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Elongation and clustering of glycosomes in *Trypanosoma brucei* overexpressing the glycosomal Pex11p

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Kinetoplastid protozoa confine large parts of glycolysis within glycosomes, which are microbodies related to peroxisomes. We cloned the gene encoding the second most abundant integral membrane protein of *Trypanosoma brucei* glycosomes. The 24 kDa protein is very basic and hydrophobic, with two predicted transmembrane domains. It is targeted to peroxisomes when expressed in mammalian cells and yeast. The protein is a functional homologue of Pex11p from *Saccharomyces cerevisiae*: *pex11*Δ mutants, which are defective in peroxisome proliferation, can be complemented by the trypanosome gene. Sequence conservation is significant in the N- and C-terminal domains of all putative Pex11p homologues known, from trypanosomes, yeasts and mammals. Several lines of evidence indicate that these domains are oriented towards the cytosol. TbPex11p can form homodimers, like its yeast counterpart. The *TbPEX11* gene is essential in trypanosomes. Inducible overexpression of the protein in *T.brucei* bloodstream forms causes growth arrest, the globular glycosomes being transformed to clusters of long tubules filling significant proportions of the cytoplasm. Reduced expression results in trypanosomes with fewer, but larger, organelles.

Keywords: glycosome/peroxin/peroxisome biogenesis/ *Saccharomyces cerevisiae*/*Trypanosoma brucei*

Introduction

Trypanosoma brucei is an extracellular protozoan parasite that causes severe diseases of livestock and humans in tropical Africa. It is transmitted between mammals by tsetse flies. Like other members of the order Kinetoplastida, *T.brucei* has compartmentalized the first seven enzymes of the glycolytic pathway inside specific organelles, the glycosomes (Clayton and Michels, 1996). We have no idea how this compartmentalization arose, and the metabolic advantages, if any, are also unclear. However, there is strong evidence that glycosomes are evolutionarily and functionally related to the peroxisomes of other eukaryotes (Michels and Hannaert, 1994). Beyond a similar ultrastructure, buoyant density and some common enzymatic constituents, glycosomes share with peroxisomes the two protein import pathways involving targeting signals, PTS1 and PTS2, that are responsible for post-translational import of peroxisomal matrix proteins (Opperdoes, 1987; Blattner *et al*., 1992, 1995; Sommer and Wang, 1994). All evidence so far indicates that the mechanism of microbody biogenesis has been conserved throughout eukaryotic evolution.

In yeast and higher eukaryotes, a growing number of peroxin (peroxisomal biogenesis, *PEX*) genes have been defined by genetic complementation screens. Several of them encode membrane proteins (Erdmann *et al*., 1997; Waterham and Cregg, 1997). It has been reported previously that membranes of *T.brucei* glycosomes contain two predominant integral membrane proteins, estimated at 24 and 26 kDa (Aman and Wang, 1987). Here we report the cloning of the gene encoding the 24 kDa (p24) protein. p24 is structurally and functionally related to the 27 kDa peroxin Pex11p in yeast. Pex11p is required for peroxisome division in *Saccharomyces cerevisiae* (Erdmann and Blobel, 1995; Marshall *et al*., 1995) and *Candida bodinii* (Sakai *et al*., 1995). Previous reports have indicated that dimerization may be important in Pex11p function and that both N- and C-termini are oriented towards the peroxisomal matrix, thereby being inaccessible to cytosolic components (Marshall *et al*., 1996).

Results

Cloning of the gene encoding the 24 kDa membrane protein

The major constituents of purified glycosomes have been identified as enzymes involved in glycolysis and glycerol metabolism (Misset *et al*., 1986). In addition, two proteins with an estimated molecular mass of 24 (p24) and 26 (p26) kDa previously have been shown to be integral membrane proteins that are expressed in both bloodstream (BF) and procyclic (Pro) forms of the parasite (Aman and Wang, 1987). We purified the 24 kDa protein, generated tryptic peptides and used the sequences to clone the corresponding cDNA and gene (see Materials and methods). The open reading frame (ORF) of 654 nucleotides predicts a protein of 218 amino acids with a molecular mass of 23 999.6 Da (Figure 1A), and includes the sequences of the p24 tryptic peptides. The calculated isoelectric point of 9.96 for p24 agrees with published estimates (Parsons and Nielsen, 1990). Using a specific antiserum raised against a predicted N-terminal peptide of p24, we performed a Western blot analysis to confirm that p24 is expressed in both life cycle stages of the parasite (see Figure 2A for BF and Figures 5 and 6

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for Pro) and is largely resistant to carbonate extraction (Figure 5A).

Southern blot analysis of genomic DNA from *T.brucei* strain 427 revealed a pattern of fragments compatible with a single p24 gene per haploid genome (data not shown). Northern blot analysis showed transcripts of 1.35 kb hybridizing with p24 probes in total RNA from both Pro and BF form trypanosomes (data not shown), confirming the Western blot data.

p24 shows homology to yeast Pex11p and a rat peroxisomal membrane protein

A blastp database search revealed similarity between *T.brucei* p24 and the peroxisomal membrane protein Pex11p from *S.cerevisiae* (ScPex11p/PMP27) in two regions at the N- and C-termini. An alignment of p24, the two yeast peroxins ScPex11p and *C.boidinii* Pex11p (CbPex11p/PMP30), and a rat peroxisomal membrane protein is shown in Figure 1A. The overall identity (similarity) between *T.brucei* p24 and ScPex11p is 23% (47%). The strongest conservation (36% identity, 64% similarity) is in a 28 amino acid stretch at the N-terminus (block 1 in Figure 1A). Other blocks (2–5) with their respective similarity values are given in Figure 1B. Interestingly, the regions most strongly conserved between p24 and ScPex11p are also conserved in the sequence of a mammalian Pex11p homologue (PMP26) from *Rattus norvegicus* (Passreiter *et al*., 1998).

Overexpression of p24 leads to growth arrest

To study the properties of p24 in more detail, we generated trypanosomes expressing p24 and its myc epitope-tagged derivatives under control of a tetracycline- (tet) regulatable promotor (see Materials and methods). These lines possess the two endogenous p24 genes plus a tet-regulatable transgene. As shown in Figure 2A, induction of p24 transgene expression by addition of tetracycline leads to a marked increase in the p24 signal in Western blots of total cellular protein (~18-fold after 20 h relative to the level in wild-type cells, as determined in a titration experiment; not shown). Induction of both the N-terminally tagged p24Nmyc and the C-terminally tagged p24Cmyc led to the appearance of an additional band at ~27 kDa that is recognized by the p24 antiserum. The level of expression of tagged p24 relative to wild-type (449) levels of p24 was ~2-fold for p24Nmyc and ~35-fold for p24Cmyc. Interestingly, at late time points of p24 induction, the amount of p26, the other major membrane protein of *T.brucei* glycosomes (Aman and Wang, 1987; P.Lorenz, A.G.Maier and C.Clayton, in preparation), is strongly reduced. The levels of glycosomal matrix enzymes aldolase (targeting signal PTS2) and phosphoglycerate kinase (PTS1) were unchanged.

After tetracycline addition to the cell lines, we observed a strong reduction in the growth rates of cells harbouring the p24 and p24Cmyc transgenes (Figure 2B). Induction of p24Nmyc only had a minor effect on cell growth. This might have been due to the smaller amount of protein produced.

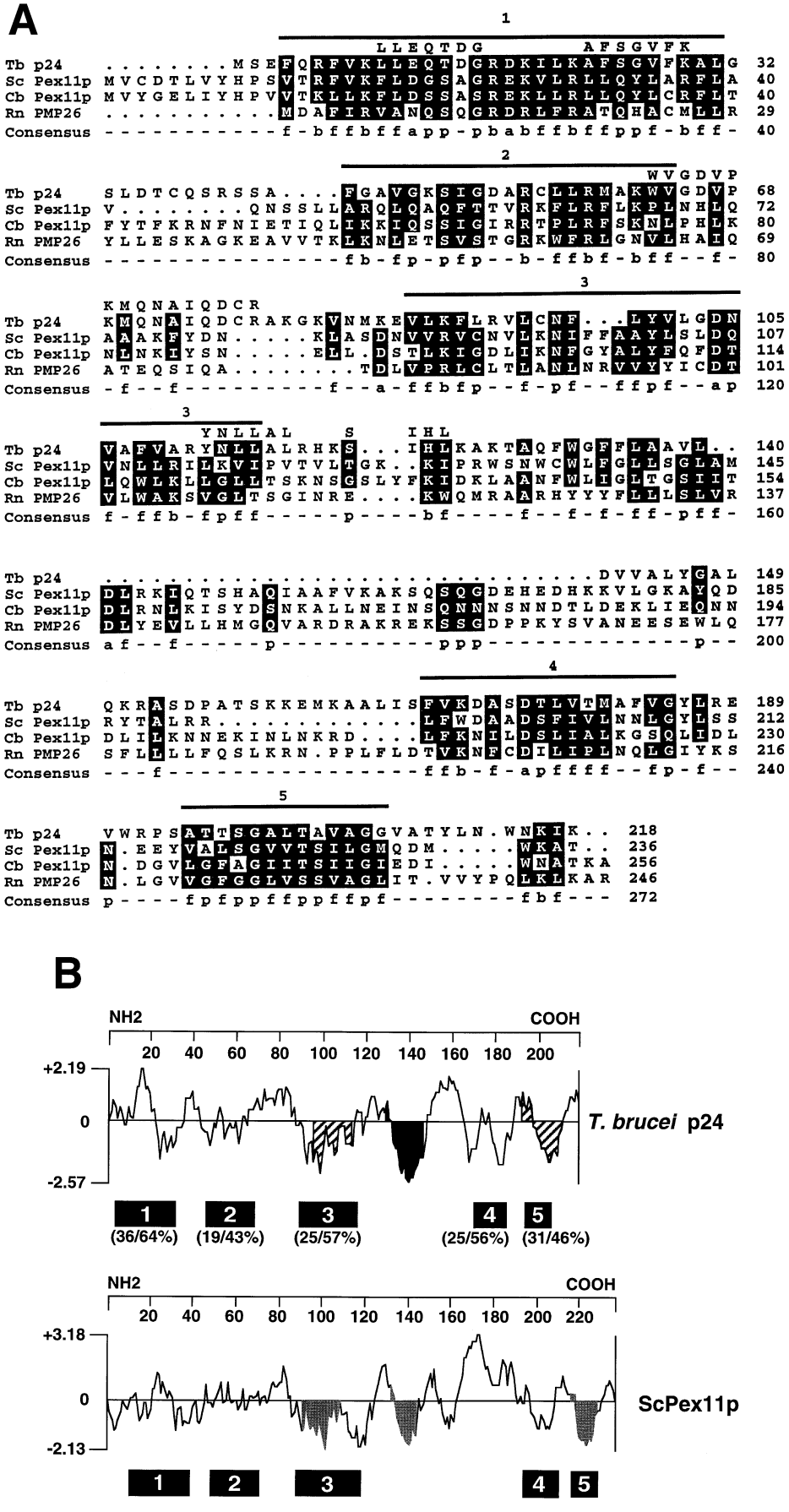
Effects of p24 overexpression on glycosome morphology

