**Summary**

We have established a procedure for isolating native peroxisomal membrane protein complexes from cultured human cells. Protein-A-tagged peroxin 14 (PEX14), a central component of the peroxisomal protein translocation machinery was genomically expressed in Flp-In-293 cells and purified from digitonin-solubilized membranes. Size-exclusion chromatography revealed the existence of distinct multimeric PEX14 assemblies at the peroxisomal membrane. Using mass spectrometric analysis, almost all known human peroxins involved in protein import were identified as constituents of the PEX14 complexes. Unexpectedly, tubulin was discovered to be the major PEX14-associated protein, and direct binding of the proteins was demonstrated. Accordingly, peroxisomal remnants in PEX14-deficient cells have lost their ability to move along microtubules. In vivo and in vitro analyses indicate that the physical binding to tubulin is mediated by the conserved N-terminal domain of PEX14. Thus, human PEX14 is a multi-tasking protein that not only facilitates peroxisomal protein import but is also required for peroxisome motility by serving as membrane anchor for microtubules.

**Key words:** Peroxisome, Microtubule, PEX14, Protein complex isolation

**Introduction**

Peroxisomes are ubiquitous, single membrane-bound cell organelles with a large variety of metabolic functions of which some are essential for humans. This is indicated by several inborn diseases caused by defects in peroxisome function. The most severe peroxisomal diseases known as the ‘spectrum of Zellweger syndrome’ are characterized by a defect in the biogenesis of peroxisomes and are lethal within the first months of life. So far, 12 complementation groups of peroxisome biogenesis disorders (PBDs) have been identified and studied in detail (Steinberg et al., 2006). Notably, with the exception of a gene coding for a protein of the peroxisomal fission machinery (Waterham et al., 2007), all genes affected in these patients encode proteins required for the import of matrix and membrane proteins into peroxisomes. Proteins that are required for peroxisome biogenesis are collectively called peroxins (PEX), and three of them, PEX3, PEX16 and PEX19, are necessary for the targeting and insertion of peroxisomal membrane proteins (Ma and Subramani, 2009). Seven peroxins that are defective in Zellweger patients belong to the peroxisomal import machinery for peroxisomes and are lethal within the first months of life. So far, 12 peroxins have been identified and studied in detail. Peroxisomes are remarkably dynamic in terms of their intracellular movements, which are mediated by either microtubules or actin filaments, depending on the species. Mammalian peroxisomes are moved along microtubule tracks by dynein–dynactin and kinesin motors (Kural et al., 2005; Schrader et al., 2000).

Most peroxins were identified by functional complementation of yeast or Chinese hamster ovary (CHO) cell mutants affected in peroxisome biogenesis (Erdmann et al., 1989; Tsukamoto et al., 1990) and the human orthologs were then identified by sequence similarity. However, current data indicate that the list of peroxins identified is far from being complete. This is due to the fact that: (1) some peroxins are present in functionally overlapping forms, like the ubiquitin-conjugating enzymes of the peroxisomal protein translocation takes place is still not known; however, the existence of a transient peroxisomal pore with properties expected for a protein-conducting channel has recently been identified (Meinecke et al., 2010). Notably, membrane-embedded PEX5 and PEX14 are major constituents of the translocation pore. After dissociation of receptor–cargo complex and release of the cargo protein into the peroxisomal lumen, the receptors are recycled for another round of import, which requires release of the receptors from the membrane (Lanyon-Hogg et al., 2010). The export of receptors is a complex process with ubiquitylation and ATP-dependent dislocation by “ATPases associated with diverse cellular activities” (AAA – peroxins) as key steps (Fujiki et al., 2008).

Other peroxins, including human PEX11 and Dlp1, play a role in peroxisome fission, proliferation and inheritance (Thoms and Erdmann, 2005). In fact, peroxisomes are remarkably dynamic in terms of their intracellular movements, which are mediated by either microtubules or actin filaments, depending on the species. Mammalian peroxisomes are moved along microtubule tracks by dynein–dynactin and kinesin motors (Kural et al., 2005; Schrader et al., 2000).

