Binding of coatomer by the PEX11 C-terminus is not required for function

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Abstract Microbodies are single membrane-bound organelles found in eukaryotes from trypanosomes to man. Although they have diverse roles in metabolism, the mechanisms and molecules involved in membrane biogenesis and matrix protein import are conserved. Similarly, the basic mechanisms and structures involved in vesicular transport are similar throughout eukaryotic evolution. The PEX11 proteins are required for the division of microbodies in trypanosomes, yeast and mammals, and a role of coatomer in this process has been suggested. We show here that the binding of trypanosome, yeast and bovine coatomers to selected peptides is identical. Coatomer binds to the C-termini of trypanosome PEX11 and rat Pex11α, but not yeast Pex11p or human Pex11β. Mutations of the C-terminus of trypanosome PEX11 that eliminated coatomer binding did not affect function in yeast or trypanosomes. Thus binding of coatomer to the C-terminus of PEX11 is not required for PEX11 function.

Key words: Trypanosome; Microbody; Glycosome; Coatomer; PEX11

1. Introduction

Microbodies are single membrane-bound organelles that contain a wide variety of metabolic pathways in different eukaryotes. For example, growth of *Saccharomyces cerevisiae* on oleate is dependent on the presence of functional peroxisomes which contain enzymes required for β-oxidation of fatty acids. In trypanosomes, the first 7–9 enzymes of glycolysis are found inside microbodies called glycosomes. The mechanisms and *PEX* genes involved in microbody biogenesis have been conserved throughout evolution [1–4]. All microbody matrix proteins and most microbody membrane proteins are incorporated post-translationally [4,5]. For a minority of peroxisomal membrane proteins, however, there are indications for routing via the endoplasmic reticulum [6–8]. One indication is the fact that ARF (ADP-ribosylation factor) and coatomer are associated with proliferating rat liver peroxisomes [9]. Coatomer is a multisubunit complex involved in trafficking of vesicles between the endoplasmic reticulum and the Golgi apparatus [10,11]. Coatomer assembly and vesicle budding depend on the presence of ARF-GTP and, probably, a membrane-bound peptide bearing a coatomer binding sequence [12]. Coatomer binding signals typically contain a K(X)KXX motif and/or a diphenylalanine motif, and are found on ER-resident proteins and on putative cargo receptors of the p24 protein family [13–15].

Pex11p is an abundant peroxisomal membrane protein that is essential for microbody division in yeast [16] and trypanosomes [17]. A rat Pex11 homologue, expressed abundantly in proliferating peroxisomes, has a C-terminal KXKXX motif [9]. It has therefore been suggested that Pex11 function may involve coatomer binding. Here we have investigated the correlation between C-terminal binding specificity and Pex11 function in trypanosomes, yeast, and mammals.

2. Materials and methods

2.1. Trypanosomes and yeast

Procyclic trypanosomes of the strain 427 were cultured and transfected as previously described [18]. In all experiments, the 'wild-type' (449) cells are trypanosomes stably expressing the tet repressor [18].

S. cerevisiae strain no. 470 (genotype Mata/Matα, ade2-1, trp1-1, can1-100, leu2-3, 112, his3-11,15, ura3, Gal, psi+) (gift of Claudia Kruze, ZMBH) was grown in YEP (1% yeast extract, 2% bactopeptone, 55 mg/l adenine, 2% glucose) to exponential phase ($0.5–2\times10^7$ cells/ml) at 30°C. *S. cerevisiae* strain W303A*pex11*Δ was grown and complemented as previously described [16].

