Compartmentation of enzymes in a microbody, the glycosome, is essential in *Trypanosoma brucei*

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Summary

All kinetoplastids contain membrane-bound microbodies known as glycosomes, in which several metabolic pathways including part of glycolysis are compartmentalized. Peroxin 2 is essential for the import of many proteins into the microbodies of yeasts and mammals. The *PEX2* gene of *Trypanosoma brucei* was identified and its expression was silenced by means of tetracycline-inducible RNA interference. Bloodstream-form trypanosomes, which rely exclusively on glycolysis for ATP generation, died rapidly upon PEX2 depletion. Insect-form (procyclic) trypanosomes do not rely solely on glycolysis for ATP synthesis. PEX2 depletion in procyclic forms resulted in relocation of most tested matrix proteins to the cytosol, and these mutants also died. Compartmentation of microbody enzymes is therefore essential for survival of bloodstream and procyclic *T. brucei*. In contrast, yeasts and cultured mammalian cells grow normally in the absence of peroxisomal membranes unless placed on selective media.

Key words: Trypanosoma, Kinetoplastida, Glycosome, Peroxisome biogenesis, Glycolysis

Introduction

The kinetoplastids are unicellular parasites that cause lethal infections of millions of people in the tropics. Chemotherapy relies on a limited selection of rather toxic drugs and resistance is increasing. The search for new drug targets focuses on unique aspects of parasite biology and metabolism. One of these is the glycosome.

Glycosomes are peroxisome-related microbodies found in all kinetoplastids; they contain the first 7-9 enzymes of glucose metabolism. The sleeping sickness trypanosome, *Trypanosoma brucei*, is entirely dependent on glycolysis for ATP generation while it multiplies in the mammal. Expression of either phosphoglycerate kinase (PGK) or triosephosphate isomerase in the cytosol inhibits parasite growth (Blattner et al., 1998; Helfert et al., 2001), suggesting that correct localisation of glycolytic enzymes is important. Various results, including metabolic modeling, suggest that in bloodstream *T. brucei*, compartmentation plays a vital role in the regulation of glycolysis (Bakker et al., 2000; Michels et al., 2000).

T. brucei is transmitted between mammals by tsetse flies. The 'procyclic' form that multiplies in the tsetse depends for ATP generation mainly on the mitochondrial metabolism of amino acids (Michels et al., 2000). Several enzymes from pathways other than glycolysis can be associated with trypanosomatid glycosomes, their activities varying considerably between parasite species and life-cycle stages (Michels et al., 2000).

Microbody biogenesis depends on peroxin (PEX) proteins, which are needed for microbody membrane formation and the import of matrix proteins from the cytosol (Baerends et al., 2000; Subramani et al., 2000; Titorenko and Rachubinski, 2001). Mutations of the PEX2 protein lead to defective import of matrix proteins into the peroxisomes of mammalian cells and yeasts (Eitzen et al., 1996; Faust and Hatten, 1997; Waterham et al., 1996); a number of other defects could be secondary to metabolic disturbance (Baerends et al., 2000). The peroxisomes of *pex2* mutants (those lacking PEX2) are reduced to empty ghosts (Hettema et al., 2000). PEX2 has two essential membrane-spanning regions and the N- and C-termini are oriented towards the cytosol (Harano et al., 1999). The C-terminus contains a RING finger motif of uncertain function (Shimozawa et al., 2000). Evidence from *Yarrowia lipolytica* indicates that PEX2 traffics through the endoplasmatic reticulum and is involved in the early stages of peroxisome formation (Titorenko et al., 2000; Titorenko and Rachubinski, 2001).

The *PEX2* gene of the kinetoplastid *Leishmania donovani* was identified by complementation of a mutant with a partial defect in glycosomal matrix protein import (Flaspohler et al., 1997). The defect in the mutant was caused by premature termination in one copy of the *PEX2* gene. This encoded a protein lacking one transmembrane domain and the RING finger (Flaspohler et al., 1997; Mannion-Henderson et al., 2000), which had a dominant-negative effect. It was not possible to delete both copies of the *LdPEX2* gene (Flaspohler et al., 1999).

Mammalian tissue culture cells survive indefinitely in the absence of PEX2 function; *pex* mutations are lethal in animals because of the accumulation of metabolites or substrates and consequent defects. Yeasts lacking peroxisomal membranes grow normally on glucose, but die when they are restricted to a carbon source that is metabolised by peroxisomal enzymes, such as oleate in *Saccharomyces cerevisiae* (Erdmann et al., 1989) or methanol in *Hansenula polymorpha* (Waterham et

al., 1994). The situation in *pex* mutants of bloodstream trypanosomes grown on glucose should be analagous to that of oleate- or methanol-grown yeasts. One would therefore predict that the glycosomal membrane should be essential in bloodstream forms of *T. brucei*, but dispensable in the procyclic forms, which do not need glycolysis to generate ATP and may thus cope without glycosomes.