**Research Article**

**Perox14 is required for microtubule-based peroxisome motility in human cells**

Pratima Bharti1,*, Wolfgang Schliebs1,*, Tanja Schievelbusch1, Alexander Neuhaus1, Christine David1, Klaus Kock2, Christian Herrmann2, Helmut E. Meyer3, Sebastian Wiese3,4, Bettina Warscheid3,4, Carsten Theiss3 and Ralf Erdmann1,†

1Institute for Physiological Chemistry, Department of Systems Biology, Faculty of Medicine, Ruhr University of Bochum, 44780 Bochum, Germany
2Department of Physical Chemistry 1, Faculty of Chemistry and Biochemistry, Ruhr University of Bochum, 44780 Bochum, Germany
3Medizinisches Proteom-Center, Faculty of Medicine, Ruhr University of Bochum, 44780 Bochum, Germany
4Faculty of Biology and BIOSS Centre for Biological Signalling Systems, University of Freiburg, 79104 Freiburg, Germany
5Institute of Anatomy and Molecular Embryology, Faculty of Medicine, Ruhr University of Bochum, 44780 Bochum, Germany

*These authors contributed equally to this paper
†Author for correspondence (Ralf.Erdmann@rub.de)

Accepted 14 January 2011
Journal of Cell Science 124, 1759-1768
© 2011, Published by The Company of Biologists Ltd
doi:10.1242/jcs.079368
Import machinery (Grou et al., 2008) and (2) other peroxins have different cellular functions in addition to their peroxisome-specific roles and thus, inactivation of the corresponding genes lead to more generalized cellular defects and severe human diseases that are not immediately recognized as PBDs. These processes include peroxisome fission, regulated degradation, inheritance and motility, which require proteins that are also necessary for the maintenance of other organelles (Fagarasanu et al., 2009; Farre et al., 2009; Waterham et al., 2007). Thus, because of the functional redundancy of peroxins and the sharing of peroxins by different organelles, other strategies have to be employed to identify such peroxins with overlapping function. To fill this gap, proteomic approaches have been applied; however, so far the complete analyses of whole peroxisomal proteomes did not result in the identification of new biogenesis factors (Saleem et al., 2006; Wiese et al., 2007). This might be explained by the low abundance of such proteins or the fact that they might be mistaken as contaminants.

Here, we identified tubulin as novel binding partner for PEX14, the key protein of the protein import machinery, and assign a new function for the protein in peroxisome motility. We established a new procedure for isolation of subcomplexes from the human peroxisomal membrane for mass spectrometric analyses. For this purpose, we generated stable human cell lines expressing genomically tagged peroxins that are functional and suitable for purpose, we generated stable human cell lines expressing new procedure for isolation of subcomplexes from the human function for the protein in peroxisome motility. We established a key protein of the protein import machinery, and assign a new fact that they might be mistaken as contaminants.

Results
Establishment of a human cell line expressing Protein-A-tagged PEX14
In order to stably express Protein-A-tagged PEX14 in human cells, the fusion gene PEX14-TEV-Protein A was cloned into the modified expression vector pcDNA5/FRT (Invitrogen). To ensure that the tag comprising a tobacco etch virus protease cleavage site (TEV) and Protein A does not affect the functionality of PEX14 during matrix protein import, the expression plasmid used with another plasmid encoding either a PTS1- or a PTS2-marker protein were co-transfected into a Zellweger patient fibroblast cell line deficient in PEX14. Functional complementation of the matrix protein import defect was visualized by fluorescence microscopy (Fig. 1A,B). Expression of the plasmid-encoded tagged PEX14 did result in the restoration of peroxisomal protein import shown by the reappearance of a punctate fluorescence pattern for the peroxisomal matrix markers (EGFP-PTS1 and PTS2-YFP) which co-localized with the fluorescence pattern for peroxisomal membrane marker PMP70 as well as PEX14. The data show that the C-terminal tagging of PEX14 did not interfere with the function of the protein in peroxisomal protein import.

After genomic integration of the fusion gene into Flp-In-293 cells, clones were selected by hygromycin resistance, Zeocine-sensitivity, lack of β-galactosidase activity and protein expression (data not shown). Neither the C-terminal tagging nor the ectopic expression affected the peroxisomal localization of PEX14-TEV-Protein A, as indicated by the observed co-localization of PEX14 and the peroxisomal marker EGFP-PTS1 (Fig. 1C).