Confocal laser scanning microscopy analysis confirmed the glycosomal location of p24 and its myc-tagged derivatives (Figure 3). Double immunofluorescence labelling of p24 and the glycosomal matrix enzymes GPDH or GAPDH revealed co-localization of both proteins on discrete globular structures in the cytoplasm (Figure 3A, B, D and E). This morphology was always seen in normal cells and was also dominant in the other cell lines when cultivated in the absence of tetracycline. In cells induced to overexpress either p24 or p24Cmyc, however, fluorescence was found in much larger, often elongated structures (Figure 3C and G). Ultrastructural analysis revealed that the overexpression of p24 and p24Cmyc in *T.brucei* resulted in an accumulation of intracellular tubules with an electron-dense matrix, bound by a single membrane and with a diameter about one-third that of normal glycosomes (Figure 4B and Table I). Clustered tubules often filled large parts of the cytoplasm. Immunogold labelling showed that the matrix of these tubules contained the glycolytic enzyme aldolase (Figure 4D–F), confirming that they were abnormal glycosomes. Their membranes were labelled specifically with an anti-p24 antiserum (Figure 4C) or, for p24myc, the anti-myc antiserum (Figure 4E and F). In addition to the clusters of tubules, some cells contained membrane whirls excluding detectable matrix and containing p24 (Figure 4G–I).

The moderate overexpression of p24Nmyc did not change glycosome morphology as dramatically as seen with p24 and p24Cmyc, consistent with the lack of other phenotypic effects. Although there was a tetracycline-induced increase in the number of cell sections with clusters (Table I), the tubules mostly had a larger diameter compared with those seen with p24 and p24Cmyc (not shown).

The N- and C-terminal ends of p24 are facing the cytosol

The p24 sequence contains two hydrophobic amino acid stretches that are predicted to form transmembrane helices (Figure 1B). To confirm the previously described membrane association of p24 (Aman and Wang, 1987), we extracted purified glycosomes successively with low salt buffer, high salt buffer and finally carbonate (Figure 5A). Whereas aldolase, a soluble matrix enzyme, is readily released into the high salt and carbonate supernatants, most of p24 remains inextractable in the carbonate pellet, suggesting membrane integration. To establish the membrane topology of p24, we performed protease protection assays using digitonin-permeabilized cells expressing the native and myc-tagged versions of p24. Native p24 completely resisted proteinase K digestion in the absence of Triton X-100, suggesting that it might reside largely on the inner side of the glycosomal membrane (Figure 5B), as suggested previously for ScPex11p (Marshall *et al*., 1996). The N- and C-terminally tagged p24myc proteins, however, behaved differently. The addition of proteinase without Triton X-100 resulted in the disappearance of the 27 kDa p24myc species and the appearance of a smear intermediate in size between p24 and p24myc. Under these conditions, the glycosomal matrix enzyme aldolase is protease resistant. p24 is degraded more strongly and aldolase is degraded to a slightly smaller protease-resistant core (Clayton, 1987) when detergent is added to the reaction (Figure 5B). This suggests that both


A
B
NH2
COOH
+2.19
0
-2.57
T. brucei p24
(36/64%)
(19/43%)
(25/57%)
(25/56%)
(31/46%)
+3.18
-2.13
ScPex11p

the N- and C-terminal ends of p24 are oriented towards the cytosol.

We next assessed the accessibility of p24myc to antibodies. The exposure of the myc epitope on glycosomes in a post-nuclear pellet fraction, and hence the cytosolic orientations of both N- and C-termini, were confirmed by an antibody capture assay. Whereas anti-myc antibodies precipitated similar amounts of p24myc from both p24Nmyc- and p24Cmyc-expressing cell lines, independently of whether they were added to the assay before or after detergent solubilization, anti-GAPDH antibodies precipitated significant amounts of GAPDH only when added after solubilization, reflecting the matrix localization of this glycolytic enzyme (Figure 5C).

p24 can form homodimers

The dimerization of Pex11p in yeast has been reported previously (Marshall *et al*., 1996). Dimerization of glycosomal p24 is readily demonstrated in cells overexpressing p24, but is also detectable in wild-type cells. Figure 6A illustrates the behaviour of p24 when total membranes were isolated from various cell lines in either the presence or absence of reducing agents. In the absence of dithiothreitol (DTT), a 40 kDa band was recognized by the anti-p24 antiserum (Figure 6A, lanes 449– and p24–). The intensity of this band was strongly reduced, but not abolished, if DTT was included in the extraction buffers (Figure 6A, lanes 449+ and p24+). In the cell lines expressing p24Nmyc, the p24 antiserum recognized three reduction-sensitive p24 bands of ~50, 45 and 40 kDa; in the p24Cmyc cell line, there were high molecular weight bands at 45, 42 and 40 kDa (Figure 6A). The 40 kDa band probably represents the p24 homodimer as it is present also in the wild-type (449) cells possessing only the endogenous p24 genes. In the lines overexpressing tagged versions of p24, one might expect two dimers larger than that, namely a p24myc homodimer and a p24–p24myc heterodimer. The top bands (running at 50 or 45 kDa) were indeed recognized by an anti-myc antibody in extracts of p24Nmyc- and p24Cmyc-expressing cell lines (data not shown). Unexpectedly, the intermediate bands (45 or 42 kDa) were not recognized; possibly the myc tag is conformationally masked in the heterodimer.

Table I. Quantification of glycosome morphology in ultrathin cell sections

Cell line[a]	Globular		Globular + tubular		Tubular (clusters)	
	+	–	+	–	+	–
449	52	50	44	46	4	4
p24	16	44	26	32	58	24
p24Nmyc	34	42	24	48	42	10
p24Cmyc	12	26	12	36	76	38

Using electron microscopy, 50 sections were evaluated for each incubation condition and grouped into three categories, depending on the morphology of their glycosomes.
[a]Cells were incubated for 20 h ± 1μg/ml tetracycline.
All results are given as percentages.

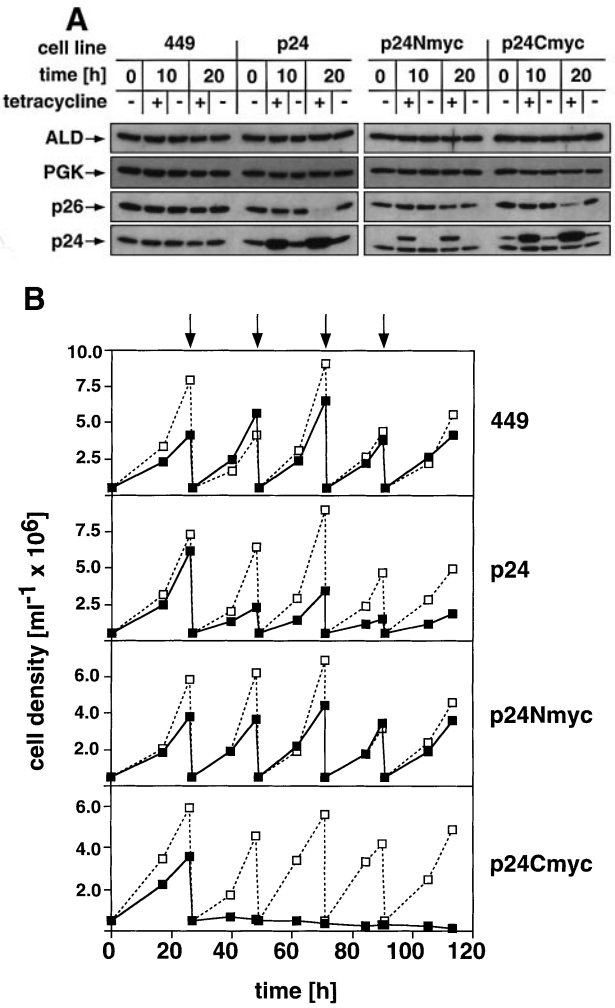


Fig. 2. Overexpression of p24 leads to growth arrest in *T.brucei*. (**A**) Wild-type (BF 449) trypanosomes and cell lines harbouring tet-inducible transgenes encoding p24, p24Nmyc or p24Cmyc were cultured for 0, 10 or 20 h with or without tetracycline before harvesting and processing for Western blot analysis. The blots (1.5×10^6 cells per lane) were probed with antisera against aldolase (ALD), phosphoglycerate kinase (PGK), p24 and p26. (**B**) The same cell lines as in (A) were grown in the presence (■) or absence (□) of tetracycline and counted at the intervals indicated. Fresh tetracycline was added after diluting cultures to 10^5 cells/ml every 24 h (arrows).