We have previously shown that downregulation of the trypanosome *PEX11* gene reduces glycosome numbers (Lorenz et al., 1998), and that depletion of the GIM5 glycosomal membrane protein is deleterious to bloodstream forms (Maier et al., 2001). However, neither of these mutations had any affect on glycosomal matrix protein import. To find out the role of glycosomal compartmentation in trypanosomes, we needed to relocate complete pathways to the cytosol. We achieved this by downregulating PEX2 expression.

Materials and Methods

Cell culture

Bloodstream and procyclic forms of *T. brucei* 427, expressing the *tet* repressor (cell line 449 (Biebinger et al., 1997)), and the bloodstream line 328.114 (Wirtz et al., 1999) expressing the repressor and T7 polymerase, were cultured and transfected as previously described (Biebinger et al., 1997). Gene expression was induced with 100 ng/ml of tetracycline (Tet).

Plasmid constructs

Clones from the bacteriophage P1 library of *T. brucei* TREU927/4 (Sarah Melville, University of Cambridge) were selected by screening with a genomic PCR product. Sequencing was done by the ZMBH sequencing facility and by TopLab (Martinsried, Germany). The complete open reading frame, and a version with a termination codon replacing the codon for glutamine 239, pex2Δr, were amplified using suitable primers and cloned into the vectors pHD676 or pHD677 (Biebinger et al., 1997) for expression from the Tet-inducible EP1 promoter, $P_{EP1}{}^{Ti}$. pHD789 (Irmer and Clayton, 2001) was used for inducible expression from the T7 promoter. In the resulting construct, the hygromycin resistance cassette could be transcribed only from the inducible T7 promoter, but the background transcription in the absence of Tet was sufficient to allow selection of hygromycin-resistant cells. The pSP72 'stuffer' fragment (Shi et al., 2000) was a gift from Elisabetta Ullu (Yale University, New Haven, CT). A 468 bp fragment from the 5′ end of *PEX2* open reading frame, Δ*pex2*, was cloned upstream of the stuffer in the sense orientation and downstream of the stuffer in the antisense (*as*) orientation. The Δ*pex2*-stuffer-Δ*pex2as* was cloned into the polylinker of the inducible trypanosomatid expression vector pHD677 [(Biebinger et al., 1997) http://www.zmbh.uni-heidelberg.de)]. Reconstructed plasmid sequences are available from the authors on request.

Northern blotting

Poly(A)+ RNA was isolated from procyclic cells using oligo(dT) cellulose (Pharmacia). Resolution of 4 μg of mRNA on agarose/formaldehyde gels, transfer to neutral nylon membranes, prehybridisation and hybridisation with radioactive probes were done using standard procedures.

Immunofluorescence and western blotting

Cells were fixed and stained as described (Maier et al., 2001) using mouse polyclonal anti-*T. brucei* glyceraldehyde phosphate dehydrogenase (GAPDH) or rabbit polyclonal anti-*T. brucei* PEX11, aldolase (ALD), hexokinase (HXK) and phosphofructokinase (PFK) antisera. Detection was with Cy2- or Cy3-linked secondary antibodies (Amersham). For Fig. 2, the anti-ALD and anti-PEX11 antibodies were purified on Protein A beads, coupled with AlexaFluor™ 488 (Molecular Probes) following the manufacturer's instructions, and applied after the PEX11 or the HXK stain. The software used was OpenLab™ (Improvision) and Adobe® PhotoShop.

For western blotting, cells were fractionated with digitonin (Helfert et al., 2001; Lorenz et al., 1998). Incubations were with rabbit polyclonal anti-*T. brucei* PEX11, PFK, GAPDH and ALD; and mouse monoclonal anti-*T. brucei* surface protein EP1 (Cedarlane, Ontario). A rabbit polyclonal antibody raised against a peptide from a *T. brucei* RNase II-like protein (accession number AJ309002) recognises an unidentified cytosolic 100 kDa protein (*Tb*CSM1), which is not the original target and was used as a cytosolic marker.

Enzyme assays

2×10^8 cells were washed in cold 25 mM Tris-HCl pH 7.8, containing 1 mM EDTA and 10% sucrose, resuspended in 0.5 ml of the same buffer with protease inhibitors (Mini-complete, Roche) and 50 mg of glass beads (212-300 μm, Sigma) and broken at 4°C by three (bloodstream) or six (procyclic) vibration pulses (5 seconds each, mini-bead beater). A post-nuclear supernatant was microfuged for 15 minutes at 20,000 ***g*** to give a cytosolic fraction and a pellet containing glycosomes and other organelles. HXK activity was measured by following the reduction of $NADP^+$ at 340 nm in 10 mM $MgCl_2$, 10 mM D-glucose, 660 μM ATP, 5.635 mM $NADP^+$, 4.75 mM $NaHCO_3$ and 5 μg/ml of glucose-6-phosphate dehydrogenase (G6PDH) in 0.1 M TEA buffer pH 7.3 at 25°C. Phosphoglucoisomerase (PGI) activity was measured in the same way, but the reaction mix contained 7 mM $MgCl_2$, 1.3 mM fructose-6-P, 400 μM $NADP^+$ and 5 μg/ml of G6PDH in 0.1 M TEA buffer.