2.2. Mutation of PEX11 and expression in trypanosomes and yeast

Wild-type *TbPEX11* (pHD810) and C-myc-tagged *TbPEX11* (pHD812) genes [17] and mutant *pex11* genes were cloned into the tet-inducible expression vector pHD678 [18]. A yeast oleate-inducible expression vector with unique *Cla*I and *Xho*I sites was constructed by inserting the *Bam*HI-*Eco*RI fragment of pPR6, containing the oleate-inducible *FOX3* (thiolase) promoter, into *Bam*HI-*Eco*RI cut pRSterm, which contains the *cyc1* terminator. Mutants of *PEX11* (Fig. 2A) were synthesised by PCR using a C-terminally myc-tagged version of the *PEX11* gene as template; the primers were cz855 (5′-UTR of plasmid) and cz854 (3′-myc tag). Internal mutating primers (amino acids deleted indicated in parentheses) were: D1 (Δ6–27): cz847; D2 (Δ44–64): cz848; D3 (Δ95–115): cz849; D4 (Δ130–147, transmembrane domain): cz850; D5 (Δ149–163, putative targeting signal): cz851; D6 (Δ170–185, transmembrane domain): cz852; D7 (Δ195–207): cz853. Mutant D8 (deletion of C-terminal eight amino acids) was made using cz855 and cz951; and D9 (mutation of C-terminal KIK to SIK) with cz855 and cz952 (Table 1).

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Abbreviations: COP, coat proteins; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; tet, tetracycline; ARF, ADP-ribosylation factor; DTT, dithiothreitol; TCA, trichloroacetic acid; PEX11 proteins are PEX11 in trypanosomes, Pex11p in *Saccharomyces cerevisiae* and Pex11 in mammals

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2.3. Antisera and immunological detection

Antibodies used for detection of PEX11 (#28680 and #28770) were as described [17]. Other antibodies were to glyceraldehydephosphatase dehydrogenase (Paul Michels, ICP, Brussels, Belgium); thiolase (Ralf Erdmann, Berlin, Germany), mammalian COP subunits (Cordula Harter, BZH) and yeast β′-COP (Rainer Duden, Cambridge, UK). The sequence of trypanosome β′-COP (AJ250726) was communicated prior to publication by Mark Carrington (University of Cambridge); corresponding antisera were generated by immunising rabbits with a trypanosome-specific N-terminal peptide, (MSSKMMSPPETLM-VANSDRVK*C) or a C-terminal sequence (C*LSASGVGVGVGG-DDDEWGEQ), each linked to keyhole limpet hemocyanin (Pierce USA). Specificity was determined using a 50-fold excess of peptide. Data shown are for the N-terminal specific antibody. Western blots and immunofluorescence assays were performed as described [17] and stained trypanosomes analysed by digital deconvolution microscopy (Improvision).

2.4. Peptide binding experiments

Bovine brain cytosol was prepared as described [19]. Trypanosomal and yeast cytosol were prepared by breaking the cells in homogenisation buffer (25 mM Tris pH 7.8; 1 mM EDTA; 10% sucrose, protease inhibitor mix complete-mini™ (1 tablet/10 ml; Roche Mannheim)) with a bead beater. Nuclei were removed (5 min; 2000×*g*) followed by a 30 min, 100 000×*g* centrifugation of the supernatant. The resulting cytosol was diluted to a final concentration of 4 mg/ml with homogenisation buffer.

Binding assays were performed as described [19]. Briefly, peptides were coupled to Thiopropyl Sepharose 6B (Pharmacia) via their NH_2-terminal Cys residues. Beads (3.3 nmol of peptide) were incubated with 300 μg of cytosolic proteins in 250 μl HEPES detergent buffer, incubation at 4°C for 2 h and then washed five times. SDS sample buffer was added, proteins separated on 7.5% SDS–polyacrylamide gels and coatomer detected by Western blotting using anti-β′-COP antibody.

3. Results

3.1. Binding specificities of coatomer from trypanosomes, yeast and mammals

We generated two antibodies to trypanosome β′-COP, by immunising with peptides corresponding to a translated genomic sequence with a predicted 39% amino acid identity with rat β′-COP (AJ250726). Both antisera reacted specifically with a 92 kDa band on Western blots, the expected size for β′-COP. The trypanosome β′-COP is present in a large complex which can be partially purified according to standard coatomer purification protocols [20]. (Details will be presented elsewhere.) Partial homologues of α-COP and γ-COP are in the trypanosome database and our completed sequences of β-COP (AJ271083) and ζ-COP (AJ271084) predict 23–29% amino acid identity with higher eukaryotic homologues.