Fig. 1. Sequence similarity of p24, RnPmp26 and yeast Pex11ps. (**A**) CLUSTAL-based multiple sequence alignment of *T.brucei* p24 (accession No. AJ005114) with peroxisomal membrane proteins. ScPEX11p (accession No. X81465) and CbPEX11p (accession No. U36243) are peroxin 11 from *S.cerevisiae* and *C.boidinii* respectively. RnPMP26 is a peroxisomal membrane protein from *Rattus norvegicus*. Shaded in black are blocks of homology (minimum 75% compliance with consensus). The N-terminal sequence of p24 is largely identical to a published *T.brucei* expressed sequence tag (accession No. W00255). The symbols used for the consensus are: a, acidic (D, E); b, basic (K, R, H); p, polar (S, T, Y, G, N, Q, C); f, hydrophobic (L, I, V, F, W, M, P, A). Shown above the alignment are the peptide sequences obtained from p24. (**B**) Kyte–Doolittle hydrophilicity plots of *T.brucei* p24 and *S.cerevisiae* Pex11p. Blocks 1–5 represent the regions of homology marked in (A) with the percentage identity/similarity calculated by the GAP program (default gap weight 3). Two transmembrane segments are predicted for p24 by each of two algorithms. Shown in black is the transmembrane domain of p24 (residues 130–147) predicted by both PredictProtein and TopPred II programs. The first hatched domain (96–113) is predicted by PredictProtein and the second hatched domain (193–213) is predicted by TopPred II. Shaded in grey are three helical domains of ScPex11p (PredictProtein) that might interact with the membrane (residues 92–110, 133–145, 217–229).

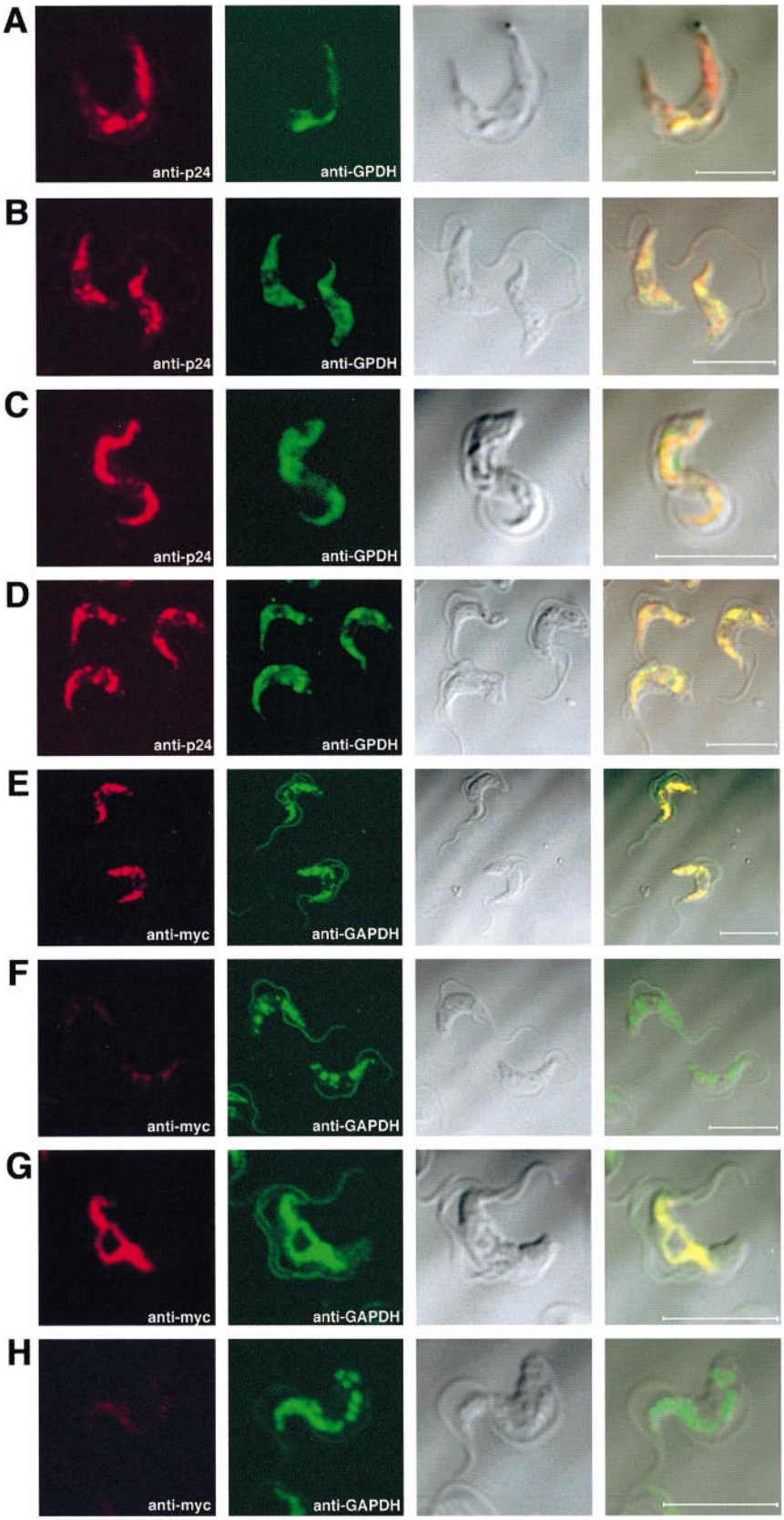


Fig. 3. Double immunofluorescence microscopy analysis of bloodstream form trypanosomes overexpressing p24. Trypanosomes were cultivated for 20 h with (A, C, E and G) or without (B, D, F and H) tetracycline before harvesting and processing for scanning laser confocal microscopy. (**A** and **B**) Wild-type (449) cells; (**C** and **D**) p24 transgenics; (**E** and **F**) p24Nmyc transgenics; (**G** and **H**) p24Cmyc transgenics. The first column in each row shows staining for p24 (Cy2, A–D) or p24myc (Cy5, E–H) using anti-p24 or anti-myc antibodies respectively. The second column shows staining for matrix markers GPDH (Cy5, A–D) or GAPDH (Cy2, E–H). The third and fourth column show interference contrast image and overlay. Scale bar 10 μm.

A second approach to investigate p24 dimerization was to test for co-immunoprecipitation of p24 from cells expressing p24myc using an anti-myc antibody. As shown in Figure 6B, anti-myc antibodies precipitate endogenous p24 only in extracts from cells also expressing p24Nmyc or p24Cmyc but not from wild-type cells expressing only p24. The other major protein in the glycosomal membrane, p26 (containing 10 cysteines in its primary structure, P.Lorenz, A.G.Maier and C.Clayton, in preparation), does not co-precipitate with p24myc, suggesting that homodimerization of p24 is specific.

To test the possibility that covalent disulfide bridge formation was an oxidation artefact occurring during isolation, we included in the extraction buffers alkylating agents known to block free sulfhydryl groups. The addition of 10 mM *N*-ethylmaleimide (NEM) led to a 90% reduction of p24 dimers in cells expressing wild-type levels of p24 (Figure 6C), suggesting that most sulfhydryl groups shown to be cross-linked in the earlier experiments were in a reduced state at the time of breaking the cells. Intriguingly, when using only 1 mM NEM, it appeared that the extra high molecular bands seen in cell lines expressing p24myc were more strongly affected than the wild-type p24 homodimer (not shown).

Heterologous expression of p24 in mammalian cells and yeast

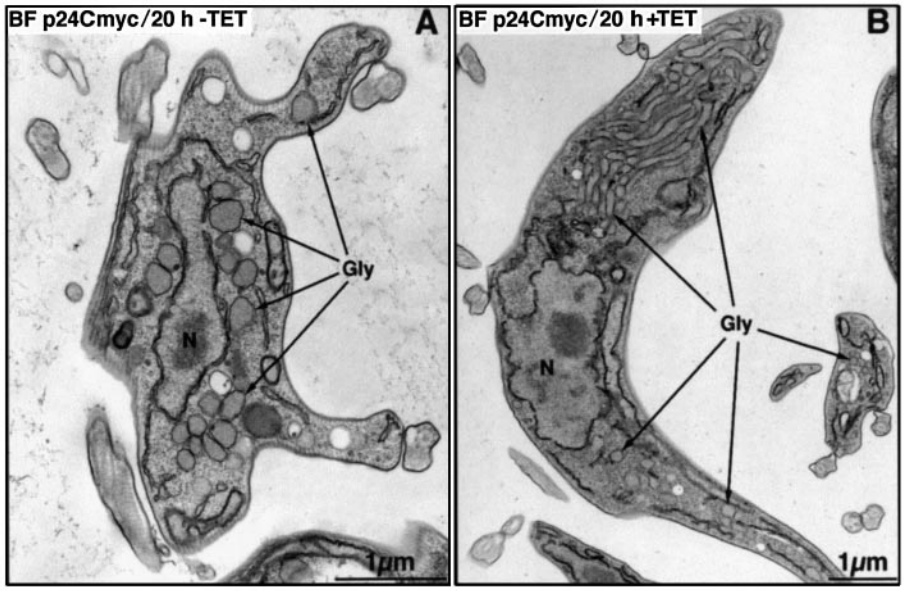
To investigate whether glycosomal sorting of p24 was mediated by an evolutionarily conserved mechanism, we transiently expressed the protein in monkey CV-1 cells. As seen in Figure 7, there was a clear co-localization of the peroxisomal matrix marker acyl-CoA oxidase and p24Cmyc on discrete spots scattered over the entire cytoplasm. Similarly, when expressed in *S.cerevisiae*, p24 was sorted to peroxisomes as evidenced by the co-localization of p24Cmyc and thiolase on discrete spots inside the cells (Figure 8B).

Prompted by the apparent sequence similarities between p24 and the yeast peroxin Pex11p, we attempted to complement functionally *pex11*Δ knockout mutants with the trypanosome gene. The *S.cerevisiae pex11*Δ mutants are characterized by a defect in peroxisome proliferation that leads to a growth defect on YNO plates containing oleate as the single carbon source and to the appearance of giant peroxisomes (Erdmann and Blobel, 1995; Marshall *et al*., 1995, 1996). As expected, introduction of *ScPEX11* restores peroxisome morphology as well as growth on YNO plates of *pex11*Δ cells (Figure 8A and B). Growth on YNO was also restored when the mutant strain was transformed with plasmids encoding p24 or p24Cmyc under the control of the *FOX3* promoter (Figure 8A). Furthermore, the heterologous expression of p24 and p24Cmyc led to the restoration of peroxisome morphology of *pex11*Δ mutant cells. Instead of the very few giant peroxisomes typical for oleate-grown *pex11*Δ mutant cells, transformants of *pex11*Δ that harbour the trypanosomal proteins showed normally sized and numbered organelles (Figure 8B). These observations suggest that trypanosomal p24 is able functionally to replace the ScPex11p in yeast peroxisome biogenesis. As p24Cmyc maintained the complementing activity, the tagging obviously did not interfere with p24 function in peroxisome biogenesis. Double immunofluorescence localization of p24Cmyc and peroxisomal thiolase (Fox3p) revealed a congruent fluorescence pattern (Figure 8B), indicating that p24Cmyc is targeted to peroxisomes in yeast. The heterologous complementation of the yeast mutant together with the peroxisomal localization of the protein in yeast and CV-1 cells strongly supports the notion that p24 is the *T.brucei* orthologue of Pex11p from yeast.

