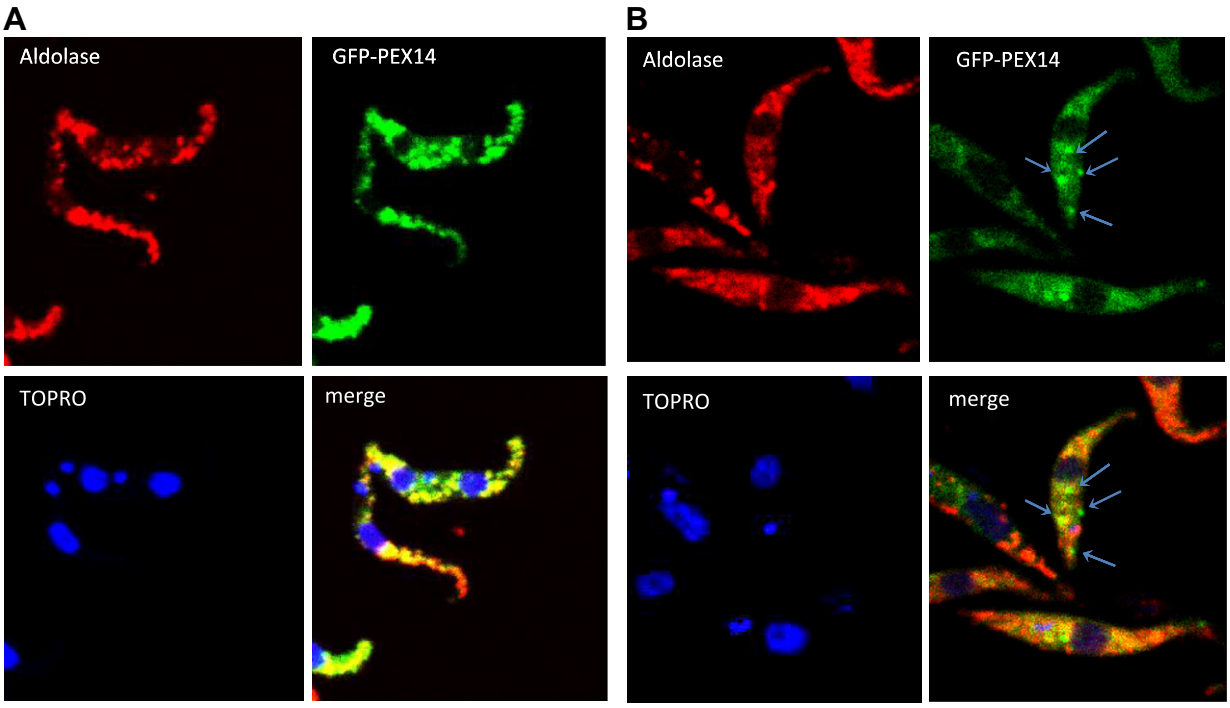

Fig. 2. Effect of the RNAi against PEX13.1 on PEX14 localization studied by immunofluorescence. (A) Procyclic trypanosomes expressing GFP-PEX14. (B) Procyclic RNAi-PEX13.1 trypanosomes expressing GFP-PEX14. Induction of GFP-PEX14 expression and RNAi against PEX13.1 was for 24 h. Arrows point to small structures with which GFP-PEX14 is associated.

by treatment with methanol and chloroform following the protocol described by Wessel and Flügge [25]. The concentrated samples thus obtained were size fractionated by SDS–PAGE, transferred to a nitrocellulose membrane and probed with antisera raised in rabbits against the purified recombinant *T. brucei* proteins PEX13 [16] at a dilution of 1:20,000, pyruvate, phosphate dikinase (PPDK – a gift of F. Bringaud) at 1:100,000 and phosphofructokinase (PFK) [5] at 1:50,000.