Purification, identification and subcellular distribution of PEX14-associated proteins
For isolation of human PEX14-associated proteins, we established a protocol that in principle was based on peroxin complex isolation described for yeast (Agne et al., 2003). A crucial step in the isolation of membrane protein complexes is the choice of detergent for solubilization. It was reported that a human peroxin complex including PEX14, PEX5 and the RING finger peroxins remains stable upon extraction with 1% (w/v) digitonin solution (Reguengas et al., 2001). Accordingly, for purification of PEX14 complexes, membranes were prepared from 25g Flp-In-293[PEX14-TEV–PA] cells (wet weight) and 1% (w/v) digitonin was applied for PEX14 complex extraction. Solubilized PEX14 complexes were affinity-purified with human IgG-Sepharose and eluted from the column by TEV protease cleavage. Isolated proteins were further separated by gradient gel electrophoresis and stained by colloidal Coomassie Blue (supplementary material Fig. S1). Non-transfected Flp-In-293 cells were treated in the same way and served as a negative control. The isolated and stained PEX14-associated proteins were subjected to in-gel tryptic digestion and peptides were identified by nano-HPLC on-line coupled to electrospray ionization tandem mass spectrometry (ESI-MS/MS).

In three independent analyses of PEX14 complexes, 22 known peroxisomal proteins were identified (supplementary material Table S1). Among these, were the known importor constituents PEX5, ubiquitin, PEX13, and the RING-finger peroxins PEX10, PEX12 and the AAA-peroxin PEX1. In addition, peroxins involved in membrane protein targeting as PEX19, PEX3 and PEX16 could be identified in association with PEX14. Thus, almost all known binding partners of human PEX14 were detected, thereby demonstrating the efficiency of the purification procedure.

In order to obtain information on the relative abundance of proteins identified in both PEX14-containing complexes and the respective mock preparations (control), we calculated their individual spectral counts according to Liu et al. (Liu et al., 2004). Fig. 2A shows the relative abundance of proteins associated with peroxisomes, microtubules and the cytoplasm as well as of proteins with other subcellular locations in the two PEX14 complexes and the respective mock preparations. In comparison with the controls, proteins associated with peroxisomes and microtubules were significantly enriched in PEX14-containing complexes. Complete lists of peroxisomal and microtubular proteins identified in PEX14 complexes, including information on accession numbers, gene names and spectral counts are provided in supplementary material Tables S1 and S2. Interestingly, microtubular proteins were up to 27.5-fold enriched (preparation #2) and accounted for the most abundant class of proteins associated with PEX14. The microtubular fraction comprised various isoforms of tubulin (predominantly β-tubulin), motor and adaptor proteins like dynein, kinesin and dynactin (DynC1H1), and subunits of the T-complex (CCT complex) proposed to act as a chaperone for tubulin polymerization (Brackley and Grantham, 2009) (supplementary material Table S2).

In order to investigate whether the high enrichment of tubulin and tubulin-associated proteins indicates a functionally relevant association, PEX14-containing complexes were purified in the presence of 100 nM nocodazole, a potent microtubule-depolymerizing agent (Vasquez et al., 1997). Relative quantitative mass spectrometric analysis revealed that under these conditions β-tubulin (TUBBx and FLJ52712), dynein (DYN1C1H1), dynactin (DCTN1) and two subunits of the T-complex (CCT5 and CCT6A)
were the only proteins with a microtubular localization that co-purified with PEX14 (Fig. 2B). The low abundance or absence of α-tubulin and MAPs in this preparation confirms the dissociation of the microtubular assemblies and suggests that association of PEX14 with the microtubules is mediated by direct binding to β-tubulin. In line with this assumption, T-complex proteins as chaperones for tubular frameworks were abundant binding partners of PEX14.

