Analysis of the Sequence Motifs Responsible for the Interactions of Peroxins 14 and 5, Which Are Involved in Glycosome Biogenesis in *Trypanosoma brucei*†

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ABSTRACT: Glycosome biogenesis in trypanosomatids occurs via a process that is homologous to peroxisome biogenesis in other eukaryotes. Glycosomal matrix proteins are synthesized in the cytosol and imported posttranslationally. The import process involves a series of protein−protein interactions starting by recognition of glycosomal matrix proteins by a receptor in the cytosol. Most proteins to be imported contain so-called PTS-1 or PTS-2 targeting sequences recognized by, respectively, the receptor proteins PEX5 and PEX7. PEX14, a protein associated with the peroxisomal membrane, has been identified as a component of the docking complex and a point of convergence of the PEX5- and PEX7-dependent import pathways. In this paper, the strength of the interactions between *Trypanosoma brucei* PEX14 and PEX5 was studied by a fluorescence assay, using (i) a panel of N-terminal regions of *Tb*PEX14 protein variants and (ii) a series of different peptides derived from *Tb*PEX5, each containing one of the three WXXXF/Y motifs present in this receptor protein. On the PEX14 side, the N-terminal region of *Tb*PEX14 including residues 1−84 appeared to be responsible for *Tb*PEX5 binding. The results from PEX14 mutants identified specific residues in the N-terminal region of *Tb*PEX14 involved in PEX5 binding and showed that in particular hydrophobic residues F35 and F52 are critical. On the PEX5 side, 13-mer peptides incorporating the first or the third WXXXF/Y motif bind to PEX14 with an affinity in the nanomolar range. However, the second WXXXF/Y motif peptide did not show any detectable affinity. Studies using variants of second and third motif peptides suggest that the α-helical content of the peptides as well as the charge of a residue at position 9 in the motif may be important for PEX14 binding. Assays with *7*-, 10-, 13-, and 16-mer third motif peptides showed that 16-mers and 13-mers have comparable binding affinity for PEX14, whereas 10-mers and 7-mers have about 10- and 100-fold lower affinity than the 16-mers, respectively. The low sequence identities of PEX14 and PEX5 between parasite and its human host, and the vital importance of proper glycosome biogenesis to the parasite, render these peroxins highly promising drug targets.

Various organisms belonging to the protozoan family Trypanosomatidae are the causative agents of several highly disabling and often fatal human diseases, especially in tropical and subtropical areas of the world. These diseases include (i) African sleeping sickness caused by two different subspecies of *Trypanosoma brucei*, *T. brucei rhodesiense* and *T. brucei gambiense*, (ii) Chagas' disease occurring in Latin America caused by *Trypanosoma cruzi*, and (iii) a spectrum of leishmaniases, widely distributed throughout the tropics, and for which different species of *Leishmania* are responsible. These diseases affect millions of people, whereas hundreds of millions are at risk to become infected. Unfortunately, current treatments are largely unsatisfactory and even worsening. Chemotherapy is the mainstay for control because effective vaccines are not available. However, most currently used drugs are inefficient and toxic, and the development and spreading of drug-resistant parasites are becoming a major problem. Therefore, there is a desperate need for new effective and safe drugs (*1*, *2*).

Glycolysis is a validated drug target of the African sleeping sickness parasite (*3*, *4*), as the trypanosomes living in the mammalian bloodstream are entirely dependent on the conversion of glucose into pyruvate through the glycolytic pathway for their ATP supply. Glycolysis also plays possibly an important role in the carbohydrate metabolism of the mammalian-infective stages of *T. cruzi* and *Leishmania* species. Although glycolytic enzymes have been well

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conserved during evolution (*5*), our research has revealed that most of the trypanosomatid enzymes of this pathway contain unique structural and kinetic features which enabled the design of potent, parasite enzyme-selective inhibitors (*4*). Some of these inhibitors stunt the growth of cultured trypanosomatids, and have no effect on mammalian cells (*6*). Several specific features of the trypanosomatid enzymes can be attributed to the unique sequestering of the major part of the glycolytic pathway within peroxisome-like organelles called glycosomes (*7*−*9*). In human cells, and almost all other organisms, glycolysis is a cytosolic process. Research on *T. brucei brucei*, a subspecies responsible for a sleeping sickness variant ("Nagana") affecting cattle in large parts of sub-Saharan Africa, and often used in the laboratory as a model trypanosomatid organism, has shown that the proper compartmentation of glycolytic enzymes inside glycosomes and the possession of intact glycosomes are equally essential for the parasite (*10*−*13*). Compounds that will interfere with glycosome biogenesis or integrity may, therefore, offer an additional possibility for therapy.

Glycosomes are distinct from peroxisomes in that only glycosomes harbor enzymes for glycolysis and purine salvage pathways, yet glycosomes share several features with peroxisomes such as a single bilayer membrane, absence of genetic material, compartmentation of metabolic pathways (β-oxidation of fatty acids and ether lipid biosynthesis) and conservation of biogenesis and protein import processes (*9*, *14*).