Table 1
Oligonucleotides used in this study

cz847	TCTGAGTTCCAACTTAAGTTTAAGGCACTT
cz848	CGCTCAAGTGCGCTTAAGGGTGATGTGCCC
cz849	TTCCTCCGCGTGCTTAAGGCTTTGCGGCAC
cz850	GCAAAAACAGCGCTTAAGGCGCTTCAGAAG
cz851	CTTTACGGTGCGCTTAAGAAGGCAGCACTT
cz852	GCACTTATCAGTCTTAAGTACCTCCGTGAA
cz853	TGGCGACCCAGCCTTAAGGTTGCAACGTAT
cz854	CTAGGATCCTCAGCCTCCGCCCAAGTCCTCTTC
cz855	AAGTAAAATTCACAAGCTTCGCGGGGCCC
cz951	CTAGGATCCTCAGCCTCCGCCCAAGTCCTCTTCAGAAATGAGC-TTTTGCTCGCCTCCGCCCTTAAGCGTTGCAACACCACC
cz952	AACCTCGAGGGATCCCTATTTGATCGAGTTCCAGTT

All oligonucleotides were synthesised at ZMBH biopolymer synthesis facilities.

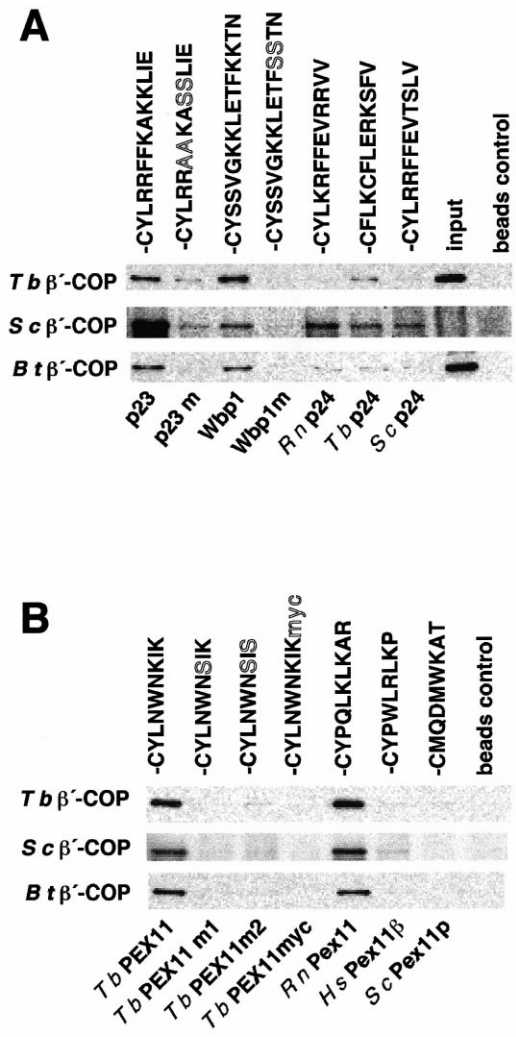
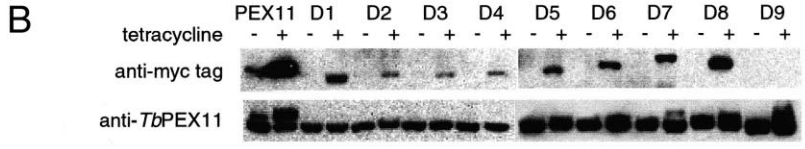


Fig. 1. Binding of coatomer to different peptides. Cytosolic fractions from yeast, cow and trypanosomes were prepared and allowed to bind to various peptides coupled to beads. The bound coatomer was detected using antibodies to β′-COP from *T. brucei* (*T.b.*), *S. cerevisiae* (*S.c.*) and *Bos taurus* (*B.t.*). The specific β′-COP bands are shown. A: p23/p24 family peptides. For explanation of proteins used, see text. *R.n.*: *Rattus norvegicus*. The absence of signal from the input in the *S. cerevisiae* lane is an artifact. B: Binding to PEX11 C-termini from various species and mutant (m) versions thereof. *H.s.*: *Homo sapiens*.