PEX14-dependent peroxisome motility

Fig. 1. PEX14–TEV–Protein A localizes to peroxisomes and is functional. (A, B) To test functionality of PEX14–TEV–Protein A during peroxisomal matrix protein import, plasmid-encoded EGFP–PTS1 (A) or PTS2–EYFP (B) was transiently expressed in PEX14-deficient (ΔPEX14T) and normal fibroblast (GM5756T) cells. Normal fibroblast cells exhibit a punctate fluorescence pattern indicating peroxisomal targeting of both PTS1- and PTS2-proteins, whereas all peroxisomal matrix proteins are mislocalized in the cytosol in PEX14-deficient cells. Functional complementation of the import defect was achieved by expression of plasmid encoded PEX14–TEV–Protein A as indicated by a punctate fluorescence pattern, which is congruent with the immunofluorescence pattern obtained for the peroxisomal membrane proteins PMP70 and PEX14. (C) Validation of the peroxisomal localization of the Protein-A-tagged genomic copy of PEX14. Cell lines Flp-In-293 cells and Flp-In-293 [PEX14–TEV–Protein A] were transiently transfected with a plasmid encoding the peroxisomal marker EGFP–PTS1. Specific immunodetection of the fusion protein PEX14–TEV–Protein A was carried out with anti-Protein A antibodies, the endogenous and the fusion protein were detected by using antibodies against PEX14. Scale bars: 10 μm.
The concurrent presence of components of the peroxisomal import machinery and the cytoskeleton raises the issue of whether the components are constituents of the same complex. To this end, the isolated PEX14-containing protein complexes were also analyzed by size-exclusion chromatography. PEX14 covered a broad range of molecular assemblies, as indicated by its presence in fractions ranging in molecular size from larger than 1000 kDa to smaller than 150 kDa (Fig. 3). One of the higher molecular weight subcomplexes might represent the so-called importomer that enables matrix protein import, as indicated by co-elution with PEX5. Another PEX14 subcomplex with a molecular size of about 800 kDa was associated with PEX19, suggesting that this complex represents PEX14 in transit. Additionally, PEX14 formed high molecular weight complexes with β-tubulin that only contained minor amounts of PEX5 and no PEX19.

PEX14 is required for the movement of peroxisomes along microtubules

To investigate the role of PEX14 as a possible peroxisomal anchor for the microtubular network, the motile behavior of peroxisomal membranes was studied using time-lapse imaging in normal fibroblast cells and PEX14-deficient Zellweger patient cell lines. These mutant cell lines lack normal peroxisomes due to the import defect but still contain peroxisomal membrane ghosts. For in vivo labeling of peroxisomes, fibroblasts were transfected with an expression plasmid encoding the peroxisomal membrane protein EGFP–PEX26. In agreement with previous literature (Rapp et al., 1996; Wiemer et al., 1997), two kinds of peroxisomal movements were observed in normal fibroblasts (Fig. 4 and supplementary material Movie 1). Most peroxisomes (87–92%) oscillated in place, bearing a slow, random, vibrational motion with a speed of 0.015±0.002 μm/second whereas a significant population of peroxisomes (8–13%) displayed fast directional, long range saltatory movement in the range of 0.1±0.02 μm/second (Fig. 4B). The long-range movement of peroxisomes was abolished when transfected cells were preincubated with 10 μg/ml colchicine, which inhibits microtubule polymerization by binding to tubulin (data not shown). Accordingly, the long-range movement was not inhibited by 10 μg/ml Cytochalasin D, which is a potent inhibitor of actin assembly (data not shown). These results confirm recent work indicating that peroxisomes in mammalian cells can either