Trypanosome p24 is involved in glycosome division

To see if the absence of p24 in trypanosomes gives a similar phenotype to the deletion of *PEX11* in yeast, we attempted to create cell lines lacking the p24 gene. Yeast *pex11* mutants are viable on rich media, showing growth defects only when grown on media that require peroxisome function for catabolism. Bloodstream trypanosomes are obligatorily dependent on the glycolytic enzymes in the glycosome for survival. Procyclic (insect) forms, in contrast, metabolize amino acids and generate energy via mitochondrial pathways and so conceivably might be able to survive without glycosomal compartmentation. We therefore attempted to delete the p24 gene from wild-type (449) Pro trypanosomes, using constructs containing the neomycin phosphotransferase (*NPT*) or hygromycin phosphotransferase (*HYG*) genes as a selectable markers. Constructs contained untranslated regions from 5′ and 3′ of p24, and were designed to enable clean replacement of the p24 gene by homologous recombination (tenAsbroek *et al*., 1990). Upon transfection with the *NPT* construct, G418-resistant clones with only a single remaining copy of the p24 gene were obtained without difficulty. After a second transfection with the *HYG* construct, half of the cells were selected for hygromycin resistance alone, and half for resistance to both drugs. Of the 19 viable clones obtained using hygromycin selection alone, none were resistant to G418, suggesting that the *HYG* gene had replaced the *NPT* gene. Only five cell lines grew out of double drug selection, but all still expressed p24 (data not shown), indicating retention of the gene. These results suggest that p24 is essential in *T.brucei*.

To assess the knockout phenotype, we created ‘inducible knockouts’ in bloodstream and procyclic cell lines containing a tet-regulatable p24 transgene (see Materials and methods). Removal of tetracycline from the medium to switch off transgene expression did not result in slowed growth, but Western blot analysis revealed that in both bloodstream and procyclic lines, p24 expression was shut off extremely slowly. After ~10 days, the amount of p24 was reduced to 10% of the wild-type level (Figure 9A) and remained stable thereafter. These observations suggest that a minimum amount of p24 is required for survival, and that the tetracycline regulation was partially lost. In both bloodstream and procyclic cells, the reduction in p24 had no effects on growth or on the levels of glycosomal matrix enzymes and p26 (Figure 9A, and data not shown). In procyclic cells with reduced p24, the only ultrastructural alteration was an increase in the frequency of large membrane whirls, but only a small minority of sections were affected, and no overall change in phospholipids was present (not shown). The BF trypanosomes expressing ~10% of the normal levels of p24, in contrast, showed alterations that were consistent with a role in glycosome division. First, the number of glycosomes in the sections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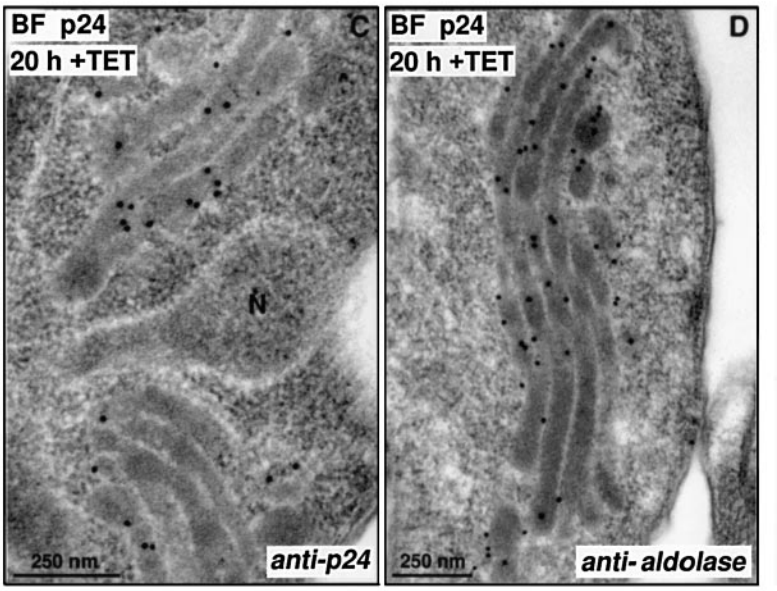
A
Gly
N
1µm
BF p24Cmyc/20 h +TET
B
Gly
N
1µm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20 h +TET
C
N
250 nm
anti-p24
BF p24
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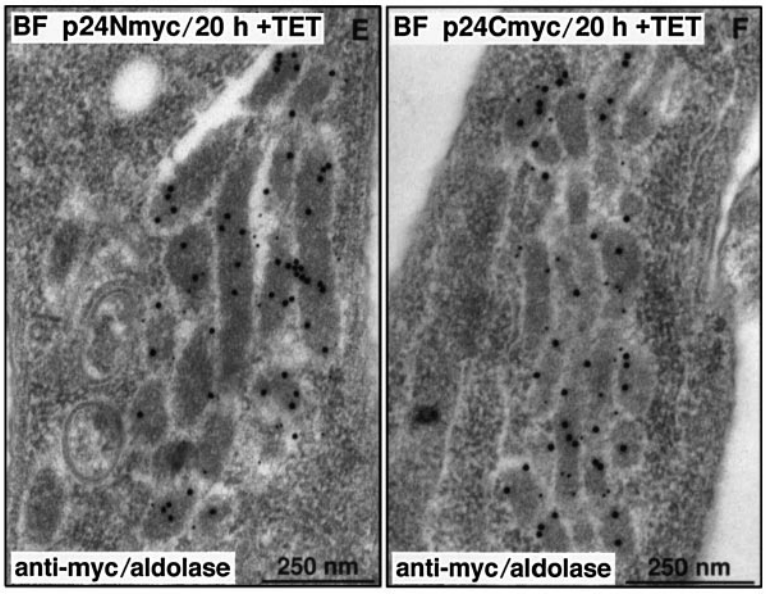
D
250 nm
anti-aldolase


BF p24Nmyc/20 h +TET
E
anti-myc/aldolase
250 nm
BF p24Cmyc/20 h +TET
F
anti-myc/aldolase
250 nm

was only two-thirds of the wild-type; and second, they were larger than normal (Figure 9B). The matrix of the enlarged organelles was less dense, consistent with a constant level of matrix enzymes. Thus p24 probably plays the same role in trypanosomes as Pex11p in yeast.

Discussion

The 24 kDa abundant membrane protein of *T.brucei* glycosomes has significant sequence similarity to the *PEX11* gene products of *S.cerevisiae* and *C.boidinii*, and the trypanosome gene functionally complements a *pex11*Δ strain of *S.cerevisiae*. A partial knockout of the p24 gene resulted in a defect in glycosome division, as seen for the *pex11* deletion in yeast. The trypanosome p24 gene can therefore be regarded as the structural and functional homologue of the yeast genes and should be designated *TbPEX11*. In normal trypanosomatid usage, the protein would be called TbPEX11, but for internal consistency we will here designate it as TbPex11p.

Implications for glycosome function

In BF trypanosomes, overexpression of TbPex11p led to growth arrest. One possible reason for this is that upon overexpression of the protein the glycosomes were transformed from a predominantly globular shape to extended tubules. These tubules aggregated into bundles often running the length of the cell and filling significant proportions of the cytoplasm; possibly this interfered with the mechanics of cell division. Alternatively, the lack of growth might have been secondary due to a slowing down in energy metabolism. If the volume occupied by the glycosomes increased in proportion to total cell volume, the enzymes of the glycolytic pathway would be diluted within the organelle, as no corresponding increase in enzymes was seen. In addition, the close apposition of membranes in the bundles of glycosomes could lead to a reduction in the effective surface to volume ratio of the organelle cluster. This might conflict with an efficient exchange of metabolites through the membrane. Additionally, the import of proteins into glycosomes within a bundle might be obstructed. This may explain the reduction of p26; alternatively, the TbPex11p overexpression level might have had a specific influence on the stability or expression of p26. The moderate overexpression of p24Nmyc caused neither a detectable reduction in p26 nor a slowing of growth, despite a significant increase in the number of elongated glycosomes and clusters (Table I). There are several possible explanations for this. Most obviously, the p24Nmyc expression may be too weak to impair growth. Alternatively, the N-terminal tag may impair function (including reducing p26) in some way. In either case, one possibility is that the growth retardation might be secondary to p26 reduction.

The overexpression of ScPex11p in *S.cerevisiae* caused a proliferation of small elongated peroxisomes (Marshall *et al*., 1995) and not the massive tubulation seen in trypanosomes overexpressing TbPex11p. This difference could be ascribed to different levels of overexpression; alternatively, the yeast protein might contain an additional activity to parcel the growing tubules into smaller units, or perhaps the glycosomes are inherently more stable in the elongated form whereas yeast peroxisome tubules spontaneously collapse. At present, we cannot distinguish between these possibilities.