2.5. Construction of a GFP-tagged PEX14, immunofluorescence studies and western blot analysis

In order to express in trypanosomes a PEX14 fused to the C-terminal side of Green Fluorescent Protein (GFP), the full-length *T. brucei PEX14* gene was amplified by PCR using *Pfu* DNA polymerase (Fermentas) from genomic DNA and the following primers: 5′-CCCAAGCTTTAATGTCTTTGCTGCTGTCG-3′ (forward) and 5′-ATGGATCCTCAAGCTGCCTCGCCGCC-3′ (reverse). The forward primer containing a *Hind*III site (underlined) corresponds to the 5′ end of the *PEX14* gene and the reverse primer is complementary to the terminal coding region of the gene followed by a *Bam*HI restriction site (underlined). The amplified fragment was purified and ligated in the pGEM-T Easy vector (Promega). After checking the sequence, the gene was liberated from the plasmid by digestion with *Hind*III and *Bam*HI and ligated in a trypanosome-specific expression vector containing already the GFP gene, pGC1, derived from plasmid pHD1336 [26]. Wild-type and PEX13 RNAi [16] procyclic cells lines were transfected with this construct. After selection of clones with 10 $\mu g\,mL^{-1}$ blasticidin (wild-type trypanosomes) or 10 $\mu g\,mL^{-1}$ blasticidin and 50 $\mu g.mL^{-1}$ hygromycin (PEX13 RNAi cells), induction of GFP-PEX14 expression in procyclic cells was done by addition of 5 $\mu g\,mL^{-1}$ Tet.

Cells for immunofluorescence analysis were grown overnight, fixed with 4% formaldehyde in phosphate-buffered saline (PBS), permeabilized with 1% Triton X-100 and spread on poly-L-lysine coated slides. The slides were then incubated for 45 min in PBS containing 5% BSA, followed by incubation in PBS with 2% BSA and the primary antiserum (i.e. rabbit polyclonal anti-*T. brucei* aldolase as glycosomal marker). After washing with PBS, cells were allowed to react with 5 $mg\,mL^{-1}$ Alexa 568 anti-rabbit IgG conjugate (Molecular Probes) and 2 μM TO-PRO-3 (Molecular Probes), washed again and mounted in Mowiol. Cells were visualized using a Zeiss Axiovert microscope coupled to an MRC-1024 confocal scanning laser imaging system (BioRad).

Cells for western blot analysis were grown for 24 h before being collected. Trypanosomes were then subjected to subcellular fractionation by differential centrifugation and 75 μg protein of each fraction were size-fractionated by SDS–PAGE and then transferred to a nitrocellulose membrane. The membrane was probed with antisera against PEX14 (dilution of 1:20,000), PEX11 (1:2500) and anti-GFP (1:400) (Millipore).

3. Results and discussion

TbPEX13.1 is an integral membrane protein [16], and its orthologues similarly predicted so in all trypanosomatids for which genome sequencing has been performed. Nonetheless, it possesses a PTS1 motif, i.e. a consensus targeting motif for a glycosomal matrix protein. Although the tripeptide TKL is rarely used for peroxisome/glycosome targeting, it has been shown to be highly efficient in routing a reporter protein to glycosomes in transient expression experiments of transfected trypanosomes [27] and is present on the *T. brucei* glycosomal protein UDP-galactose-4′-epimerase [28]. What could be the reason that uniquely in these protists one of the PEX13 isoforms possesses a PTS1? It is tempting to invoke a function in facilitating the release of PTS1 proteins into the glycosomal matrix, after the translocation event, by competition with the PTS1 cargo for the PTS1-binding domain of PEX5. This would be analogous to what has been proposed for yeast PEX8, a periph-