Results

Identification of PEX2 in *T. brucei*

The *T. brucei PEX2* gene was identified by homology screening of the sequence database; the sequence was completed (accession number AJ316008) and also later released as part of chromosome III (EMBL:AC091330). The sequence contained a 999-bp open reading frame encoding a protein of 37.8 kDa. Comparison with the predicted amino acid sequences of known or probable *PEX2* genes from *Leishmania donovani*, *Arabidopsis thaliana*, *Yarrowia lipolytica*, *Rattus norvegicus* and *Drosophila melanogaster* revealed that only 7% of the amino acids were identical, and 20% similar, in all species. The identity score was highest (48%) for *Leishmania*; for other individual species it ranged from 22 to 25%. All PEX2 sequences, including the one of *T. brucei*, contain two (in some cases, three) predicted trans-membrane domains and the RING finger. A glutamic acid residue (E55 in the human sequence) whose mutation in humans results in infantile Rhesum's disease (Fujiwara et al., 2000) is universally conserved.

The *PEX2* mRNA was detectable on northern blots as two bands of 1.3 kb and 1.7 kb (Fig. 1A, –Tet), and at similar abundance in poly(A)+ RNA from bloodstream and procyclic trypanosomes. These bands were extremely faint or undetectable when total RNA was used. We do not know the reason for the presence of the two bands. Attempts to map polyadenylation sites and splice sites by reverse transcription and PCR failed to yield specific products. By restriction digestion and Southern blot hybridisation, we found just two

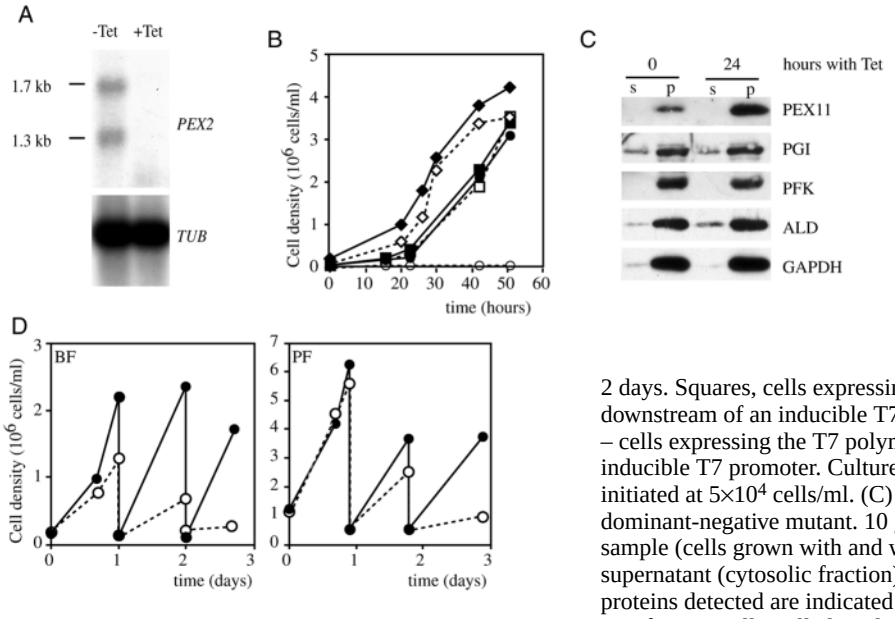

Fig. 1. (A) Northern blot. Double-stranded RNA causes destruction of *PEX2* mRNA. Each lane on the blot contains 10 μg poly(A)+ RNA from the procyclic form RNAi cell line. Cells were grown either without Tet (–), or for 13 hours in the presence of 100 ng/ml Tet (+). (B) Growth curves. Filled figures and unbroken lines, cells grown without Tet; open figures and dashed lines, cells grown in the presence of 100 ng/ml Tet. Diamonds, cells with *pex2Δr* downstream of the inducible RNA polymerase I promoter. The cultures were initiated at 1.5×10^5 cells/ml and followed for 2 days. Squares, cells expressing the T7 polymerase and with a full-length PEX2 downstream of an inducible T7 promoter. Circles, the dominant-negative mutant – cells expressing the T7 polymerase and with the *pex2Δr* gene downstream of an inducible T7 promoter. Cultures of cells expressing the T7 polymerase were initiated at 5×10^4 cells/ml. (C) Western blot. Digitonin treatment of the dominant-negative mutant. 10 μg of protein ($1\text{-}2\times10^6$ cells) were taken from each sample (cells grown with and without Tet) for treatment with 6 μg of digitonin. s, supernatant (cytosolic fraction); p, pellet (glycosomal fraction). The glycosomal proteins detected are indicated on the right. (D) Growth curves of RNA interference cells. Filled circles and unbroken lines, control – cells grown without Tet; open circles and broken lines, cells in the presence of 100 ng/ml Tet. Bloodstream cultures were initiated at 1×10^5 cells/ml and daily diluted to the same concentration. Procyclic cultures were daily diluted to 5×10^5 cells/ml.

apparently identical allelic copies of *PEX2* in genomic DNA (data not shown; C. Guerra-Giraldez, PhD Thesis, University of Heidelberg, 2001), so the different mRNAs could come from alternative splicing or polyadenylation.