We were quite surprised to discover that *TbPEX11* is essential, even in Pro trypanosomes, as a morphologically glycosomeless (but unstable) mutant of procyclic forms has been described previously (Sommer *et al*., 1996). However, there is evidence that glycosomes are essential

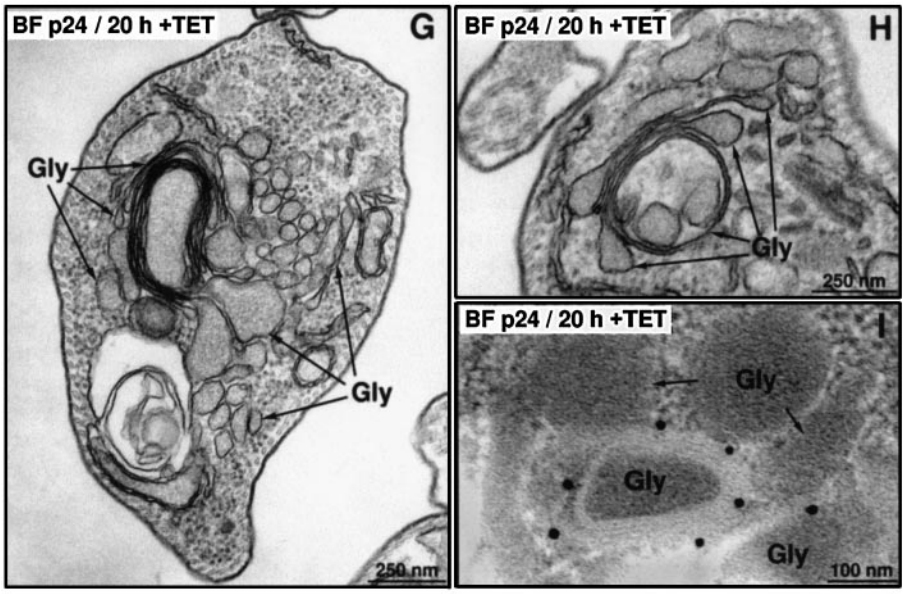


Fig. 4. Overexpression of p24 in *T.brucei* leads to the proliferation of glycosomal tubules. (**A** and **B**) The ultrastructure of p24Cmyc transgenic bloodstream form trypanosomes cultured for 20 h in the absence (A) or presence (B) of tetracycline. The tubules were also found in cells overexpressing p24 (**C** and **D**) and less frequently in cells expressing p24Nmyc (E), and could be decorated with antibodies against the glycosomal matrix marker aldolase (D) and p24 (C). (**E** and **F**) Co-localization of p24myc (6 nm gold) and aldolase (15 nm gold) on the same tubules in cells expressing p24Nmyc and p24Cmyc. Overexpression of p24 sometimes resulted in the formation of membrane whirls and club-shaped structures reflecting membrane proliferation (**G** and **H**, arrows). These whirls could also be labelled with anti-p24 gold complexes (**I**). A quantification of glycosome morphology on electron microscopical sections is shown in Table I. Gly: glycosomes, N: nucleus.

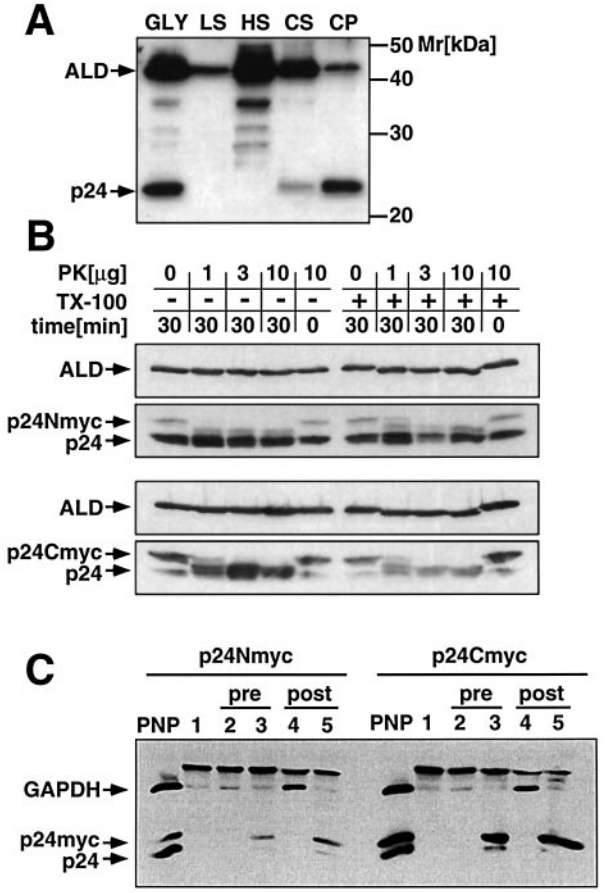


Fig. 5. p24 is an integral membrane protein with both the N- and C-termini facing the cytoplasm. (**A**) Carbonate extraction. Purified bloodstream form glycosomes (25 μg of protein) were extracted successively with low salt buffer, high salt buffer and sodium carbonate. After centrifugation, the resulting supernatants (LS, HS and CS) and the carbonate pellet were processed for Western blotting and probed with antisera against aldolase (ALD) and p24. (**B**) Protease protection assay. Procyclic trypanosomes were permeablized with digitonin. Increasing amounts of proteinase K (PK) were added to permeabilized cells on ice for either 0 or 30 min with or without Triton X-100. Separated proteins were detected with antisera to aldolase and p24. The top two panels show cells expressing p24Nmyc, the lower panels cells expressing p24Cmyc. (**C**) Antibodies have access to both N- and C-termini of p24 in a crude post-nuclear pellet fraction. Crude extracts of cells expressing either p24Nmyc or p24Cmyc received mouse antibodies to GAPDH (lanes 2 and 4) or myc (lanes 3 and 5) either pre- (lanes 2 and 3) or post-solubilization (lanes 4 and 5) followed by immunoprecipitation with protein A beads. After SDS–PAGE and Western blotting, detection was with rabbit anti-GAPDH and anti-p24 antisera. PNP, untreated post-nuclear pellet fraction; lanes 1, control precipitation using beads but no antibodies.

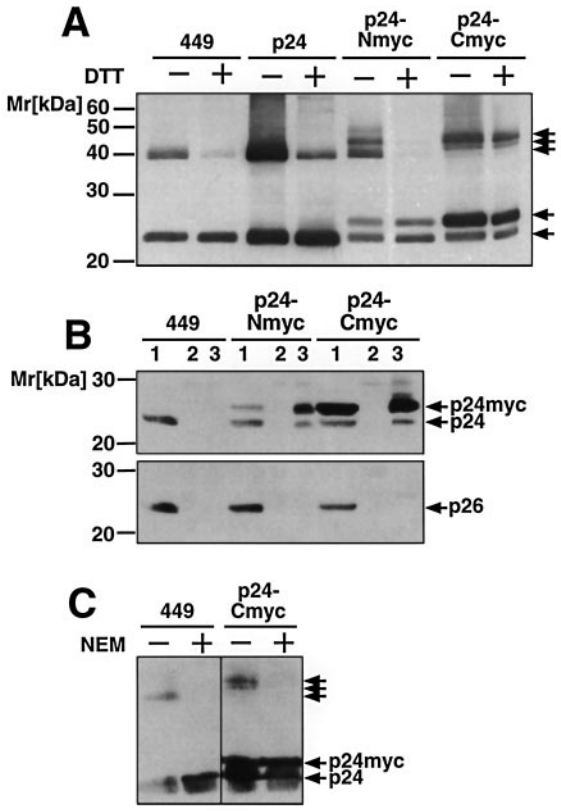


Fig. 6. p24 can homodimerize, yet covalent disulfide bonds are largely isolation artefacts. (**A**) High molecular weight forms of p24 are reduction sensitive. Total membranes of 10^7 procyclic trypanosomes were extracted with carbonate either in the presence or absence of DTT, separated by non-reducing SDS–PAGE and subjected to Western blot analysis (anti-p24 antiserum). Arrows indicate 24 (p24) and 27 kDa (p24myc) monomers and the high molecular weight dimers. (**B**) Co-immunoprecipitation of p24 and p24myc from 2×10^7 detergent-solubilized procyclic cells using antibodies against the myc epitope. The blot was probed with anti-p24 (upper panel) or anti-p26 antisera (lower panel). Lanes 1, total protein; lanes 2, pre-clear (protein A beads only); lanes 3, immunoprecipitate (beads plus anti-myc antibodies). (**C**) Most p24 high molecular weight forms are lost when free thiol groups are blocked. Western blot analysis of samples treated as in (A), but with 10 mM NEM included in the extraction buffers.

in *Leishmania major* promastigotes, which have an energy metabolism that it not dissimilar to that of Pro trypanosomes: here the *GIM1* gene (probably the *Leishmania* equivalent of yeast *PEX2*) was cloned and again it was not possible to generate double knockouts (Flaspohler *et al.*, 1997). The glycosome could be essential because it is required for energy metabolism, or for other pathways, such as pyrimidine or ether lipid biosynthesis (Opperdoes, 1987).

Conserved features of PEX11 proteins

All *PEX11* gene products described to date are small (24–29 kDa), very basic (isoelectric points >9) and membrane associated (≥40% hydrophobic amino acids), sharing conserved primary structures at their N- and C-termini. All are associated with the membranes of peroxisomes and related microbodies. The best-studied members of this family are its representatives in *S.cerevisiae* (ScPex11p/PMP27; Erdmann and Blobel, 1995; Marshall *et al.*, 1995) and *C.boidinii* (CbPex11p/PMP30; Sakai *et al.*, 1995). ScPex11p is the most abundant protein in the membrane of *S.cerevisiae* (Erdmann and Blobel, 1995), and TbPex11p is also highly abundant in the glycosomal membrane. Overexpression of ScPex11p in *S.cerevisiae* results in clusters of small peroxisomes, having a tendency to assume tubular shapes (Marshall *et al.*, 1996), similar to, but not as extreme as, what is seen with glycosomes in trypanosomes overexpressing TbPex11p (Figure 4B). Likewise, strong overexpression of Pex11p from a multicopy plasmid in *C.boidinii* was toxic (Sakai *et al.*, 1995), as is overexpression of TbPex11p in trypanosomes. Overexpression of Pex15p, another peroxisomal membrane protein in yeast (Elgersma *et al.*, 1997), leads to prolifer-

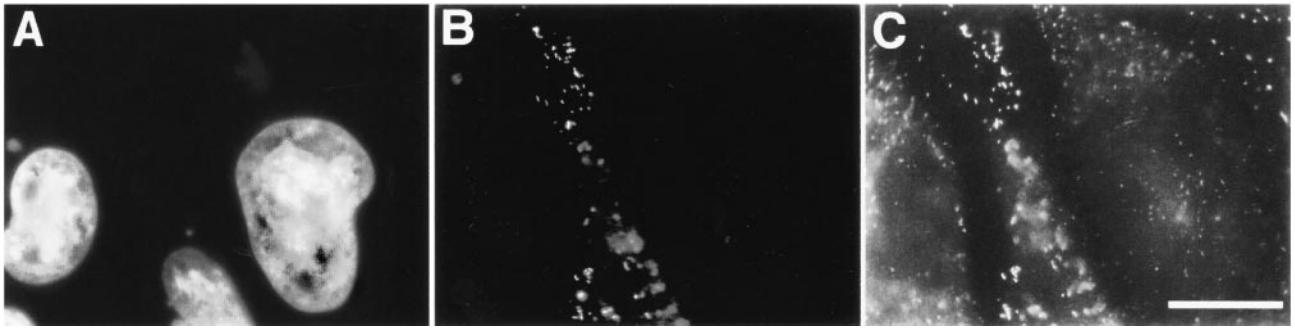


Fig. 7. p24 is targeted to peroxisomes in monkey CV-1 cells. Double immunofluorescence labelling of transiently transfected CV-1 cells showing p24Cmyc co-localizing with acyl-CoA oxidase. (**A**) DAPI staining of nuclei, (**B**) Cy2-coupled anti-myc staining for p24Cmyc, (**C**) Cy3-coupled anti-acyl-CoA oxidase staining. Only the cell in the middle of the panels expresses p24Cmyc. Scale bar 12 μm.