---

**Fig. 2. PEX14 associates with microtubules.** (A) PEX14 complexes (#1 and #2) and the respective mock preparations (control #1 and #2) were analyzed by tandem mass spectrometry, and spectral counts were calculated in order to obtain information on the relative abundance of proteins. Pie charts display the summed percentage abundance of proteins that are located at peroxisomes, at microtubules or in the cytoplasm, as well as proteins with other subcellular locations. Subcellular protein localizations are generally based on information provided by the SwissProt database. (B) List of microtubule-associated proteins and their relative abundance in PEX14-containing complexes that were isolated in the absence or presence of nocodazole as microtubule-depolymerizing agent. -, not detected; +, detected with spectral counts (SC) of 1–100; ++, 101–200; ++++, 201–300; ++++, 301–400; ++++, 401–500. All identified peroxisomal and microtubules-associated proteins are listed in supplementary material Tables S1 and S2.
associate to actin filaments or to the microtubular cytoskeleton to move for short or long distances, respectively (Schollenberger et al., 2010). Remarkably, PEX14-deficient cell lines were drastically affected in the long-range directional movement of peroxisomal structures (Fig. 4 and supplementary material Movie 2). The GFP-labeled remnant peroxisomes still showed some short-range motions, which could be completely blocked with Cytochalasin D demonstrating that the residual movement of peroxisomal structures in PEX14-deficient cells depends on the actin filaments (data not shown). To test whether the defect in microtubule-dependent motility is a feature of peroxisomal ghosts per se, as suggested previously (Nguyen et al., 2006), or whether it is specifically caused by the absence of PEX14, we conducted time-lapse imaging in mutant cell lines deficient in PEX5 or PEX1, other components of the peroxisomal import machinery. In both mutant cells, the peroxisomal ghosts displayed a vibrational and directional motion of the peroxisomal membranes similar to that in normal fibroblasts (Fig. 4 and supplementary material Movies 3, 4). Statistical analysis revealed that PEX5- as well as PEX1-deficient fibroblasts displayed wild-type-like motility with 8–13% of the peroxisomes exhibiting a fast, directional movement and 87–92% of peroxisomal population exhibiting slow and microtubular-independent oscillatory movement with little or no net displacement. By contrast, PEX14-deficient cell lines showed no long-range saltatory movement of peroxisomes, but 100% of the peroxisomal population only exhibited slow vibrational movement (Fig. 4B).

**Restoration of the peroxisomal motility of PEX14-deficient cells by complementation**

The peroxisome motility defect in PEX14-deficient cells could be rescued by expression of plasmid-encoded full-length PEX14 (Fig. 5 and supplementary material Movie 5). The plasmid was co-transfected together with the peroxisomal membrane marker EGFP–PEX26. The peroxisome motility mutant as well as complemented PEX14-deficient cell lines were assessed 48 hours after transfection by live cell imaging. The observed restoration of peroxisome motility by the expression plasmid confirmed that the mutant phenotype was indeed caused by the deficiency in PEX14 and not by a second site mutation.

To identify the minimal regions of PEX14 responsible for functional complementation, various truncated forms of PEX14 were tested for their ability to complement the defect in peroxisome
motility. A C-terminal truncated version, PEX14(1–260) including the conserved N-terminal region and the transmembrane domain (Fig. 5A), could restore the peroxisomal directional movement (Fig. 5 and supplementary material Movie 6). However, a predicted coiled-coil region ranging from amino acid 141 to amino acid 180 was also shown to be dispensable for complementation because the directional movement of peroxisomes along microtubular tracks could be restored by expression of the fragment PEX14(1–140) (Fig. 5 and supplementary material Movie 7). On the other hand, an N-terminal truncated form, PEX14(79–376) containing transmembrane domain and coiled-coil region, could not restore the peroxisomal directional movement (Fig. 5 and supplementary material Movie 8). Taken together, the data show that the N-terminal region is essential for the PEX14-dependent movement of peroxisomes and that the minimal fragment to perform that function comprises the conserved N-terminal domain together with a peroxisomal transmembrane region.

Physical interaction between the N-terminal conserved domain of PEX14 and microtubules

Tubulin was purified from porcine brain (supplementary material Fig. S2) and incubated with glutathione-Sepharose beads coated with GST–PEX14(1–78) or GST alone. Most of tubulin fraction (more than 60%) was retained by GST–PEX14(1–78) whereas only minor amounts (less than 5%) were bound by GST alone (Fig. 6A). The result of this in vitro binding assay demonstrates that the N-terminal domain of PEX14 directly interacts with tubulin. Remarkably, PEX5 as a known binding partner of PEX14(1–78) can compete with tubulin for binding to PEX14. For the competition analysis, a synthetic peptide representing the first N-terminal Wxxx[F/Y] motif (WAQEF) of the human PEX5 sequence was used. It is well established that the di-aromatic pentapeptide motif docks with high affinity to a groove at the surface of PEX14(1–78) (Neufeld et al., 2009). As shown in Fig. 6B (left panel), concomitant incubation of the GST–PEX14(1–78)-coated-beads with tubulin and an excess of a single Wxxx[F/Y] peptide (PEX5–W1 peptide) decreased the amount of bound tubulin to the level of the negative control. Moreover, incubation of a preformed GST–PEX14(1–78)–tubulin complex with PEX5–W1 peptide led to dissociation of the complex (Fig. 6B, right panel). These data demonstrate that PEX14(1–78) binds tubulin and PEX5 directly and that both proteins compete for PEX14 binding.