For detection of PEX2 protein, rabbits were immunized with two peptides, corresponding to amino acids 151-168 and 253-278. None of the four sera obtained recognised any protein of the expected size, either in total cell extracts or in purified glycosomes, although they all detected a *Tb*PEX2-maltose-binding-protein fusion overexpressed in *E. coli* (C. Guerra-Giraldez, PhD Thesis, University of Heidelberg, 2001). This suggests that PEX2 might be present at rather low abundance in trypanosomes.

To overexpress PEX2 in trypanosomes, the open reading frame was cloned downstream of the Tet-inducible RNA polymerase I promoter, $P_{EP1}{}^{Ti}$. The construct was transfected into transgenic bloodstream trypanosomes expressing the *Tet* repressor (449 cells) and clonal cell lines selected. When Tet was added, the expression of two *PEX2* mRNAs, slightly bigger than the endogenous ones, was strongly induced, but no protein was detected with the anti-*Tb*PEX2 antibodies. Finally, a myc tag was added to the inducible copy of *PEX2*; upon addition of Tet no myc-tagged protein was detectable, either in total lysates or in the organellar pellet made by digitonin or carbonate fractionation. We also failed to detect PEX2 inducibly expressed from a bacteriophage T7 promoter in trypanosomes expressing T7 polymerase. Attempts to detect the PEX2 protein after pulse-labeling with [^{35}S]methionine were similarly unsuccessful. The addition of tetracycline to all these inducible cell lines had no effect at all on cell growth (in Fig. 1B, open squares show the growth of cells inducibly overexpressing PEX2; filled squares correspond to the same cells grown without Tet) and morphology, suggesting that overproduction of PEX2, if it occurs, has no deleterious consequences.

These results suggest that PEX2 is expressed at low abundance in trypanosomes, and expression of high levels is not possible. The most likely explanation is that PEX2 can occupy a limited number of sites on the glycosomal membrane and any superfluous PEX2 is degraded.

Overexpression of truncated PEX2 is lethal in bloodstream forms

Flaspohler, Parsons and colleagues had previously shown that expression of a version of PEX2 that lacked one transmembrane domain and the C-terminal RING finger caused a partial defect in glycosomal protein localization in *L. major*, even with a background of normal PEX2 expression (Flaspohler et al., 1999; Flaspohler et al., 1997). These studies were done when no inducible expression system was available for *Leishmania*, and the cell lines showed no growth defects. We therefore thought it might be possible to see rather more severe effects in trypanosomes using an inducible gene expression system. To construct a gene encoding the truncated trypanosome PEX2, we replaced the codon for glutamine 239 with a termination codon, just as had been done for *Leishmania*. The truncated gene *pex2Δr* was then placed downstream of the Tet-inducible RNA polymerase I promoter, $P_{EP1}{}^{Ti}$. The construct was transfected into 449 bloodstream trypanosomes and clonal cell lines selected. When Tet was added, the expression of *pex2Δr* mRNA was strongly induced and a very marginal decrease in the growth rate of the parasites was seen (Fig. 1B, open diamonds).

We next placed the truncated gene downstream of a Tet-inducible T7 polymerase promoter, transfected the construct into bloodstream trypanosomes expressing T7 polymerase, and selected clones in the absence of Tet. Upon addition of Tet, the growth of the cells was severely impaired (Fig. 1B, open circles), although even here the truncated pex2Δr could not be detected. Examination of the cells by immunofluorescence, using anti-PEX11 antibodies, revealed only a low number of