We first compared the binding specificities of trypanosome, *S. cerevisiae* and bovine coatomer to control peptides (Fig. 1A). Cytosolic fractions from all three species were prepared and allowed to bind to various peptides coupled to Sepharose beads. These were washed, the bound proteins eluted and coatomer detected by Western blotting using antibodies to β′-COP, a canonical mammalian coatomer binding sequence, with a diphenylalanine and a dilysine motif, is found at the C-terminus of the putative cargo receptor p23 [19]; a mutant version was a negative control. For yeast we used the C-terminus of Wbp1, the *N*-oligosaccharyltransferase, with the corresponding non-binding mutant [19]. The C-termini of putative p24 family cargo receptors from rat and yeast (Emp24p) were also included [13]. As a control from trypanosomes, we used the C-terminal sequence of a possible p24 family homologue from the TIGR database (43D23.TF M13 and 28A8TJ). Coatomers from yeast, trypanosomes and cow showed similar

specificity with all peptides tested. As expected, binding to the rat p23 peptide and yeast Wbp1 was diminished by mutation of the binding motifs; and weak binding to the three p24 peptides was observed (Fig. 1A).

We now examined binding to PEX11 C-terminal peptides (Fig. 1B). Again, specificity was conserved throughout. The C-terminus of *Tb*PEX11, terminating in -KIK, bound coatomer despite the total absence of a consensus motif, as did rat Pex11α, terminating in -KLKAR. Mutation of the penultimate lysine residue in *Tb*PEX11 to a serine (peptide *Tb*PEX11 ml, equivalent to mutant D9), or addition of a myc tag, abolished coatomer binding. The C-termini of yeast Pex11p and human Pex11β (an orthologue of Pex11) [21] did not bind coatomer.