Glycosome biogenesis in trypanosomatids occurs via a process that is homologous to peroxisome biogenesis in other eukaryotes (*14*, *15*). Peroxisome biogenesis has been studied extensively in mammalian cells and various yeasts (*16*−*19*). To date, 25 proteins called peroxins (acronym PEX)[1] have been identified as factors involved in the biogenesis of peroxisomes. Most of them are involved in the import of matrix proteins, some in other aspects of peroxisome propagation (synthesis of peroxisomal membranes, inheritance and division of peroxisomes, etc.) that are as yet less well understood. Peroxisomal matrix proteins are encoded by nuclear genes, synthesized on free ribosomes in the cytosol and imported posttranslationally. The import is a multistep process involving recognition of the cargo protein by a receptor in the cytosol, docking of the receptor−cargo complex at the membrane, translocation across the membrane, cargo release inside the organelle, and receptor cycling (*16*−*19*). Each of these steps involves specific protein−protein interactions. Most glycosomal matrix proteins, involved in glycolysis or other processes, contain the common PTS-1 or PTS-2 sequences (*9*). The proteins to be imported are recognized by a cytosolic receptor, either PEX5 or PEX7. PEX5 binds cargo proteins with a PTS-1 signal, which is a C-terminal tripeptide -SKL or a variation thereof (*20*−*22*), and PEX7 binds those with a PTS-2 signal, a motif -R/K-L/V/I-X_5-H/Q-A/L- close to the N-terminus (*23*−*25*).

PEX14, a protein associated with the peroxisomal membrane, has been identified in various yeasts, mammalian cells, and plants as a component of the docking complex and a point of convergence of the PEX5- and PEX7-dependent import pathways (*26*−*28*). Both cargo-loaded receptors interact with PEX14 to form the docking complex together with PEX13 and PEX17 (the latter protein has, so far, only been identified in yeasts). The N-terminal part of PEX14, which is more conserved than the C-terminal region, interacts with so-called "WXXXF/Y motifs" located in the N-terminal part of PEX5 (*29*−*33*). In addition, the N-terminal domain of PEX14 interacts with the SH3 domain of PEX13 through a proline-rich motif (*26*, *27*, *34*) (Figure 1). Interactions of PEX14 with PEX7 have also been demonstrated (*27*), but the precise nature of the interaction is still unclear. In PEX14, there is a putative membrane binding region that consists of 16 consecutive hydrophobic residues spanning residues 148−163 in *T. brucei*. In mammalian cells and *Saccharomyces cerevisiae*, it was shown that both the N-terminal and the C-terminal domain are at the cytosolic face of the peroxisomal membrane (*27*, *28*, *35*). The C-terminal part of PEX14 is very diverse in sequence (Figure 1) and its role is not yet known.

Previously, we have cloned and characterized PEX5 and PEX14 of *T. brucei (21, 43)*. Both proteins (*Tb*PEX5 and *Tb*PEX14) have a low degree of overall amino acid sequence identity with their human counterparts (*Hs*PEX5 and *Hs*PEX14): 26 and 31%, respectively. The low sequence identities of these peroxins between parasite and its human host, and the vital importance of proper glycosome biogenesis to the parasite as discussed above, render these peroxins highly promising drug targets. In this paper, we present a detailed analysis of the sequences in *Tb*PEX14 that are responsible for the interaction with each of the three WXXXF/Y motif repeats present in *Tb*PEX5 (Figure 2). We also investigated the affinities of peptide variants of the three WXXXF/Y motifs for *Tb*PEX14 and discovered remarkable differences among the peptides. This specific information regarding the PEX5−PEX14 interactions in trypanosomes forms a solid basis for future inhibitor design.

MATERIALS AND METHODS

Preparation of Proteins and Synthetic Peptides. Three N-terminal constructs of *Tb*PEX14, *Tb*PEX14(84), *Tb*PEX14(128), and *Tb*PEX14(146,H), which include amino acids 1−84, 1−128, and 1−146, respectively, were used for binding studies. *Tb*PEX14(146,H) also contains a C-terminal 6-histidine tag with a 16-amino acid-long linker. Single and double mutants of *Tb*PEX14(128) were generated by introducing point mutations in the DNA using the Quik-change protocol from Stratagene. All mutations were confirmed by DNA sequencing. The wild-type and 10 mutant proteins were expressed using *Escherichia coli* grown in LB medium at 37 °C, and the proteins were purified through a cation-exchange column (HS20) followed by size-exclusion chromatography (Superdex 200). However, these expression conditions did not yield sufficient amount of protein of two mutants, *Tb*PEX14(128)K56A and *Tb*PEX14(128)K56A/G57A, to perform binding assays. This may be an indication that Lys56 is important for folding and/or that the K56A substitution causes the formation of large insoluble PEX14-(128) aggregates in the bacteria. For the other alanine mutants made it is likely that the amino acid substitutions made had minor if any effects on the conformation of the protein since

[1] Abbreviations: *At*PEX5, *Arabidopsis thaliana* PEX5; *At*PEX14, *Arabidopsis thaliana* PEX14; BCA, bicinchonic acid; *Hs*PEX5, *Homo sapiens* PEX5; PEX5, peroxin 5; PEX14, peroxin 14; PTS, peroxisome-targeting signal; *Tb*PEX5, *Trypanosoma brucei* PEX5; *Tb*PEX14, *Trypanosoma brucei* PEX14.