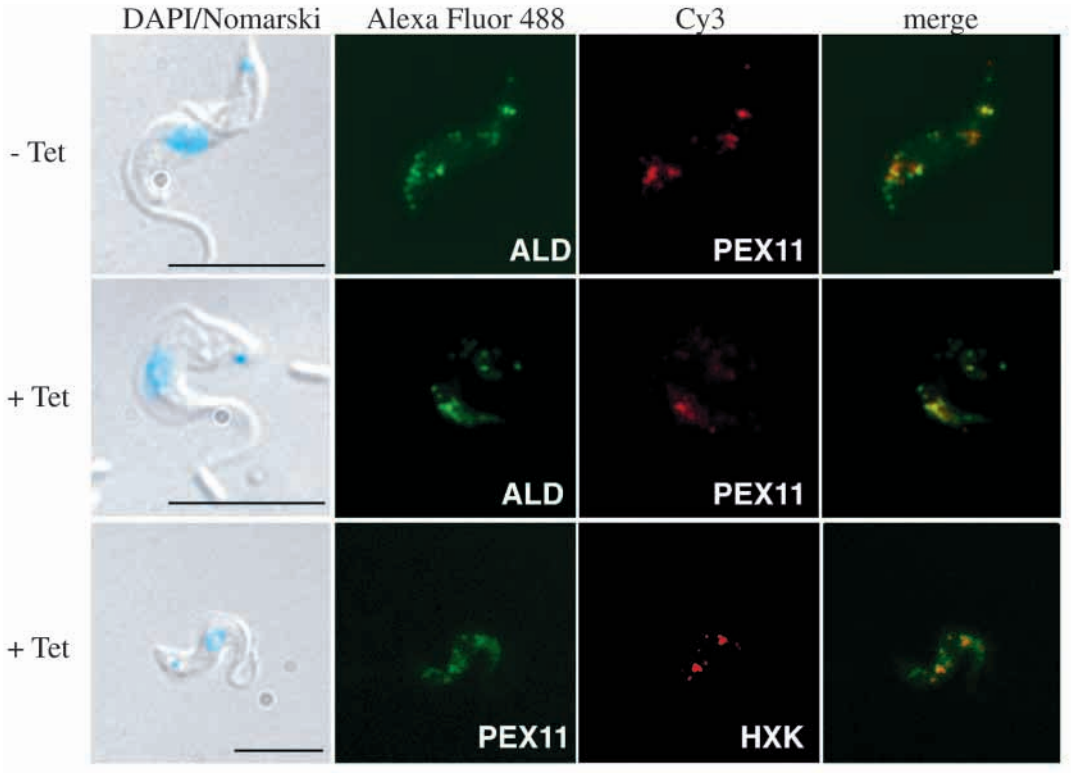

Fig. 2. Effects of PEX2 depletion on matrix protein import and glycosome morphology in bloodstream form trypanosomes. Cells were grown without Tet (control, –Tet) or in the presence of 100 ng/ml Tet for 24 hours (+Tet). For the top and middle rows, ALD was stained with AlexaFluor 488 and PEX11 was detected by a Cy3-coupled secondary antibody. In the bottom row, PEX11 was Alexa-stained and HXK detected by Cy3. Corresponding Nomarski images and DAPI staining are shown on the left. Bar, 10 μm.

surviving trypanosomes with intact glycosomes (punctate pattern), and cell fractionation studies with digitonin revealed no mislocalised glycosomal enzymes after 24 hours (Fig. 1C); samples of cells grown with Tet for longer times were difficult to obtain.

Destruction of *PEX2* mRNA causes cell-growth arrest in both forms of the parasite

The experiments with dominant-negative PEX2 had not resulted in the creation of living trypanosomes with glycolytic enzymes in the cytosol, as we had expected from the results previously obtained with *Leishmania*. We adopted an alternative approach, attempting to reduce the level of PEX2 expression. Attempts to knock out both genes by homologous recombination failed, as had previously been reported with *Leishmania*. We therefore expressed a *PEX2* double-stranded RNA (dsRNA) under the control of a Tet-inducible promoter. The expression of dsRNA in trypanosomes causes RNA interference (RNAi): highly sequence-specific destruction of the homologous mRNA (Ngô et al., 1998). In normal cells, or in the transfected cells grown without Tet, two *PEX2* mRNAs were present at low abundance. When Tet was added, these RNAs became undetectable within 13 hours in procyclic forms (Fig. 1A); a control mRNA (tubulin) was unaffected.

In bloodstream trypanosomes the destruction of *PEX2* mRNA resulted in reduced growth within 24 hours (Fig. 1D). (We were unable to analyse the RNA in these cells because even after only 24 hours, it was not possible to isolate sufficient good-quality mRNA.) By the second day many cells looked abnormal and growth was strongly impaired. Procyclic trypanosomes grew relatively normally for about 2 days, but by the third day, cells of both stages were dying.

The computer model of trypanosome glycolysis predicts that bloodstream trypanosomes lacking glycosomal membranes and grown in 5 mM glucose should be able to maintain glycolysis, whereas parasites grown in 25 mM glucose (normal bloodstream-form medium) will undergo lethal runaway hexose phosphorylation (Bakker et al., 2000). We tested the growth of the bloodstream forms over 3 days after Tet addition, in a range of glucose concentrations (1, 2, 4, 5, 10, 25 mM). Growth inhibition was similar at all concentrations (not shown). Control of glycolytic flux is clearly not the only role of the glycosomal membrane in bloodstream trypanosomes.