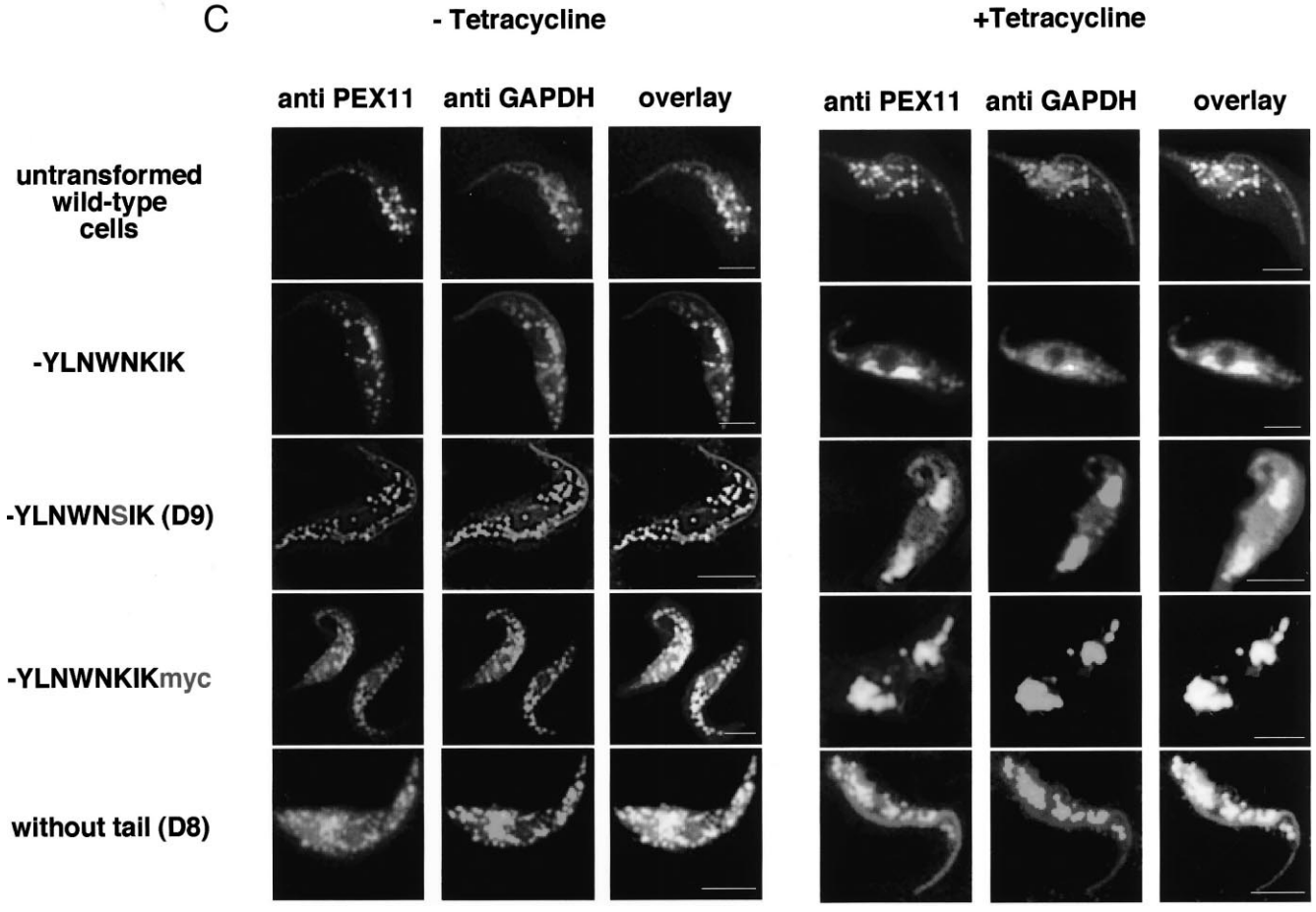


Fig. 2. A: Scheme of deletion mutations in *Tb*PEX11. Mutants D1, D3 and D7 affect regions that are conserved in all known PEX11 proteins. Mutants D4 and D6 delete the probable transmembrane domains, and D5 a putative peroxisomal membrane targeting signal. D8 has a deleted C-terminus and D9 (not shown) a mutation of the penultimate lysine to serine. B: Western blot of extracts from transformed trypanosome lines, grown in the presence (+) and absence (−) of tet. Transgenic PEX11 myc was detected via myc antibody (except for D9, which lacks the tag), and transgenic and wild-type PEX11 using antibody #28680 against *Tb*PEX11. C: Immunofluorescence of glycosomes in trypanosomes over-expressing mutant versions of PEX11. The panels on the left show representative trypanosomes with normal levels of wild-type PEX11, grown in the absence of tet and stained with antibody to PEX11 and the glycosomal marker GAPDH. The cells shown on the right side are grown in the presence of tetracycline to induce over-expression of the inducible *PEX11* genes. Scale bar 5 μm.