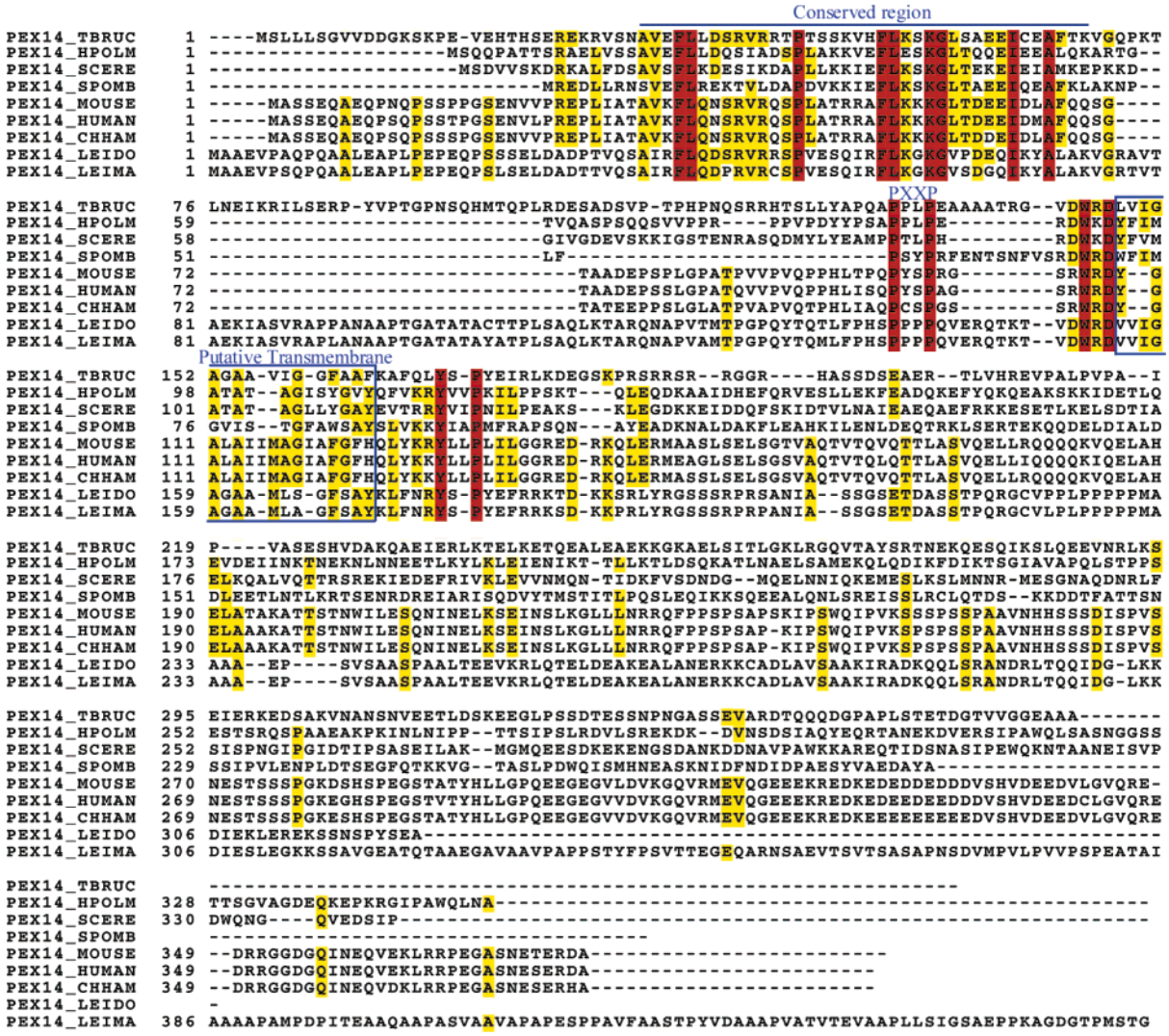

FIGURE 1: Sequence alignment of PEX14s by CLUSTALW. Completely conserved residues are colored red, more than 50% conserved residues are yellow. The conserved region at the N-terminal region, the PXXP motif, and the putative transmembrane region (blue box) are also indicated. The sequences used in the alignment are *Hansenula polymorpha* (Swiss Prot code: P78723), *Saccharomyces cerevisiae* (P53112), *Schizosaccharomyces pombe*, *Mus musculus* (Q9P0A0), Human (O75381), Chinese hamster (Q9Z2Z3), *Leishmania donovani* (AAN03593), *Leishmania major* (AL163505), and *Trypanosoma brucei* (AJ512212).

the mutants could be purified easily and were as well-behaving during the purification and concentration steps as wild-type protein.

The *Tb*PEX14(84) construct was made by introducing a stop codon using the Quik-change kit and expressed and purified in the same way as *Tb*PEX14(128). The expression and purification of *Tb*PEX14(146,H) were carried out as described previously (*43*). The homogeneity of the purified protein was checked using SDS−PAGE. The protein concentrations were measured using a BCA-based colorimetric assay (Pierce) with bovine serum albumin as a standard if the protein does not contain tryptophan; for the tryptophan-containing *Tb*PEX14(146,H), UV absorbance at 280 nm was used.

All the WXXXF/Y motif peptides used in this study are derived from *Tb*PEX5. The 13-mer peptides were purchased from United Biochemical Research, Inc., and the purity was checked by HPLC and mass spectrometry. Fluorescein-tagged peptide was synthesized by coupling 5-(and-6)-carboxyfluorescein succinimidyl ester to the N-terminal amino group of the peptide. The concentration of the peptides without fluorescein was measured using the absorbance at 280 nm and the extinction coefficient calculated from the sequence. For fluorescein-tagged peptide the absorbance at 494 nm was measured at pH 9, and the peptide concentration was obtained using an extinction coefficient of 77 000 M^{-1} cm^{-1}.