eral membrane protein at the matrix face that possesses both a PTS1 and PTS2 [29]. Such a role would require the exposure of the PEX13 C-terminus in the matrix [30]. However, in all other organisms, the SH3 domain is cytosolically orientated [20,31,32]. In order to investigate the probable function of this motif, we determined the topology of the C-terminal half of *T. brucei* PEX13 in the glycosomal membrane. A PEX13.1-specific antiserum raised against a fragment of the protein from the second predicted transmembrane segment (comprising residues 285–306 of the TbPEX13.1 polypeptide) to the C-terminus allowed us to determine the orientation of the SH3 domain and the immediately adjacent PTS1 motif in a protease protection assay (Fig. 1A). To that end, an organellar fraction enriched in intact glycosomes was, prepared by differential centrifugation of a lysate from procyclic trypanosomes, and subsequently incubated in the presence of proteinase K. The susceptibility of PEX13.1-SH3 – and glycosomal matrix proteins as controls – for the protease was compared under conditions where the integrity of the glycosomal membrane was affected by addition of Triton X-100 or not. Fig. 1B shows that, upon permeabilization of the membrane by the detergent, PEX13.1 as well as the matrix proteins phosphofructokinase (PFK) and pyruvate, phosphate dikinase (PPDK) were degraded (largely and completely, respectively) by the protease (lane 4). However, when the glycosomal membrane was left intact, only the band detected by the PEX13.1 antiserum in the control condition disappeared upon addition of the protease while PFK and PPDK were still present (lane 3). These results prove that, also in trypanosomes, the SH3 domain of PEX13.1 is present at the cytosolic face of the membrane and thus also the PTS1 sequence. This excludes a role of the motif in discharging the cargo-loaded PEX5 after its translocation. Future research should reveal if the TKL sequence serves to establish an interaction with PEX5 and if so, what would be the function of such an interaction. Or alternatively, if the conserved TKL motif at the C-terminus of PEX13.1 of all trypanosomatids plays an entirely different function.

PEX14, the other docking complex protein of the glycosomal membrane, is peripherally associated with the cytosolic side of this membrane. It may need an integral membrane protein as anchor for its attachment and/or correct positioning at the membrane. Data from studies with yeast and mammalian peroxisomes suggested that PEX13 plays such a role in those cells [4,19,20]. To determine if, also in trypanosomes, PEX13.1 is important for the correct topology of PEX14, the subcellular localization of newly synthesized PEX14 was compared in cell lines in which glycosome biogenesis is intact (Fig. 2A) or impaired by RNAi-dependent depletion of PEX13.1 (Fig. 2B). Newly synthesized PEX14 was followed by additionally expressing an N-terminally Green-Fluorescent Protein (GFP)-tagged copy of this peroxin (GFP-PEX14). In cells induced for only expressing this recombinant PEX14 (Fig. 2A), a punctate pattern of the GFP-PEX14 signal colocalized with the signal obtained by immunostaining of the glycosomal matrix protein aldolase. This indicates that the GFP-PEX14 is correctly addressed to glycosomes and that routing of glycosomal proteins is not affected by expression of the construct. When the localization of GFP-PEX14 was evaluated in the PEX13.1 RNAi cell line (Fig. 2B), a different pattern was observed after induction of RNAi. The fluorescence of GFP-PEX14 is mainly cytosolic but also associated with few small structures (indicated by arrows). The signal of aldolase in this cell line has a dual localization with a presence in the cytosol and association with punctate structures, glycosomes, similarly as published previously [16], indicating that newly synthesized aldolase cannot be imported anymore. Superimposition of both fluorescence signals showed that the small structures with which GFP-PEX14 is associated in this RNAi cell line do not correspond to glycosomes neither to the single, large mitochondrion of procyclic trypanosomes (not shown).

The consequences of PEX13.1's expression level decrease on newly synthesized PEX14 localization were also evaluated by western blot analysis of subcellular fractions obtained by differential centrifugation (Fig. 3). In wild-type (Fig. 3A) and GFP-PEX14 (Fig. 3B) expressing cells, the distribution of endogenous PEX14 was determined with a PEX14-specific antiserum. With this serum, identical distribution patterns were observed for both cell lines with the majority of PEX14 present in small (glycosomes enriched) and large granular fractions as well as a partial cytosolic location. The distribution of GFP-PEX14, determined with anti-GFP, in an intact glycosome biogenesis background (Fig. 3B) showed a main association with the small granules. But an appreciable amount of the protein was also found associated with the large granules and microsomes. As for endogenous PEX14, a small amount of the recombinant protein was cytosolic. When the biogenesis of glycosomes was impaired by depletion of PEX13.1 by RNAi (Fig. 3C), only a little part of GFP-PEX14 remained associated with the small granules, most of it was relocated to the microsomes, while again a small amount was also present in the cytosol. Another glycosomal membrane protein, PEX11 that is not part of the docking complex, appeared not to be affected by the absence of PEX13, as indicated by the similar subcellular distribution of PEX11 in all three cell lines, with its predominant presence in the small granular fraction.