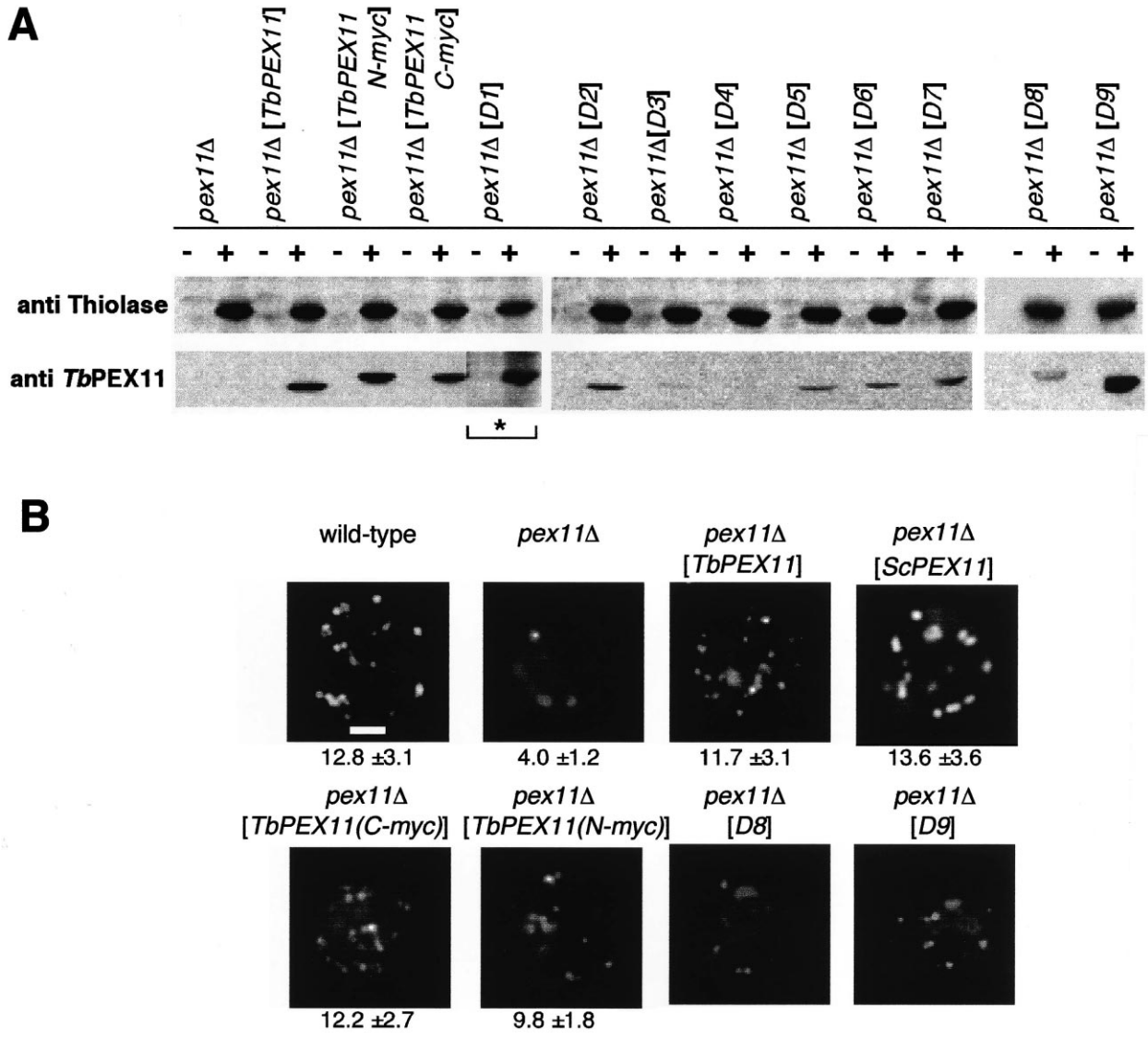


Fig. 3. Expression of mutant versions of PEX11 in *S. cerevisiae*. A: Western blot of extracts from transformed yeast lines, grown in glucose (−) or oleate (+) medium. The proteins detected are *Tb*PEX11 and the peroxisomal marker protein thiolase. Longer exposure of the Western blot (not shown) revealed low expression of D3 and D4. For pex11D1 antibody #28770 was used instead of #28680. B: Immunofluorescence of peroxisomes in yeast expressing various versions of *Tb*PEX11. Only the versions that were well expressed are shown. *Tb*PEX11, *Tb*PEX11 C-myc, *Tb*PEX11 N-myc and *Tb*PEX11D9 restored proliferation of peroxisomes in *pex11*Δ yeast cell lines. Scale bar 3 μm. The numbers of peroxisomes in several of the cell lines are given below the pictures as the mean and standard deviation for 20 cells.