Fluorescence Assay. The binding of WXXXF/Y peptides to *Tb*PEX14(128) was monitored through the intrinsic fluorescence of tryptophan using an excitation wavelength of 295 nm, and the emission was monitored at 350 nm. An initial peptide concentration in the range of 20−500 nM was used for the assays. The peptides were titrated by adding an increasing amount of PEX14 until no significant increase of fluorescence signal was reached or until a protein concentration of 40 μM was reached. All assays were performed using a SPEX 1681 spectrometer in a 3-mL cuvette with 50 mM sodium phosphate pH 7.0 at 20 °C with constant stirring. Although the PEX14(84) and PEX14(128) used for the assay do not contain tryptophan, they gave an increasing fluorescence signal even in the absence of peptide presumably due

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PEX5_TBRUC   1 MDCGAGFALGQQLAKDALHMQGGVRPGTTGNVEQDALMTGMMVPPTGPMED--WAQHFAAHQHHHQQHQQMMMQRQHNDA
PEX5_LEIMA   1 MDCNTGMQLGQQFSKDVTMMHGGV-PMSGAMSEQDALMVSAQVGGASPMMAAQWAQNFQQQQAMQAMRQQHEMEQAFQNS
PEX5_LEIDO   1 MDCNTGMQLGQQFSKDATMMHGGV-PMSGAMSEQDALMVGAQVAGANPMMAAQWAQNFQQQQAMQAMRQQHEMEQAFQNS

PEX5_TBRUC  80 LMIQQQHRDMEEAFRASARAGAPQQANAGPLMMPPGPMMMAGGMAPMMHAGGFMMGGMPQMMPCAPMGMNMGMAPVATMS
PEX5_LEIMA  80 ---QQQQHAAVAQSGQMLGMAGPQQQQFMVQQQQQASMMNAAMMSQGMMTANMGFG---MMMPRTQY------QPLPNLS
PEX5_LEIDO  79 ---QQQQ-AAAAQSRQMLGMAGPQQQQFMAQQQQ-ASMMNAAMMSQGMMAANMGLG---MMMPRTQY------QPLPNLS

PEX5_TBRUC 148 PATTNTVSGAREGATAVSSAAPGVVDLGGDSAWAEKLHQAEWGQDYKDVEVHTVEGSTAQTVEEHAKTSKFYEFMDKIRK
PEX5_LEIMA 146 VLQPNQQQQQQQQLVNLAPAVQ-------DSAWADQLSQQQWSTDYSQVQTFSAPGMEDKTVEERIKDSEFYKFMDQVKN
PEX5_LEIDO 159 ALQP----KQQQPLANLAPAAQ-------DSAWADQLSQQQWSTDYSQVQTFSAPGMEDKTVEERIKDSEFYKFMDQVKN

PEX5_TBRUC 221 KELLVDEDSGEVVQGPGPDPDVEADTEYLARLAAMEGINVPPSVMDHMQGQDGVQRGTDEDMEGMMGDDVYDPSADVEQW
PEX5_LEIMA 215 KEVLIDEEKGELVQGPGPEVGVPEDAEYLRHWAEMEGLNMPEGVFQPPPPASAMMTPENGDPDTYI-KEMDMAENDVEDW
PEX5_LEIDO 239 KEVLIDEEKGELVQGPGPEVGVPEDAEYLRHWAEMEGLHMPESVFQSPPPASAMTSPENGDPDAYV-KEMDMAANDVEDW

PEX5_TBRUC 300 AQEYAQMQAMQERLQNNTDYPFEANNPYMYHENPMEEGLSMLKLANLAEAALAFEAVCQKEPEREEAWRSLGLTQAENEK
PEX5_LEIMA 294 AQEYAEMQERLQKVTNNTDYPFEPNNPYMFHDFPFDEGMEMLQLGNLAEAALAFEAVCHKDSSNEKAWQILGTTQAENEK
PEX5_LEIDO 319 AQEYAEMQERLQKVTNSTDYPFEPNNPYMFHDFPFDEGMEMLQLGNLAEAALAFEAVCHKDSSNEKAWQILGTTQAENEK

PEX5_TBRUC 380 DGLAIIALNHARMLDPKDIAVHAALAVSHTNEHNANAALASLRAWLLSQPQYEQLGSVNLQADVDIDDLNVQSEDFFFAA
PEX5_LEIMA 374 DGLAIIALNNARKLNIRNLEVHAALSVSHTNERNADAAMDSLKAWLVNHPEYEQLASVSIPPDAELD----VQETFFFAD
PEX5_LEIDO 399 DGLAIIALNNARKLNPRNLEVHAALSVSHTNERNADAAMDSLKAWLINHPEYEQLASVSIPPNAELD----VQETFFFAD

PEX5_TBRUC 456 PNEYRECRTLLHAALEMNPNDAQLHASLGVLYNLSNNYDSAAANLRRAVELRPDDAQLWNKLGATLANGNRPQEALDAYN
PEX5_LEIMA 450 PSRMREARTLYEAAIEMNPSDSQLFTNLGVLHNVAHEFDEAAECFRKAVALHPDDPKMWNKLGATLANGGHPDQALEAYN
PEX5_LEIDO 479 PSRMREARTLYEAAIEMNPSDSQLFTNLGVLHNVAHEFDEAAECFRKAVALHPDDPKMWNKLGATLANGGHPDQALEAYN