Depletion of *PEX2* reduces the number of glycosomes in both stages

We next examined the distributions of glycosomal matrix enzymes and a glycosomal membrane marker, PEX11, by immunofluorescence. 24 hours after Tet addition, most bloodstream-form cells were normal but some had fewer glycosomes than usual (Fig. 2). The matrix enzymes ALD, HXK (Fig. 2), PFK and GAPDH (not shown) still showed punctuate fluorescence; cytosolic staining for matrix enzymes was never observed. Both enzymes colocalised with the glycosomal membrane marker PEX11. Attempts to repeat these experiments at later time points yielded essentially

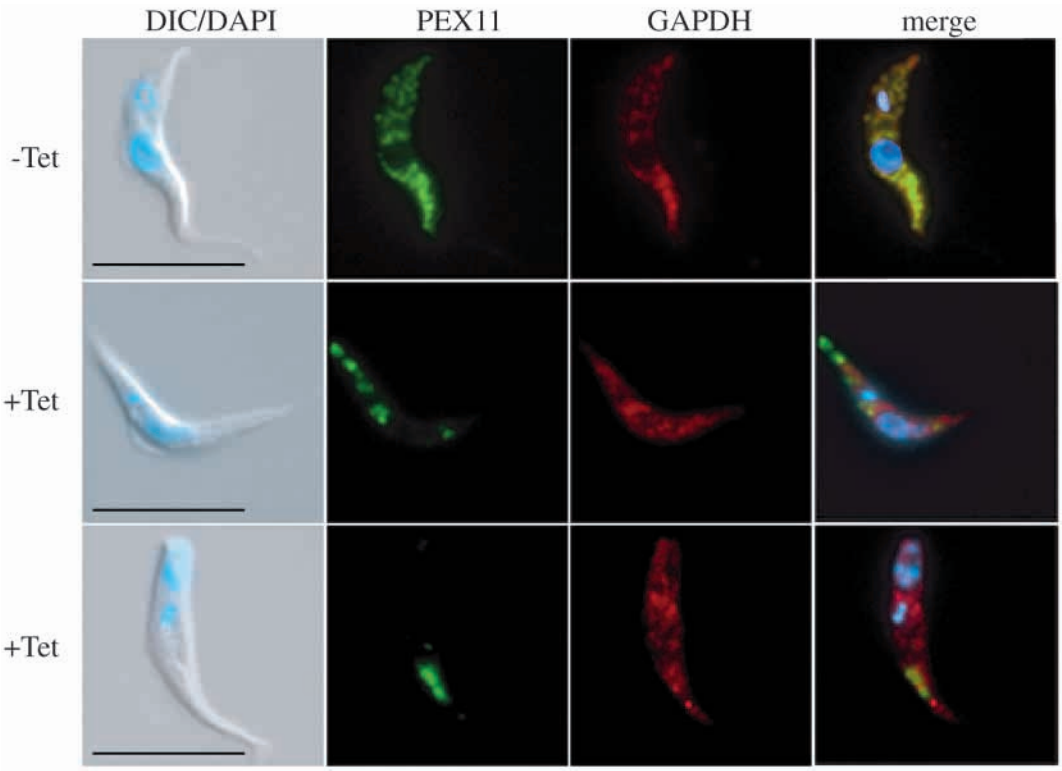

Fig. 3. Effects of PEX2 depletion on matrix protein import and glycosome morphology in procyclic trypanosomes. DAPI staining is shown in blue, PEX11 in green and GAPDH in red. Cells were grown in the presence (+Tet) or absence (–Tet) of 100 ng/ml Tet for 3 days. Bar, 10 μm.

similar results, but were much more problematic because of the number of dead cells. Transmission electron microscopy of PEX2-depleted bloodstream trypanosomes was also difficult because hardly any of them survived the fixation. The few intact cells looked normal and the rest were lysed (not shown). These results suggested that in all the living bloodstream-form trypanosomes, the glycosomal matrix enzymes remained predominantly compartmented within the organelle. A short treatment (5 minutes) with digitonin allowed the glycosomal compartment to be left intact before immunofluorescence. In this case, there was no staining of glycosomal enzymes (not shown), confirming that these were not cytosolic.

Examination of PEX2-depleted procyclic trypanosomes, in contrast, showed that the mutation had the effects that were expected from a 'silenced' PEX2. Trypanosomes grown in the absence of Tet showed the punctate PEX11 and GAPDH staining pattern typical for cells with intact glycosomes (Fig. 3, top row). After 60 hours of RNAi induction, the number of PEX11-labeled structures was drastically reduced in most of the cells and the GAPDH signal was spread throughout the cell and excluded from the glycosomal remnants (Fig. 3, middle and bottom rows). This suggests that PEX2 depletion does indeed prevent proper targeting of glycosomal matrix enzymes in trypanosomes.