3.2. Function of mutated PEX11 in trypanosomes

Both *Tb*PEX11 and a C-terminally myc-tagged version can complement a yeast Δ*pex11* mutant [17]. This suggests that function is independent of coatomer binding. To confirm this, and to locate other functional domains, we generated mutant versions of *Tb*PEX11 containing short deletions (Fig. 2A). These were expressed in trypanosomes using a tet-inducible expression vector. In almost all cases, very little mutant protein was detected after induction (Fig. 2B). Although the mutant proteins were associated with the particulate fraction during digitonin fractionation, the low expression precluded exact Localisation by immunofluorescence. We therefore cannot exclude the possibility that D1–D4 and D6–D8 proteins sediment because of aggregation, although the low expression makes this unlikely. The mutant pex11D5, which lacks the putative peroxisomal targeting signal, comigrated with glycosomes during sucrose gradient centrifugation and was an integral membrane protein as assessed by carbonate extraction (not shown).

Over-expression of wild-type *Tb*PEX11, or of the C-terminally myc-tagged version, leads to proliferation of glycosomal membrane, resulting in many adjacent glycosomal tubules which appear as large clusters by immunofluorescence microscopy [17] (Fig. 2C). The N-terminally myc-tagged version [17], and mutant D8, which has a C-terminal deletion preceding the C-terminal myc tag, were not well expressed but still altered the glycosomal staining pattern (Fig. 2C). The mutant D9 (PEX11-KIK → SIK), which cannot bind coatomer, was well expressed and gave the same phenotype as wild-type PEX11 (Fig. 2C). Thus the phenotype obtained by over-expression of *Tb*PEX11 in trypanosomes does not require coatomer binding by the C-terminus.

3.3. Function of mutated PEX11 in yeast

To determine whether the various mutants were functional in mediating peroxisome division we assessed the ability of the genes to complement an *S. cerevisiae* Δ*pex11* mutant. To measure synthesis of the proteins, glucose-grown cultures were washed, resuspended in oleate medium to induce transcription, and the cells harvested after 16 h. The full-length trypanosome PEX11, and the myc-tagged versions, were able to restore the ability of pex11Δ yeast to grow on oleate (not shown) and to restore peroxisome division such that the numbers of peroxisomes were indistinguishable from wild-type (Fig. 3B). Expression of nearly all the mutant proteins, including D8, was so poor that complementation would be unlikely (Fig. 3A). Only the non-coatomer binding mutant D9 (PEX11K→S) was synthesised in levels approaching those of the wild-type *Trypanosoma brucei* protein and capable of complementing the mutant.

4. Discussion

4.1. Evolutionary conservation of coatomer binding specificity

Our results suggest that the specificity of coatomer binding is conserved throughout evolution. Previously described motifs with strong coatomer binding were restricted to K(X)KXX and a diphenylalanine (FF) motif, either of which can independently mediate binding [19]. Weak binding was observed to RFFEVRRVV and a sequence containing MRK [13]. This study extended the range of possible coatomer binding sequences to include the motif KIK, and the weakly binding CFLERKSFV.

4.2. What is the role of coatomer in peroxisome division?

A coatomer binding motif at the C-terminus of PEX11 is not required for the protein to function in peroxisome division. *Tb*PEX11 mutants which showed no C-terminal coatomer binding induced proliferation and elongation of microbodies in trypanosomes and were able to complement yeast *pex11*Δ. Yeast has only one *PEX11* gene: the C-terminus of the encoded protein lacked coatomer binding activity. In fact, no known *S. cerevisiae* Pex protein bears an identifiable coatomer binding motif. Mammals have two Pex11 proteins: Pex11α binds coatomer and is expressed in cells which show strong induction of peroxisome proliferation, whereas Pex11β, which is capable of mediating peroxisome proliferation and division but lacks C-terminal coatomer binding, is constitutively expressed [21]. Our results would therefore be consistent with the possibility that the involvement of coatomer in peroxisome proliferation is restricted to specific situations on mammalian cells.

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