PEX5_TBRUC 536 RALDINPGYVRVMYNMAVSYSNMSQYDLAAKQLVRAIYMQVGGTTPTGEASREATRSMWDFFRMLLNVMNRPDLVELTYA
PEX5_LEIMA 530 RALDINPGYVRAMYNMAVAYSNMSQYNMAARQIVKAIASQQGGTKPSGEGSIMATRNMWDLLRMTLNLMDRDDLVQLTYN
PEX5_LEIDO 559 RALDINPGYVRAMYNMAVAYSNMSQYNMAARQIVKAIASQQGGTKPSGEGSIMATRNMWDLLRMTLNLMDRDDLVQLTYN

PEX5_TBRUC 616 QNVEPFAKEFGLQSMLL
PEX5_LEIMA 610 EQLEPFVKEFGLEGHV-
PEX5_LEIDO 639 EQLEPFVKEFGLEGH--
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FIGURE 2: Sequence alignment of Trypanosomatid PEX5's. Completely conserved residues are colored yellow and the WXXXF/Y motifs are boxed in red. The sequences used are *Trypanosoma brucei* (Swiss Prot code: Q9U7C3), *Leishmania major*, and *Leishmania donovani* (Q9NIR9).

to contaminant proteins that have tryptophan residues. The fluorescence increase due to contaminants was subtracted from the fluorescence change obtained from the titration experiment. The relative fluorescence intensity change versus protein concentration was analyzed by the program Prism version 3.0 and the K_d's were calculated using the following equations.

$$K_d = C_{P14}C_{pep}/C_{complex}$$

$$C_{P14} = C_{P14,total} - C_{complex}$$

$$C_{pep} = C_{pep,0} - C_{complex}$$

$$F = f_{P14}C_{P14} + f_{pep}C_{pep} + f_{complex}C_{complex}$$

where F is the total observed fluorescence, f_{P14} is the molar fluorescence coefficient of the protein solution, f_{pep} is the molar fluorescence coefficient of the peptide, $f_{complex}$ is the molar fluorescence coefficient of the complex, $C_{P14,total}$ is the total added PEX14 concentration, $C_{pep,0}$ is the initial peptide concentration, and $C_{complex}$ is the concentration of the peptide−protein complex.

In the case of fluorescein-tagged peptides, an excitation wavelength of 492 nm and an emission wavelength of 523 nm were used with an initial peptide concentration of 20 nM. With this choice of wavelengths, the contribution of the fluorescence signal from the protein solution was negligible and omitted from the calculation of the K_d.

RESULTS

Affinity of the Three 13-mer WXXXF/Y Motif Peptides from TbPEX5 for TbPEX14. *Tb*PEX5 contains three WXXXF/Y pentapeptide motifs in its N-terminal half that can potentially bind *Tb*PEX14 (Figure 2). Three different 13-mer peptides, each containing one of the three WXXXF/Y motifs in the *Tb*PEX5 sequence, were used to determine their binding

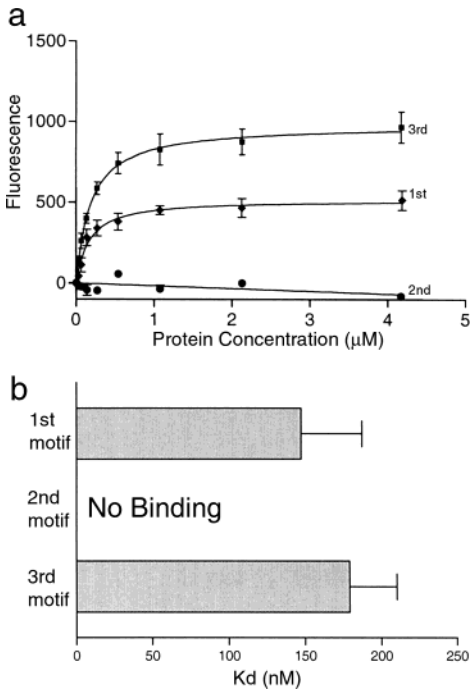

FIGURE 3: Fluorescence assays using three WXXXF/Y motifs of *Tb*PEX5 and wild type *Tb*PEX14(128) protein. The intrinsic fluorescence signal of tryptophan was used: (a) Fluorescence data and standard deviations from independent measurements; (b) calculated K_d's and standard deviations using the computer program PRISM.

affinity for *Tb*PEX14(128) using the intrinsic fluorescence of the peptides (Figure 3). Equilibrium-binding constants calculated from the titration curves showed that the first and third motifs have similar affinities for *Tb*PEX14(128) with K_d's of 147 and 179 nM, respectively (Table 1). Surprisingly, the second motif did not show any significant affinity at the conditions used. The purity of the second motif peptide was carefully checked and appeared to be at least as high as that

Table 1: Binding Affinity between the Three WXXXF/Y Motifs of *Tb*PEX5 and *Tb*PEX14(128) Wild-Type Protein[a]

Peptide	Protein [1)]	number of experiments	K_d(nM)	σ(nM)
ED**WAQHF**AAHQHH (1st motif)	*Tb*PEX14(128) WT	3	147	40
AE**WGQDY**KDVEVH (2nd motif)	*Tb*PEX14(128) WT	2	-*	NA
EQ**WAQEY**AQMQAM (3rd motif)	*Tb*PEX14(128) WT	3	179	40

[a] Asterisk (*): This motif did not increase the fluorescence signal and the K_d cannot be determined. The binding affinity is therefore assumed to be very low. [1]*Tb*PEX14(128) includes the first 128 residues of *T. brucei* PEX14.