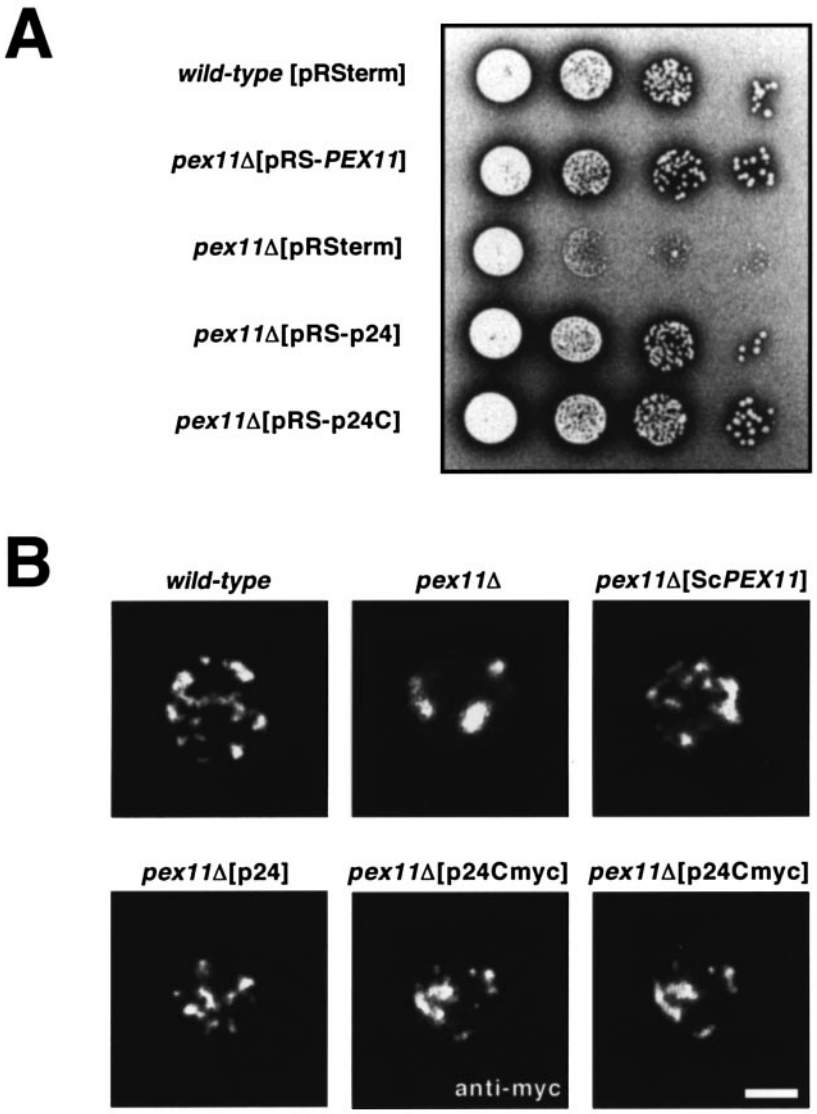


Fig. 8. Expression of p24 functionally complements the *pex11*Δ mutation of *S.cerevisiae*. Yeast *pex11*Δ mutants were transformed with the low copy plasmid pRSterm or pRSterm containing the genes encoding Sc*PEX11* (pRS-*PEX11*), p24 (pRS-p24) or p24Cmyc (pRS-p24C) under the control of the yeast *FOX3* promoter. Similar results were obtained for both UTL7A and W303A strains. (**A**) p24 restores growth of UTL7A *pex11*Δ cells on oleate. Wild-type, *pex11*Δ cells and the indicated transformants were plated on YNO at different densities. (**B**) p24 is targeted to yeast peroxisomes and restores normal peroxisome morphology of oleic acid-induced *pex11*Δ cells. Immunofluorescence localization of thiolase (in W303A wild-type and *pex11*Δ harbouring pRSterm, pRS-*PEX11*, pRS-p24) or thiolase plus myc-tagged p24 (in UTL7A *pex11*Δ harbouring pRS-p24C as indicated). Scale bar 5 μm.

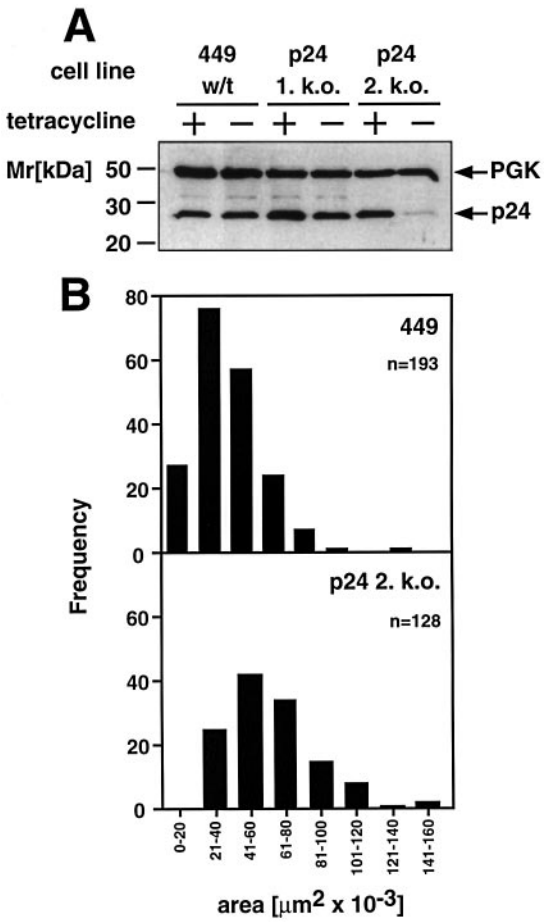


Fig. 9. Phenotype of bloodstream trypanosomes expressing low levels of p24. (**A**) Western blot of different cell lines grown for 13 days in the presence and absence of tetracycline. The nature of the cell line is shown above the lanes. (**B**) The inducible knockout cells, grown for 17 days without tetracycline, were compared with the wild-type cells by electron microscopy. For each cell line, the glycosomes from 50 sections were counted and their area measured. The total number of glycosomes (n) is shown, together with their size distribution.

ation of membranes resembling endoplasmatic reticulum; we were unable to completely rule out the possibility of a relationship with this organelle as we have no appropriate membrane markers for trypanosomes. In contrast, the co-localization of the post-translationally imported glycosomal matrix marker aldolase (Clayton, 1987) with all the elongated organelles in the overexpressing cells and their electron-dense appearance indicates that they are in fact glycosomes. Passreiter *et al*. (1998) have shown recently that coatomer can specifically associate with rat peroxisomes. Rat Pex11p (PMP26) has a C-terminal K-x-K-x-x sequence (Figure 1A), which is related to the consensus K-K-x-x signal mediating coatomer binding (Schekman and Orci, 1996). The trypanosome and yeast homologues lack such a C-terminal motif. Moreover, C-terminally tagged versions of the trypanosome (myc-tag; this study) and yeast (HA-tag; Erdmann and Blobel, 1995) proteins are functional, ruling out any requirement for a precise motif at the C-terminus.

Topology of PEX11 gene products

The nature of the Pex11p membrane association in yeast is controversial. Although a difference between species is possible, it is difficult to reconcile this with the strong functional conservation observed. Different degrees of resistance to alkali extraction have been described. Erdmann and Blobel (1995) reported ScPex11p to be predominantly carbonate resistant, whereas Marshall *et al*. (1995) could extract most of the protein when using a crude post-nuclear pellet as starting material, thus suggesting that carbonate resistance could be an artefact of peroxisome purification. We found TbPex11p to be largely carbonate resistant, as previously reported by others (Aman and Wang, 1987), whether we were using purified organelles (Figure 5A) or whole cells (Figure 6A) as starting material. In contrast to the yeast proteins which have no strongly predicted transmembrane domains, TbPex11p and RnPex11p (PMP26) have two hydrophobic segments predicted to form transmembrane helices (Figure 1B).

In TbPex11p, the two predicted transmembrane domains leave two topological options available for the N- and C-termini: both outside or both inside. Marshall *et al*. (1996) reported that Pex11p is completely resistant to low levels of protease; digestion was only seen at protease concentrations that also clipped a peroxisomal matrix protein. We confirmed this observation for TbPex11p, which was also resistant to levels of protease that efficiently attack matrix proteins in the presence of detergents. Indeed, even in the presence of Triton X-100, the untagged TbPex11p often appeared at least partially protease resistant (Figure 5B). Experiments with N- or C-terminally tagged TbPex11p, however, yielded different results: the tags were accessible to both antibodies and proteases, under conditions where matrix markers were completely inaccessible, indicating that they were exposed on the cytosolic side. Interestingly, after protease digestion, only the tags were removed, the body of TbPex11p remaining intact. The easiest interpretation of these results is that native TbPex11p is indeed located with 'both ends out' but is so tightly folded, and, as a highly basic protein, so closely apposed to the acidic head groups of the membrane lipids that it is protease resistant. The alternative, that tag addition disrupts the topology, is possible but unlikely, as the C-terminally tagged version is functional *in vivo*.