Discussion

Here we present a novel function for the human peroxin PEX14. Besides its well-established role as a central constituent of translocation machinery for matrix proteins, we discovered that PEX14 also serves as a membrane anchor for microtubules and is required for peroxisome motility in human cells. The existence of a peroxisomal protein that might anchor peroxisomes to microtubules had been proposed earlier (Schrader et al., 1996) and it is known that peroxisomes can move along microtubular tracks in mammalian cells (Rapp et al., 1996; Schrader et al., 1996; Wiemer et al., 1997). However, the mechanistic details of this movement, especially the nature of the peroxisomal interface and its functional relevance, remained unclear. Some light on the role of peroxisome–microtubule association was shed by the application of microtubule-disrupting drugs, which led to clustering and non-mobility of mature peroxisomes (Wiemer et al., 1997), suggesting that microtubules are required for the spatial organization of peroxisomes.

Microtubular association also was reported to be crucial for the formation of peroxisomal precursors (Brocard et al., 2005) and the regulated degradation of peroxisomes under starvation conditions (Hara-Kuge and Fujiki, 2008). In plant cells, the close association of microtubules and peroxisomes is thought to improve the efficiency of import of several matrix enzymes with microtubule-
binding domains (Chuong et al., 2005). The application of microtubule-disrupting drugs has the disadvantage of provoking general defects that might interfere with peroxisome morphology and abundance. The finding that PEX14 is required for microtubule-association of peroxisomes now allows study of the phenotypic effects of the non-motility of peroxisomes without harming other cytoskeleton-dependent cellular processes.

The existence of peroxisomal ghosts in PEX14-deficient cells apparently contradicts the previous assumption that the peroxisome–microtubule interaction plays a crucial role in the earliest stages of peroxisome biogenesis, namely the PEX16-dependent de novo synthesis of peroxisomal membranes (Brocard et al., 2005). It might still be possible that early states of peroxisomes, so-called pre-peroxisomes, possess additional PEX14-independent microtubule-binding sites; however, our data are clear in that mature peroxisomal membranes rely on the PEX14-mediated association with microtubules.

There are an increasing number of findings in support of a role for PEX14 in the autophagic degradation of peroxisomes. The importance of PEX14 for pexophagy has been demonstrated in yeast (Bellu et al., 2001; Zutphen et al., 2008) and indirect evidence to the peroxisomal membrane (Fransen et al., 2004; Itoh and Fujiki, 2006). The existence of peroxisomal ghosts in PEX14-deficient cells might reflect a possible mechanism of regulation. When the high-expression of PEX14 is required for pexophagy (Hara-Kuge et al., 2006). In this respect, it is noteworthy that pexophagy in yeast involves the actin filaments rather than microtubules (Reggiori et al., 2005). PEX14 is not required for non-selective autophagic peroxisome degradation (micropexophagy) (de Vries et al., 2006), a process that does not necessarily involve microtubules or microfilament attachment of peroxisomes (Kirisako et al., 1999; Kunz et al., 2004).

Our data are clear in that PEX14 is required for peroxisome long-range motility and that the protein serves as the connecting link for anchoring peroxisomes to microtubules. Analysis of the molecular basis of the PEX14-association with microtubules revealed two surprising findings. First, the interaction between tubulin and PEX14 turned out to be direct and, second, the C-terminal region of PEX14, which is required for its function in peroxisomal protein import, is dispensable for tubulin binding and its role in peroxisome motility. In fact, the 140 amino acid segment that maintained the activity does comprise the N-terminal tubulin-binding fragment (amino acids 1–78) and the peroxisomal membrane anchor sequence. This region also contains the binding sites for PEX5 and PEX19, raising the question whether they all exist in the same complex. An answer to this question is provided by Fig. 3, which indicates that PEX14 forms three distinct complexes with its binding partners. For PEX19 and PEX5, it has already been shown that they both occupy the same binding site of PEX14 (Neufeld et al., 2009). The finding that PEX5 competes with tubulin for binding to PEX14 (Fig. 6) provides a molecular rationale for the existence of distinct functional subpopulations of PEX14 at the peroxisomal membrane.