Table 2: Affinity of Second and Third WXXXF/Y Motif Variants of *Tb*PEX5 for Wild-Type *Tb*PEX14(128)[a]

	Name	Sequence	Protein	number of experiments	K_d(nM)	σ(nM)
	Position	123456789				
	1(wt)	AE**WGQDY**KDVEVH	*Tb*PEX14(128) WT	2	-*	NA
	2	AE**WGQDY**KDV<u>Q</u>VH	*Tb*PEX14(128) WT	2	-*	NA
2nd motif	3	AE**W<u>A</u>QDY**KDVEVH	*Tb*PEX14(128) WT	2	-*	NA
	4	AE**W<u>A</u>QDY**KDV<u>Q</u>VH	*Tb*PEX14(128) WT	2	57200	30000
	5	AE**W<u>A</u>QDY**<u>A</u>DV<u>Q</u>VH	*Tb*PEX14(128) WT	3	6000	1230
	6(wt)	EQ**WAQEY**AQMQAM	*Tb*PEX14(128) WT	3	179	31
3rd motif	7	EQ**WAQEY**AQM<u>E</u>AM	*Tb*PEX14(128) WT	3	826	99
	8	EQ**W<u>G</u>QEY**AQMQAM	*Tb*PEX14(128) WT	2	8800	991

[a] Asterisk (*): These peptides did not increase the fluorescence signal and a K_d value could not be determined.

of the other two peptides. Its sequence was confirmed by mass-fragmentation experiments (data not shown). The possible reason for the lack of binding of the second motif peptide is addressed later. The binding affinities between the *Tb*PEX5 WXXXF/Y pentapeptide repeats and *Tb*PEX14-(128) are lower than the reported values between human counterparts where K_d's are in the low nanomolar to 100 nM range (*30*).

Testing Variants of the Second and Third WXXXF/Y Motif Peptides for PEX14 Affinity. To investigate the reason the second motif peptide did not bind PEX14, variants of the second and third WXXXF/Y motif peptides were obtained and evaluated in the binding assays. Two positions were chosen to be altered in the various peptides. The first one was the residue immediately after the conserved tryptophan (a glycine in the second motif and an alanine in the third motif) and the second one was the ninth residue from the conserved tryptophan (a glutamate in the second motif and a glutamine in the third motif) (see Table 2). The first position was chosen because it has been suggested that the WXXXF/Y motif adopts an α-helical conformation to present the conserved W and F/Y at the same side of the helix (*22*, *23*). To estimate the effect of the propensity of the peptides to form an α-helical conformation on the binding affinity, the glycine at the second position in the second motif was changed to an alanine and the alanine at the second position in the third motif was replaced by a glycine. The rationale for these changes is that the presence of a glycine would lead to a lower tendency to form an α-helix, whereas peptides having an alanine have an increased tendency to form an α-helix and, hence, the largest effect on the secondary structure was expected with these substitutions (*36*−*38*). The experimental results showed that the second motif peptide with the glycine-to-alanine change (peptide 3 in Table 2) still did not have any measurable affinity for PEX5. The third motif peptide with the alanine-to-glycine substitution (peptide 8 in Table 2) exhibited about 50 times reduced binding affinity demonstrating the importance of alanine at this position in the third motif peptide.

The second position that was varied was the ninth residue from the conserved tryptophan (a glutamate in the second motif and a glutamine in the third motif). In yeast species, several PEX5 WXXXF/Y motifs with negatively charged residues at the ninth position from the tryptophan are known to bind only to PEX13 and not to PEX14 (*32*, *33*, *39*, *40*). Interestingly, the second WXXXF/Y motif of *Tb*PEX5 has a negatively charged residue (E) at motif position 9, and did not show any binding to *Tb*PEX14. To test if this residue is important in *Tb*PEX5-*Tb*PEX14 binding, we used peptides where the residue at motif position 9 was changed from glutamate to glutamine in the second motif and, conversely, from glutamine to glutamate in the third motif (Table 2). The fluorescence assays showed that the second motif with an E to Q change did not have measurable affinity for PEX5, while the Q to E substitution in the third motif decreased the affinity by 4.6-fold. These results suggested that whether the residue at the ninth position is negatively charged or not does not play a critical role, at least per se, in *T. brucei*

Table 3: Binding Affinity between Different Lengths of the Third WXXXF/Y Motif of *Tb*PEX5 and *Tb*PEX14(128) Wild-Type Protein

Peptide containing *Tb*PEX5 3rd motif		Protein	number of experiments	K_d(nM)	σ(nM)
ADVEQ**WAQEY**AQMQAM	(16mer)	*Tb*PEX14(128)WT	3	214	19
EQ**WAQEY**AQMQAM	(13mer)	*Tb*PEX14(128)WT	3	179	31
Q**WAQEY**AQMQ	(10mer)	*Tb*PEX14(128)WT	3	1520	175
Q**WAQEY**A	(7mer)	*Tb*PEX14(128)WT	2	23000	4900

PEX14-PEX5 interaction, although the introduction of a negative charge reduced the binding affinity significantly as shown with peptide 7 in Table 2.

To obtain more insight into the role of various PEX5 motif side chains in the PEX5−PEX14 interaction, several additional variants of the second motif peptide were tested. Peptide 4, a second motif variant with two changes compared to the wild-type peptide, increased the binding affinity and showed interaction with *Tb*PEX14(128). When one more alanine is introduced in place of lysine at the sixth position (peptide 5 in Table 2), the binding affinity is increased about 10 times compared to peptide 4. The results obtained with these variants suggest that the low tendency to form an α-helix and the charge at the ninth position may explain the absence of binding of the second motif peptide. Comparison of the results with peptides 5 and 8 on one hand and peptides 4 and 7 on the other also showed that the tendency to form an α-helix is more important for PEX14 binding than the absence of charge at the ninth position. The results also suggest that the ninth residue from the conserved tryptophan (glutamate in the second motif and glutamine in the third motif) is also involved in PEX14 interactions even though it is outside the WXXXF/Y motif.