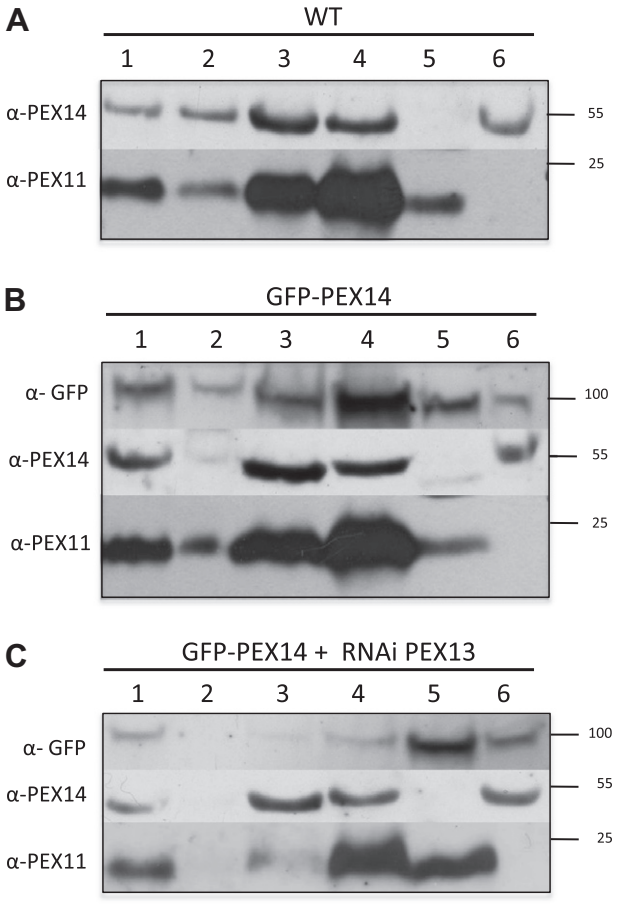

Fig. 3. Effect of the RNAi against PEX13.1 on the PEX14 localization studied by subcellular fractionation by differential centrifugation. 75 μg of proteins of each fraction from procyclic wild-type trypanosomes (A), cells expressing GFP-PEX14 (B) and cells expressing both GFP-PEX14 and the PEX13.1 RNAi construct (C) were size-fractionated by SDS–PAGE, blotted to a nitrocellulose filter and probed with antisera against PEX14, GFP and PEX11. Lane 1, post-nuclear supernatant; lane 2, nuclear fraction; lane 3, large granular fraction; lane 4, small granular fraction; lane 5, microsomal fraction; lane 6, cytosol.

This preliminary, qualitative analysis of the fate of PEX14 in a virtually PEX13.1-depleted cell line, using confocal microscopy and western blotting, suggests that this latter peroxin is involved, at least partially, in realizing the glycosomal localization of PEX14. Similar results were very recently obtained in trypanosomes in which PEX13.2 was depleted [17], indicating that, despite the very different primary structures of the two isoforms, they exert similar functions, not only by an involvement in the import of glycosomal matrix proteins, but also in correctly positioning PEX14 in the membrane. However the precise role of both TbPEX13 isoforms in the glycosomal membrane association of PEX14 remains to be determined. Do the TbPEX13s serve as anchors that retain PEX14 to the membrane or do they serve as a docking platform to insert PEX14 into the correct membrane? We have previously shown that both *T. brucei* PEX13 isoforms interact, in yeast two-hybrid studies, with each other and with PEX14 [16,17].

The identity of the structures with which GFP-PEX14 is associated in the absence of PEX13 has not yet been identified precisely, but they seem to belong to the microsomal fraction. Importantly, this fraction contains also a considerable amount of the glycosomal membrane protein TbPEX11 (Fig. 3C). Small vesicles derived from the endoplasmic reticulum (ER) are part of this heterogeneous organellar fraction. Since there is good evidence that the peroxisomal membrane originates (at least in part) from (specialized areas of) the ER [33–35], it seems reasonable to assume that PEX14 is inserted in this membrane when routing to the glycosomes is prevented.

The phenotype observed after RNAi against each of the PEX13s thus suggests that both PEX13 isoforms in trypanosomes have a dual function: they are essential components of the glycosomal matrix-protein import machinery and they also serve to ascertain the correct association of PEX14 with the glycosomal membrane.

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