Interestingly, our findings might also explain the previously observed perturbed microtubule association of peroxisomal remnants in PEX13-deficient cells (Nguyen et al., 2006) because PEX13 was shown to be required for correct targeting of PEX14 to the peroxisomal membrane (Fransen et al., 2004; Itoh and Fujiki, 2006).

The fact that PEX5 and tubulin are not in the same complex might reflect a possible mechanism of regulation. When the high-
affinity binding partner PEX5 is present in its cargo-loaded form, matrix protein import will occur and microtubule binding might be diminished. By contrast, upon starvation, less cargo-loaded receptor might bind to PEX14, thus opening the way for tubulin association and regulated degradation.

It has been suggested that cytosolic linker proteins (CLIPs), which attach organelle membranes to microtubules, might also play a role in connecting peroxisomes to microtubules (Rickard and Kreis, 1996). CLIPs are components of a general mechanism that promotes microtubule growth and organizes the dynein–dynactin complex (Galgart, 2005). Because these motor proteins and other CLIP-interacting proteins like IQGAP1 were found as constituents of the PEX14 complex (see supplementary material Table S2), general steps in movements along microtubule tracks might be used jointly by the peroxisomes and other organelles.

Materials and Methods

Plasmids
Expression/integration plasmid pPEX14-TEV-PA encoding human PEX14 fused to a TEV protease cleavable Protein A tag was constructed in three subsequent steps. First, the DNA-sequence encoding the TEV-protease cleavable Protein A was PCR-amplified from plasmid pYM8 (Knop et al., 1999) using primer pair 5'-TACCAcGTATGCGTACGTCGAGTTCG-3' (EcoRV/NotI sites are underlined) and subcloned into pCDNA5/FRT (Invitrogen). Second, an adapter generated by oligonucleotides 5'-CTAAGCACGTATGCGTACGTCGAGTTCG-3', 5'-AAATGGATCAGCTGATCGAGTTCG-3' was subcloned into NotI/EcoRV digested vector, introducing new EcoRV and HindIII cloning sites. Third, PEX14 was PCR-amplified from pDNA3-HsPEX14 (Will et al., 1999) using primer pair 5'-TAGATACTAGCGGTCCATGGACGACG-3', 5'-ACAACCGCTGTCGTCTACGCCTGT-3' (EcoRV/HindIII sites are underlined) and subcloned. For complementation assays, truncated fragments of PEX14 were PCR-amplified and subcloned into pCDNA3 expression vector either digested by KpnI/XbaI (PEX14[79–377] or HindIII/NotI [PEX14[1–140] and PEX14[1–260]). The full-length PEX14 expression plasmid pDNA3-HsPEX14 (Will et al., 1999), E.coli GST–PEX14[1–78] expression plasmid (Schleibs et al., 1999), as well as the human GFP–PEX26 expression plasmid (Halbach et al., 2006) and pEGFP-PTS1 (Stanley et al., 2006) have been described previously. The expression plasmid encoding PTS2-EYFP was kindly provided by Gabriele Dobt (University of Tübingen, Germany).

Generation and selection of stable cell lines expressing PEX14–TEV–ProteinA
Flp-In system (Invitrogen, Carlsbad, CA, USA) was used for the generation of a stable PEX14–TEV–Protein A expression cell line Flp-In-293[PEX14–TEV–PA]. Flp-In-293 cells (Invitrogen) were co-transfected with expression vector pPEX14–TEV–PA and the FLP recombinase vector (pOG44) resulting in a stable integration of the gene of interest at the ‘FLP Recombination Target’ (FRT) site in the genome (O’Gorman et al., 1991). For the selective growth test, individual cell colonies were grown in duplicate on six-well plates and exposed to a confluence of 25%. The culture medium was supplemented with hygromycin (200 μg/ml) or Zeocin (100 μg/ml). The growth of possible integrants and Flp-In-293 cells as a control was monitored over a period of 7 days. Hygromycin-resistant and Zeocin-sensitive cells were further assayed for the lack of β-galactosidase activity. The expression of PEX14–TEV–ProteinA was verified by immunoblot analysis using anti-PEX14 antiseraum (Will et al., 1999).