Affinity of 7-, 10-, 13-, and 16-mer WXXXF/Y Peptides for TbPEX14. To investigate the dependence of peptide length on the affinity for PEX14, a series of 7-, 10-, 13-, and 16-mer peptides, each containing the *Tb*PEX5 third WXXXF/Y motif (Table 3), were used to measure the affinities for *Tb*PEX14(128). The 16- and 13-mer peptides showed comparable affinities around 200 nM (Table 3). The 10-mer peptide had an approximately 7-fold lower affinity than the 16-mer peptide, and the 7-mer peptide showed a K_d of 23 μM, an affinity that is about 100 times lower than that of the 16-mer peptide.

Affinity of TbPEX14 Variants for the Third WXXXF/Y Motif of TbPEX5. Turning to the other partner of the complex under investigation, point mutations were introduced in *Tb*PEX14(128) to determine the critical residues in *Tb*PEX14 for *Tb*PEX5 binding. From the sequence alignment of eight different species, nine completely conserved residues in the N-terminal region of PEX14 were selected for mutagenesis (Figure 1). All these residues except Ala66 were mutated to alanine, while Ala66 was mutated to a methionine. Three additional double mutants were made including F35A/L36A, F52A/L53A, and K56A/G57A to explore the role of these consecutively conserved residues. All the mutated and wild-type PEX14(128) proteins were tested for affinity except for mutants K56A and K56A/G57A, for which insufficient protein could be produced to perform the fluorescence assay. All the mutants showed lower binding affinities for the third motif 13-mer peptide than the wild-type *Tb*PEX14(128) (Table 4). The P45A and G57A PEX14(128) mutants retained about 30% affinity, and the L36A, L53A, I63A, and A66M mutants had less than 10% affinity compared to the wild-type protein. Especially mutants F35A, F35A/L36A, F52A, and F52A/L53A showed no significant increase in fluorescence signal, indicating that their binding affinities were very low. This suggested that the two completely conserved phenylalanine residues (F35 and F52) play a critical role in the interactions with PEX5. The observed binding affinities of the mutants agree with a previous study in *Arabidopsis thaliana* PEX14, where the binding affinities of mutant *At*PEX14s with *At*PEX5 were measured using a β-galactosidase activity-coupled assay (*41*). Our study confirms that the conserved amino acids in the N-terminal region are important for PEX5-PEX14 binding in *T. brucei*, and also provides quantitative data about the contribution of the individual amino acids to the interaction between these two key proteins in glycosome biogenesis.

Affinity of Three N-Terminal Constructs of TbPEX14 for the Third Motif 13-mer Peptide from TbPEX5. The N-terminal part of PEX14 is responsible for binding to PEX5 (*29*, *41*). To study the affinities of PEX14 of various lengths, three C-terminally truncated *Tb*PEX14 constructs, *Tb*PEX14-(84), *Tb*PEX14(128), and *Tb*PEX14(146,H), were used in a fluorescence-based assay with the third motif 13-mer peptide. The rationale for this study was to see if the length of *Tb*PEX14 has an influence on the affinity. The shortest construct, *Tb*PEX14(85), would in principle be more suitable for an assay using the intrinsic fluorescence signal of Trp-containing peptides because it contains no tryptophan or tyrosine residue. However, in that case its affinity for the WXXXF/Y motif should not be dramatically different from that of the longer PEX14 constructs. Fortunately, it appears indeed that the binding affinities of the different constructs to the 13-mer peptide are comparable to each other (Table 5). These results agree with the study of *A. thaliana* where two regions of *At*PEX14, $I^{58}-L^{65}$ and $R^{78}-R^{97}$, were shown to be responsible for *At*PEX5 binding. These regions correspond to $V^{29}-L^{36}$ and $K^{49}-T^{68}$ in *Tb*PEX14, which are part of the shortest construct, *Tb*PEX14(84).

DISCUSSION

Highly specific information has been obtained regarding the PEX5 and PEX14 interactions in *T. brucei*, the causative agent of sleeping sickness. Affinities between various WXXXF/Y motif peptides from *Tb*PEX5 and several N-terminal *Tb*PEX14 constructs were measured using a steady-state fluorescence assay.

Table 4: Binding Affinity between the Third WXXXF/Y Motif of *Tb*PEX5 and *Tb*PEX14(128) Mutants[a]

Peptide	Protein	number of experiments	K_d(nM)	σ(nM)
EQ**WAQEY**AQMQAM (3rd motif)	*Tb*PEX14(128) WT	3	179	31
	*Tb*PEX14(128) F35A	1	-*	NA
	*Tb*PEX14(128) L36A	2	2981	641
	*Tb*PEX14(128) F35A/L36A	2	-*	NA
	*Tb*PEX14(128) P45A	3	768	208
	*Tb*PEX14(128) F52A	1	-*	NA
	*Tb*PEX14(128) L53A	2	3439	451
	*Tb*PEX14(128) F52A/L53A	1	-*	NA
	*Tb*PEX14(128) K56A	0	N/D**	NA
	*Tb*PEX14(128) G57A	3	510	90
	*Tb*PEX14(128) K56A/G57A	0	N/D**	NA
	*Tb*PEX14(128) I63A	2	5314	1740
	*Tb*PEX14(128) A66M	3	4483	1179

[a] Single asterisk (*): These mutants did not increase the fluorescence signal, and the K_d cannot be determined. The binding affinity therefore most likely is very low. Double asterisk (**): The K_d cannot be determined because these mutants did not yield sufficient amount of protein for the assay.