Implications of orientation for Pex11p function

The topology of Pex11p has implications for targeting, the chemical environment of the protein and, most importantly, its potential to interact with other cellular components. First, TbPex11p has a potential peroxisomal membrane targeting signal (x-x-[K/R]-[K/R]-x(3–7)-[T/S]-x-x-[D/E]-x) similar to that shown to be important for targeting of *C.boidinii* Pmp47 (Dyer *et al*., 1996), immediately downstream of the predicted transmembrane domain at residues 130–147 (Figure 1A). This is the only known peroxisomal targeting signal sequence present in the protein. Either this signal or others are capable of directing TbPex11p not only to the membranes of trypanosome glycosomes, but also to the membranes of simian and yeast peroxisomes (Figures 7 and 8B). Depending on the algorithm used to predict the topology, this targeting signal would be either on the cytosolic side, or, as found in CbPmp47 and the other known membrane targeting signals in *S.cerevisiae* (Hohfeld *et al*., 1991; Dyer *et al*., 1996; Elgersma *et al*., 1997), on the matrix side. However, Pex11p orthologues from other organisms,

and the other abundant glycosomal membrane protein p26, lack such a signal, so its presence in TbPex11p may be fortuitous.

Second, we found that TbPex11p, like ScPex11p, is capable of forming disulfide-linked homodimers. The dimerization is specific, as heterodimers with the abundant membrane protein p26 were not detected. It is not clear, however, whether disufide links are formed *in vivo*. When alkylating agents such as NEM were included in the extraction buffers, up to 90% of the covalently linked dimers were lost, indicating that they were isolation artefacts. Nevertheless, the ease of disulfide bridge formation and its specificity support the view of a very close, non-covalent homotypic interaction *in vivo*. If, as we think, the bulk of the protein is oriented towards the cytosol, disulfide bridge formation would be improbable as the environment is strongly reducing (S.Krieger and C.Clayton, in preparation). Should our assessment of the orientation be incorrect, a covalent link between intra-glycosomal parts of the protein might be feasible (the redox state within the glycosome is unknown). The specific N-terminal sulfhydryl group in ScPex11p, postulated by Marshall *et al*. (1996) to be involved in regulation of peroxisome proliferation, is not conserved in any other Pex11 protein.

Lastly, the orientation of the protein determines absolutely the molecules with which it can interact. Inwards orientation restricts heterologous interactions to matrix components and other membrane proteins. An outward-facing orientation would allow interactions with the whole battery of proteins involved in membrane biogenesis and vesicular transport in the cytoplasm. Given the accumulating evidence for cross-talk between the proteins involved in peroxisome biogenesis and components involved in vesicular transport (Titorenko *et al*., 1997; Passreiter *et al*., 1998), a cytosolic orientation for a protein involved in peroxisome division is very attractive.

Materials and methods

Cloning of p24 and construction of trypanosome expression vectors

Glycosomes were purified from BF trypanosomes (Aman *et al*., 1985) and resuspended in TEDS (25 mM Tris–HCl, pH 7.8, 1 mM EDTA, 1 mM DTT, 250 mM sucrose). The 24 kDa protein (p24) was isolated from 600 μg of total glycosomal protein by two rounds of denaturing SDS–PAGE; the 24 kDa band was processed for in-gel tryptic cleavage (Jeno *et al*., 1995), and derived peptide sequences (Figure 1A) were used to design degenerate PCR primers. The 5′ end of the gene was amplified from bloodstream form cDNA using oligos Z891039, directed against the *T.brucei* 5′-spliced leader sequence, and CZ580, a primer corresponding to the sequence MQNAIQDS. The 3′ end was amplified using a second specific oligo (CZ577) and oligo(dT) (CZ585). Finally, the full-length coding region was amplified from genomic DNA with Vent polymerase (New England Biolabs, Eschborn) using 5′-oligo CZ584 and 3′-oligo CZ599 and cloned into pBSII (Stratagene, Heidelberg) for sequencing (pBS-p24). A 3.2 kb *Bam*HI fragment from a corresponding genomic clone was isolated from a *T.brucei* λ-pGEM11-library (a gift of P.Michels, ICP Brussels). The coding sequences of the genomic clone and PCR product were identical. Six amino acid sequences obtained from the tryptic peptides were identified in the ORF, confirming that we had cloned the gene corresponding to the purified protein. To generate a construct to knock out the p24 gene, sequences flanking the p24 coding region 5′ (515 bp, using oligos CZ680/681) and 3′ (297 bp, using oligos CZ682/684) were amplified by PCR and cloned upstream and downstream of the *NPT* and puromycin resistance (*PUR*) genes.

For expression in trypanosomes, the p24 coding region was cloned into tet-inducible expression vectors pHD677 (to yield pHD804 for BF) and pHD678 (to yield pHD810 for Pro) (Biebinger *et al*., 1997). In addition, triglycyl-flanked myc epitope tags were fused to the N- (p24Nmyc) and C-terminus (p24Cmyc) of p24. For the N-terminal tags, annealed and kinased oligos CZ628 and CZ633 were ligated into *Apa*I-cut pHD804 and pHD810. For the introduction of a C-terminal myc tag, a PCR of full-length p24 was performed using Vent polymerase to introduce a unique 3′ *Bam*HI site (oligonucleotides CZ584 and CZ650). The *Apa*I–*Bam*HI product was cloned into pHD677 and pHD678 to yield pHD806 and pHD812 respectively.

All oligonucleotides (see Table II for details) were synthesized at ZMBH synthesis facilities (Dr Rainer Frank). Details of all vectors used in the experiments are available from the authors, and the *TbPEX11* sequence is deposited under accession No. AJ005114.

Trypanosome strains, culture conditions and recombinant cell lines

Pro (insect) form trypanosomes were derived from the 427 strain and BF trypanosomes were derived from the MITat 1.2 strain. Cell lines inducibly expressing p24 were generated as described (Beverley and Clayton, 1993; Biebinger *et al*., 1997). In all experiments, the 'wild-type' (449) cells are BF or Pro trypanosomes, as appropriate, stably expressing the tet repressor from the plasmid pHD449 (Biebinger *et al*., 1997). For puromycin selection of Pro trypanosomes, cells were transfected with 25 μg of linearized DNA. Puromycin was added to 1 μg/ml after 24 h, when the cells were cloned by limiting dilution; several clones had a homozygous (double) knockout. To knock out the endogenous p24 genes in BF trypanosomes, the first copy was replaced by *NPT* using G418 selection at 0.5 μg/ml, and the second copy replaced through loss of heterozygosity (Gueiros-Filho and Beverley, 1996), (S.Krieger, in preparation) by gradually increasing the G418 concentration to 50 μg/ml. The gene replacements were confirmed by Southern blot analysis.

Sequence analysis

DNA and protein sequences were analysed using the DNAStar software package (DNAStar Madison, WI) and the GAP alignment program (Genetics Computer Group Inc. Madison, WI). Homology searches were performed by blastp analysis (Altschul *et al*., 1990). Hydrophilicity plots (Kyte and Doolittle, 1982) were done using a nine amino acid window size. Secondary structure and transmembrane domain predictions were performed by the PredictProtein program available at EMBL, Heidelberg (Rost *et al*., 1995) and the TopPred II program (Claros and vonHeijne, 1994).

Electrophoresis and Western blotting

Proteins were separated on 12% SDS–PAGE gels. Western blotting onto nitrocellulose (0.45 μm, Schleicher und Schuell, Germany) was performed according to standard protocols (Towbin *et al*., 1979) and blots were processed for antigen detection with a chemiluminescence system (ECL, Amersham Buchler Braunschweig). The antibodies used for detection were mouse monoclonal anti-myc (9E10 at 1/1000; Evan *et al*., 1985, a gift of Dr M.Eilers, ZMBH) and the following rabbit antisera against *T.brucei* proteins: anti-p24 (#28680 dil. 1/1000), anti-p26 (#28612 at 1/1000), anti-aldolase (at 1/4000), anti-phosphoglycerate kinase C (at 1/3000; a gift of Dr P.Michels, ICP Brussels) and anti-glyceraldehydephosphate dehydrogenase (GAPDH, at 1/4000; Dr P.Michels). The antiserum to p24 was generated by immunizing rabbits with the N-terminal 21 amino acids of p24 (MSEFQRFVKLLEQT-DGRDKILC*; the C-terminal cysteine was added for coupling) linked to keyhole limpet haemocyanin (KLH, Pierce USA). The antiserum to p26 will be described elsewhere.

Digitonin fractionation and protease protection

Trypanosomes were washed once in ice-cold phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4), once in STE (250 mM sucrose, 25 mM Tris–HCl pH 7.4, 1 mM EDTA) and resuspended in ice-cold STEN (STE containing 150 mM NaCl), 0.1 mM phenylmethylsulfonyl fluoride (PMSF). A sample containing 10 μg of trypanosome protein was filled up to 98 μl with STEN (25°C). After adding 2 μl of digitonin (in dimethylformamide, DMF; final concentration 0.2 mg/mg protein) and 5 s vortexing, the cells were incubated at 25°C for 4 min, then centrifuged (2 min, 20 000 *g*). For protease protection assays, the pellets (resuspended in 85 μl of STEN) either received 10 μl of water or Triton X-100 (1% v/v final) and either 5 μl of water or protease solution (containing 1–10 μg of proteinase K). Incubations were stopped after 30 min on ice by adding 10% w/v trichloroacetic acid (TCA) (controls were stopped before adding protease). Protein precipitates were harvested by centrifugation, washed with acetone and analysed by SDS–PAGE and Western blotting.