Glycolytic enzymes are abnormally distributed in cells lacking PEX2

To confirm the effects of PEX2 depletion on glycosomal matrix enzymes, we subjected the mutants to cell fractionation. In no case was the total enzyme level affected by PEX2 reduction. Fig. 4A shows the effects on the distribution of HXK and PGI activities. HXK activity was always found mainly in the organellar pellet fraction, consistent with the results from the immunofluorescence in bloodstream forms (Fig. 2). In contrast, PGI activity showed a clear shift to the cytosolic fractions of both bloodstream and procyclic cells. Unfortunately we could not confirm this by immunofluorescence because suitable antibodies are not available. The mis-localisation of PGI could therefore be due either to intrinsic cytosolic location, or leakage from weakened organelles (or remnants) during the fractionation procedure.

Western blots of three other glycosomal enzymes from digitonin-fractionated procyclic cells are shown in Fig. 4B. The *pex2* procyclic forms were defective in targeting of ALD, PFK and GAPDH. No redistribution of any of these enzymes to the cytosol was detected in the bloodstream mutants, as seen by immunoflourescence (ALD shown in Fig. 2).

In *Y. lipolytica pex2* mutants, levels of a surface and a secreted protein were reduced by 80% (Titorenko et al., 1997). We checked the synthesis and processing of the variant surface glycoprotein (VSG) in bloodstream cells by pulse chase (5 minutes pulse, 60 minutes chase); no differences could be detected between mutants grown in the absence or the presence of Tet (data not shown; C. Guerra Giraldez, PhD Thesis, University of Heidelberg, 2001). For procyclics, we examined the level of the major surface protein EP for up to 3 days after addition of Tet; the *pex2* mutation also had no effect (Fig. 4C).

Discussion

The effects of PEX2 reduction in procyclic trypanosomes were analogous to those seen in yeast and mammalian deletion mutants: the import of matrix proteins was severely impaired, resulting in an accumulation of glycosomal enzymes in the cytoplasm, but remnants of glycosomal membrane persisted

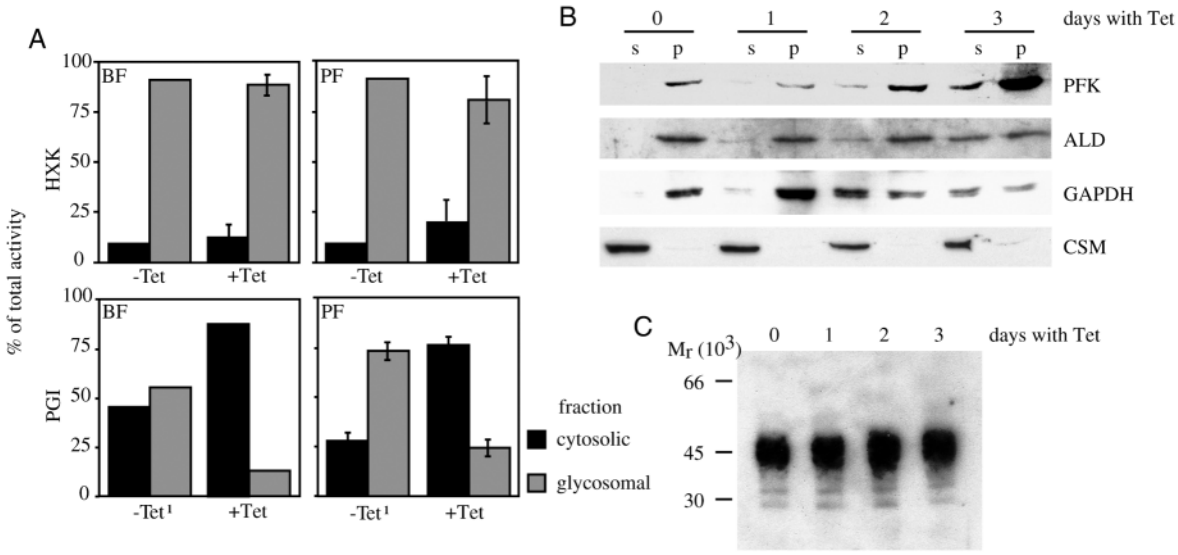

Fig. 4. (A) Effects of PEX2 depletion on the location of HXK and PGI activities. Bloodstream and procyclic *pex2* cells were disrupted using glass beads. Enzymatic activities were measured in the cytosolic (black) and organellar pellet (gray) fractions. The +Tet columns are from cells grown in the presence of 100 ng/ml Tet for 36 hours; –Tet columns are the controls. Measurements were made with three independent cell extracts; the activities shown are the mean and standard deviation from two to five readings. (B) Western blots showing the distribution of proteins in procyclic mutants, 0, 1, 2 and 3 days after Tet addition. Cells were separated into digitonin-soluble (s) and pellet (p) fractions. The proteins detected are indicated on the right. (C) Western blot showing the expression of EP1 in procyclic mutants grown in the absence and in the presence of Tet for up to 3 days. Each lane was loaded with a total protein extract from 5×10^5 cells.