Table 5: Affinity of the Third WXXXF/Y Motif Peptides of *Tb*PEX5 for Constructs of *Tb*PEX14 Wild-type Protein with Different Lengths

Peptide (*Tb*PEX5 3rd motif)	Protein	number of experiments	K_d(nM)	σ(nM)
EQ**WAQEY**AQMQAM	*Tb*PEX14(84) WT	3	230	86
EQ**WAQEY**AQMQAM	*Tb*PEX14(128) WT	3	179	31
Fluoresein-EQ**WAQEY**AQMQAM	*Tb*PEX14(146) WT	3	307	25

The data obtained from *Tb*PEX14(128) mutants showed that all mutants had a lower affinity than wild-type *Tb*-PEX14(128) for the third WXXXF/Y motif peptide of *Tb*PEX5, indicating that the eight conserved residues in PEX14 are important for the PEX14−PEX5 interaction. The hydrophobic residues of PEX14 including Phe35, Leu36, Phe52, Leu53, and Ile63 have larger effects on affinity than residues such as Pro45, Gly57, and Ala66. Especially, mutation of two phenylalanine residues (Phe35 and Phe52) to alanine almost completely abolished the binding (Table 4). These data on *Tb*PEX14 mutants agree with the results reported for *A. thaliana* PEX14 where mutation of, among others, residues Phe64, Leu65, Phe81, Leu82, and Ile92 disrupted the interaction between *At*PEX14 and *At*PEX5, and mutation of Pro74 did not affect the binding (*41*). The study of *A. thaliana* PEX14 also suggested that less strictly conserved amino acids such as Ala61, Leu87, and Thr88 might be involved in the interaction.

The blue shift in the fluorescence of the *Tb*PEX5 third WXXXF/Y motif peptide upon binding to *Tb*PEX14 suggests that the tryptophan of the peptide is placed in a nonpolar environment (*42*). The mutation studies imply that hydrophobic residues of PEX14 such as phenylalanine and leucine may interact with the tryptophan and phenylalanine/tyrosine residues of the WXXXF/Y motif, in particular, since these hydrophobic side chains can face the same side of the peptide if the latter adopts an α-helical conformation. There are two completely conserved phenylalanine residues (Phe35 and Phe52) separated by 16 amino acids in the N-terminal region of PEX14's (Figure 1). Interestingly, phenylalanine-to-alanine mutations in either positions (F35A or F52A) are sufficient to completely disrupt the binding. This suggests that these phenylalanine residues might be a critical part of the hydrophobic PEX14−PEX5 interaction and may form a single binding site for PEX5.

Among the three WXXXF/Y pentapeptide motifs in *Tb*PEX5, the second motif did not show any affinity for *Tb*PEX14(128) (Table 1, Figure 3). On the basis of the results obtained with variants of the second and third motif peptides, there seem to be two factors that can explain the lack of binding of the second motif peptide. The first one is the low propensity of the second motif peptide to adopt an α-helical conformation. Introduction of an amino acid such as alanine that confers on the peptide a high tendency to form an α-helix, augments the binding affinity as demonstrated by peptides 4, 5, and 8 in Table 2. The second factor is the

negative charge on the ninth residue from the conserved tryptophan as inferred from the results obtained with peptides 4, 5, and 7 in Table 2. The WXXXF/Y motif that has a negatively charged residue at the ninth position has been shown to interact with PEX13 in Chinese hamster, *Pichia pastoris*, and *S. cerevisiae* (*32*, *39*, *40*), suggesting that this residue plays a key role in determining whether PEX5 interacts with PEX14 or PEX13. In the case of *T. brucei* PEX14−PEX5 interaction, the introduction of a negative charge at the ninth position in the third motif did not completely abolish the binding, although it reduced the affinity of the peptide, and removal of charge in the second motif, in conjunction with increasing the propensity of the peptide to adopt a helical conformation increased the affinity (Table 2). These results indicate that the ninth residue is involved in PEX14−PEX5 interaction, but it does not play a critical role in determining the binding partner of the WXXXF/Y motifs in *Tb*PEX5. Although the 13-mer peptide containing the second WXXXF/Y motif of *Tb*PEX5 did not show any affinity for *Tb*PEX14 in an in vitro assay, it remains to be studied whether the second motif as part of the *Tb*PEX5 contributes to the binding of this PTS-receptor to *Tb*PEX14.

The fluorescence assay between 7-, 10-, 13-, and 16-mer peptides and *Tb*PEX14(128) showed the dependence of affinity on peptide length. Especially the 7-mer peptide had an approximately 100-fold lower affinity than the 16-mer peptide. These results suggest that either residues outside the WXXXF/Y motif might be involved in the interaction or, alternatively, that shorter peptides may have a lower tendency to form a helical conformation which seems important to make the tryptophan and phenylalanine/tyrosine residues face to the same side of the helix. Finding the minimum length of the peptide that retains most of the affinity and understanding the reason for decreased affinity of shorter peptides can have great implications for the design of peptidomimetic inhibitors that can potentially interfere with the PEX14−PEX5 interaction.

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