Table II. Oligonucleotides used in this study

CZ577	5′-ATGGAATTCATGCA[AG]AA[CT]GCIAT[CT]CA[AG]GAT-3′
CZ580	5′-ATGGGATCCIGA[AG]TC[CT]TG[AG]ATIGC[AG]TT[CT]TGCAT-3′
CZ584	5′-TCCCCCGGGGGGCCCATGTCTGAGTTCCAAAGGTTTG-3′
CZ585	5′-CGGAATTCCTTTTTTTTTTTTTTTT[ACG][ACGT][ACGT]-3′
CZ599	5′-CGGAATTCCTATTTGATCTTGTTCCAGTTCAAA-3′
CZ628	5′-CACCATG*GGCGGAGGCGAGCAAAAGCTCATTTCTGAAGAGGA CTTGGGCGGAGGC*GGGCC-3′
CZ633	5′-C*GCCTCCGCCCAAGTCCTCTTCAGAAATGAGCTTTTGCTCGCC TCCGCC*CATGGTGGGCC-3′
CZ650	5′-CTAGGATCCTCA*GCCTCCGCCCAAGTCCTCTTCAGAAATGAG CTTTTGCTCGCCTCCGCC*TTTGATCTTGTTCCAGTTCAAATAC-3′
CZ680	5′-ATCGGGCCCAGCTAACGTAACGGCAGAGC-3′
CZ681	5′-ATCCTCGAGACTGCTGTTATCGAATACAA-3′
CZ682	5′-ATCGGATCCACAAGGGTCACAGGCTGCCA-3′
CZ684	5′-ATCTCTAGATGAGTAAATAAATATGCGCA-3′
CZ721	5′-ATCGGGCCCATGGTCTGTGATACACTGGT-3′
CZ722	5′-ATCGTCGACGGATCCCTATGTAGCTTTCCACATGT-3′
Z891039	5′-CGGAATTCAACGCTATTATTAGAACAGTTTCTGTACT-3′

Underlined are restriction sites introduced for cloning purposes. myc-tag coding sequences are in italics.

Immunoprecipitation

Trypanosomes (2×10^7 cells) were washed once and then resuspended in 50 µl of ice-cold PBS. Cells were broken by adding 50 µl of solubilization buffer (50 mM Tris–HCl pH 7.5, 0.5 M NaCl, 2% v/v IGEPAL CA-630, 10 µg/ml leupeptin, 0.2 mM PMSF) followed by incubation on ice for 30 min with 10 s vortexing every 10 min and centrifugation (5 min, 20 000 *g*). For precipitations, 90 µl of supernatant were made up to 200 µl with water before pre-clearing by adding 30 µl of bead slurry (Pharmacia protein A CL-4B Sepharose 1:1 v/v in buffer A: 10 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.2% v/v IGEPAL CA-630, 2 mM EDTA), rolling incubation (1 h, 4°C) and centrifugation. The supernatant was incubated with 10 µl of anti-myc antibodies (2 h, 4°C) before adding beads and incubation as above. Beads were washed three times in buffer A, twice in buffer B (10 mM Tris–HCl pH 7.5, 0.5 M NaCl, 0.2% v/v IGEPAL CA-630, 2 mM EDTA) and once in buffer C (10 mM Tris–HCl pH 7.5) before removing bound material by boiling in 30 µl of 2× sample buffer and processing for SDS–PAGE and Western blotting.

For the antibody accessibility studies, a post-nuclear pellet was prepared from 7×10^8 cells (Aman *et al*., 1985), resuspended in TESS (25 mM Tris–HCl pH 7.8, 1 mM EDTA, 0.25 M sucrose, 0.15 M NaCl, 4 µg/ml leupeptin) and split into five samples of 45 µl. Samples 2 and 3 received 5 µl of antibodies (mouse anti-GAPDH; a gift of Dr F.Opperdoes, ICP Brussels or anti-myc). After 1 h on ice, all samples were washed twice with 1 ml of TESS and dissolved in 45 µl of TESS, 1% v/v IGEPAL CA-630. After a further 30 min on ice, they were centrifuged to remove insolubles (20 min, 125 000 *g*), and samples 4 and 5 received 5 µl of antibodies for incubation as above. After 1 h, all samples received 200 µl of TESS, 1% v/v IGEPAL CA-630 and 30 µl of bead slurry for 1 h rolling incubation before the beads were processed for SDS–PAGE and Western blot as above.

Carbonate extraction of membranes

For extracting total cell membranes, 10^7 trypanosomes were incubated for 15 min in 300 µl of ice-cold low salt buffer (5 mM Tris–HCl pH 7.8, 1 mM EDTA, 0.1 mM PMSF, 4 µg/ml leupeptin), passed through a micropipette tip (10×) then centrifuged (30 min, 20 000 *g*, 4°C). The pellet was resuspended in 300 µl of high salt buffer (25 mM Tris–HCl pH 7.8, 0.5 M KCl, 1 mM EDTA, 0.1 mM PMSF, 4 µg/ml leupeptin), incubated for 15 min on ice and centrifuged as above. The resulting pellet finally was resuspended in 300 µl of 0.1 M Na_2CO_3 (Fujiki *et al*., 1982) and, after 30 min on ice, was centrifuged for 1 h (125 000 *g*, 4°C) through a cushion of 500 µl of 0.1 M Na_2CO_3, 0.25 M sucrose. Protein was precipitated from supernatants by adding 10 µl/ml of 0.15% w/v sodium deoxycholate and 10% w/v TCA. Before SDS–PAGE, the pH was adjusted with 3 M Tris base as necessary. Optionally, the low salt and high salt buffers received 1 and 10 mM DTT respectively. In some experiments, all buffers contained 10 mM NEM. For extraction of glycosomal membranes, 20 µl (~100 µg of protein) of glycosomes in TEDS were diluted into 480 µl of ice-cold low salt buffer, left on ice for 6 min, subjected to five freeze–thaw cycles and then centrifuged for 30 min at 125 000 *g*, 4°C. The resulting pellet was resusupended in 500 µl of high salt buffer. After 30 min on ice, the suspension was centrifuged for 30 min at 125 000 *g*, 4°C. The resuspending of the resulting high salt pellet in sodium carbonate and all subsequent steps were as described above for the whole-cell membrane extraction except that the incubation on ice was for 60 min.

Immunofluorescence and electron microscopy

For light microscopy, trypanosomes were washed once in ice-cold PBS and fixed 1 h at 4°C in 1% v/v formaldehyde in PBS. The cells then were immobilized by spinning onto poly-L-lysine-coated chamberslides (200 *g*, 10 min, this and all following steps at room temperature) and permeabilized for 25 min in PBS, 0.2% v/v Triton X-100. Incubation with primary antibodies was for 1 h in PBS, 10% fetal calf serum (FCS) (dilutions: rabbit anti-p24 1/100, mouse anti-GPDH 1/200; a gift of Dr F.R.Opperdoes, ICP Brussels, mouse anti-myc 1/200). The secondary antibodies were Cy2-labelled goat anti-rabbit IgG and Cy5-labelled goat anti-mouse IgG both 1/800 (Amersham Buchler GmbH, Germany). Coverslips were mounted with 90% glycerol in PBS, 0.5% w/v 4′,6-diamidino-2-phenylindole (DAPI) and 5% w/v 1,4-diazabicyclo[2.2.2]octane (DABCO).

Adherent CV1 cells were fixed for 15 min in fresh 3.7% w/v paraformaldehyde in PBS (all procedures at room temperature). After washing (2× 10 min in 0.1 M glycine, PBS), permeabilizing (2× 5 min in PBS, 0.1% v/v IGEPAL CA-630) and blocking (45 min in PBS, 0.5% v/v IGEPAL CA-630, 5% FCS), the cells were incubated for 45 min with the primary antibodies (mouse anti-myc, 1/50; rabbit anti-rat acyl-CoA oxidase 1/200) in blocking solution. After washing with blocking solution (3× 5 min), cells were incubated with the secondary antibodies (45 min, Cy2-labelled goat anti-rabbit IgG 1/500, Cy3-labelled goat anti-mouse IgG 1/300; Amersham Buchler Braunschweig) before a final wash (3× 5 min in PBS, 0.5% v/v IGEPAL CA-630) and mounting. Yeast cells were processed for immunofluorescence microscopy as described (Erdmann, 1994).

For ultrastructural analysis, trypanosomes were fixed for 60 min in 4% paraformaldehyde, 0.05% glutaraldehyde, 2% sucrose in PBS. For routine electron microscopy, cells were post-fixed with reduced osmium and embedded in Epon 812. For immunocytochemistry, cells were embedded directly in LR White. Immunocytochemical localization of glycosomal antigens was done by post-embedding labelling on LR White sections and detection of antigen–antibody complexes with gold-labelled secondary antibodies (Baumgart, 1994).

Expression of p24 in yeast and monkey CV-1 cells

For the expression of p24Cmyc in monkey cells, the dihydrofolate reductase gene was removed with *Hin*dIII–*Bgl*II from pSV2-DHFR (Subramani *et al*., 1981) and replaced by the p24Cmyc gene excised with *Apa*I–*Bam*HI from pHD806. The resulting plasmid was designated pSV2-p24C. CV-1 cells were transfected at 80% confluency using LipofectAce (Gibco Life Technologies) following the instructions of the manufacturer.

For stable expression in yeast, the ORFs for p24 and p24Cmyc were excised from pBS-p24 and pHD806 with *Apa*I–*Eco*RI and *Apa*I–*Bam*HI respectively, blunted and inserted into *Eco*RV-cut pPR6/39 (Rehling, 1996) downstream of a thiolase promotor (*FoxP*). *PEX11* from *S.cerevisiae* was PCR-amplified from pCSp27 (Erdmann and Blobel, 1995) using oligos CZ721 and CZ722 and Vent polymerase, cut with *Apa*I–*Bam*HI and inserted blunt into *Eco*RV-cut pPR6/39. The *FoxP* gene fusions were

checked for correct orientation, excised with *Bam*HI–*Xho*I, blunted and subcloned into *Eco*RV-cut pBSII to invert restriction site orientation. Finally, they were excised with *Xho*I–*Bam*HI and cloned in front of the CYC1-terminator in pRSterm. For the construction of pRSterm, the *Xho*I–*Ase*I fragment of pYES2.0 (Invitrogen) containing the CYC1-terminator was first subcloned into pSL1190 (Pharmacia) and subsequently with *Xho*I–*Kpn*I into pRS316 (Sikorski and Hieter, 1989).

Transformation and cultivation of *S.cerevisiae* wild-type strains UTL7A (*MATa, ura3-52, trp1/his3-11,15, leu2-3,112*) and W303A (*MATa, ura3-52, trp1, his3-11,15, leu2-3,112, can1-100*) and mutant strains UTL7A *pex11*Δ (*MATa, ura3-52, trp1/his3-11,15, leu2-3,112, pex11::LEU2*) and W303A *pex11*Δ (*MATa, ura3-52, trp1, his3-11,15, leu2-3,112, can1-100, pex11::LEU2*) were performed according to Erdmann and Blobel (1995).

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