(Waterham et al., 1996; Faust and Hatten, 1997). Enzymes bearing two types of import signal, called peroxisomal targeting signals (PTS), PTS-1 (PFK, GAPDH, PGI) and PTS-2 (ALD), were equally affected by the import defect. This is consistent with the evolutionarily conserved function of PEX2 in microbody biogenesis (Shimozawa et al., 1999; Subramani et al., 2000; Tsukamoto et al., 1994). We did not detect any impairment of the secretory pathway. In the *Leishmania* mutants previously described, which expressed a truncated dominant-negative form of PEX2, most matrix proteins retained glycosomal localisation, whereas PGK and luciferase bearing an -SKL sequence (PTS-1) were partially cytosolic (Flaspohler et al., 1997). Thus our inducible mutant trypanosomes have a much more severe phenotype.

ALD, GAPDH and PFK have basic isoelectric points and associate in high-molecular-weight complexes (‘cores’) which sediment during centrifugation even after dissolution of the glycosomal membrane (Opperdoes, 1987). Their cytosolic location in *pex2* procyclic trypanosomes clearly indicated an import defect. PGI has a neutral pI (Parsons and Nielsen, 1990), does not associate with cores and is readily released from glycosomes during fractionation [(Opperdoes, 1987) and see Fig. 4, –Tet columns]. The partitioning of PGI into the soluble fraction in *pex2* cells could therefore reflect either mis-targeting or organelle fragility. HXK was always associated with the pellet, even in the mutants, and retained a punctate distribution by immunofluorescence. HXK has a PTS-2 signal (de Walque et al., 1999); it is unlikely to be imported into the PEX2-defective glycosomes via this signal, so the most likely explanation is that an alternative signal is present. This could be a membrane-targeting signal, or hexokinase might be imported attached to a membrane protein; HXK has a strong tendency to aggregate and stick to membranes (Opperdoes, 1987) (P. Michels, personal communication).

Procyclic trypanosomes depend mainly upon amino acids and the mitochondrion for ATP generation. (Although there is some glucose in our standard procyclic media, proline is supplied as the principal carbon source.) The fact that depletion of PEX2 is nevertheless lethal implies that pathways other than glycolysis are essential and require compartmentation. Enzymes of fatty acid beta oxidation, ether lipid biosynthesis, purine salvage, sterol synthesis and pyrimidine synthesis are found in kinetoplastid glycosomes (Michels et al., 2000), although some of these are probably absent in bloodstream trypanosomes. A properly assembled, intact organelle could be essential for the functioning of these pathways or enzymes. As the procyclic parasites survive for at least 24 hours with defective compartmentation, the growth impairment seems likely to be caused by gradual depletion of critical products or accumulation of toxic intermediates.

In bloodstream trypanosomes, the effects of PEX2 depletion were much more severe than in procyclics. Some parasites with abnormally low numbers of glycosomes were seen but neither ALD, PFK nor GAPDH was detected in the cytosol. Very similar results were obtained when we overexpressed a C-terminally truncated, dominant-negative version of PEX2 in bloodstream trypanosomes, as had been done in *Leishmania* (Flaspohler et al., 1999). Cell growth rapidly ceased and the resulting population was a mixture of few normal-looking and mostly dead cells; no matrix enzyme relocalisation was detectable. The most likely explanation for these observations is that in PEX2-depleted bloodstream trypanosomes, a decrease in the number of glycosomes can be tolerated, but as soon as overall import of enzymes fails – or the level of cytosolic enzymes rises above a critical threshold – the parasites die. The

most likely cause of death is a failure of ATP production. It may be that glycolysis cannot be maintained at an adequate rate when the enzymes are in the cytosol. It is possible that import into the glycosome is required for the glycolytic enzymes to assemble into a multienzyme complex, allowing metabolite channeling, but no evidence of such channeling has ever been found. The results of metabolic modeling indicate that dilution of the glycolytic enzymes into the whole cell volume would have no effect at all on the glycolytic rate. However, the regulatory and kinetic properties of trypanosome glycolytic enzymes are unusual (for example, no feed-back control is exerted on HXK or PFK) and may be adapted so that adequate glycolytic flux can be sustained only when the enzymes are in a specialised (glycosomal) environment. It has, for example, been suggested that if hexokinase and phosphofructokinase were exposed to the ATP/ADP ratio of the cytosol, runaway phosphorylation would ensue (Bakker et al., 2000). An alternative explanation is afforded by our observation that hexokinase appears to remain glycosome-associated in *pex2* cells. If the glycosomal membrane represents a permeability barrier for hexose phophates, the retention of hexokinase in the glycosomes would result in a break in the pathway after the formation of glucose-6-phosphate.

Even minor mis-localisation of glycosomal enzymes is lethal in bloodstream trypanosomes. Furthermore, our results imply that compartmentation of glycosomal enzymes is essential for survival of procyclic *T. brucei*. Given the low sequence conservation between the PEX proteins of kinetoplastids and mammals, it will be worthwhile to look for specific inhibitors of glycosomal biogenesis.

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