Isolation of PEX14 complex
For complex isolation, cells were cultured on triple flasks (Nunc 500 cm² each) on DMEM medium supplemented with hygromycin. Approximately 25 g (wet weight) were disrupted by vortexing with three volumina glass beads (diameter 0.5 mm) in 20 mM HEPES pH 7.5, 100 mM potassium acetate and selected protease inhibitors. Glass beads and cell debris were removed by centrifugation. Cellular membranes were collected by centrifugation at 100,000 g for one hour. Membrane proteins (5 mg/ml) were solubilized with 1% (w/v) digitonin at a detergent/protein ratio of 2 for one hour. Insoluble material was removed by centrifugation at 100,000 g for one hour. The supernatant was incubated with 10 μl of IgG Sepharose beads. Beads were concentrated by centrifugation, thoroughly washed and incubated with TEV protease in lysis buffer containing 0.1% (w/v) digitonin at 16°C for 2 hours. After centrifugation, the beads were washed with lysis buffer containing 200 mM potassium acetate and 0.1% (w/v) digitonin. Eluates were combined and further analyzed by size-exclusion chromatography or mass spectrometry.

For size-exclusion chromatography, TEV–protease eluate was separated on a calibrated Superose 6 PC 3.2/30 column (GE Healthcare). Molecular mass markers were blue dextran (Vₑ=void volume, larger than 2000 kDa), thyroglobulin (669 kDa), ferritin (440 kDa) and aldolase (158 kDa). Fractions of 80 μl were collected and subjected to immunoblot analysis using monoclonal anti-PEX19 antibodies (BD Biosciences, Heidelberg), monoclonal anti-HIF-α (Sigma-Aldrich) and anti-HIF-PEX14 (Will et al., 1999). Polyclonal rabbit anti-HIF-PEX5 antibodies were raised against recombinant His-tagged PEX5p, which was expressed in E.coli and purified as described previously (Schleibs et al., 1999).

Mass spectrometry
For mass spectrometric analyses, samples were suspended in LDS sample buffer (Invitrogen, Carlsbad, CA) and loaded onto NuPAGE Novex 4–12% acrylamide Bis-Tris gels (Invitrogen) or 12% acrylamide Tris-glycine gels. Gel electrophoresis was carried out according to the manufacturer’s protocol and subsequent staining was performed with colloidal Coomassie Brilliant Blue G-250. Processing of gels, in-gel tryptic digestion and online reversed-phase capillary HPLC/ESI-MS/MS analyses of peptide samples were performed as described (Wiese et al., 2007). In brief, mass spectrometric analyses were performed on a Bruker Daltonics HCT plus ion trap instrument (Bremen, Germany). The three most abundant ions were selected for fragmentation by low-energy collision-induced dissociation. Exclusion limits were automatically placed on previously selected mass-to-charge (m/z) ratios for 1.2 minutes. MS spectra were the sum of seven integral scans ranging from m/z 300–1500 with a scanning speed of 8.100 (m/z)/second; and MS/MS spectra were the sum of four scans ranging from m/z 100–2200 at a scan rate of 26,000 (m/z)/second. For peptide identification, peaks were correlated with the human IPI database (http://www.ebi.ac.uk) and a duplicate of the same database, in which the amino acid sequence of each protein entry was randomly shuffled (Neufeld et al., 2004). All searches were performed with trypsin specificity allowing two missed cleavages. Oxidation of methionine was considered as variable modification, as well as mass tolerances of 1.2 Da and 0.4 Da for MS and MS2 experiments, respectively. Proteins were assembled on the basis of peptide identifications using the ProteinExtractor tool in ProteinScape (version 1.5.4.2; Bruker Daltonics, Bremen, Germany). ProteinExtractor was used with a Mascot score threshold of 15. Subsequent to the assembly of proteins, hits up to an accumulated false positive rate of 5% were considered as true-positive protein identifications. For the relative quantification of proteins, spectral counts were calculated using in-house developed software (Liu et al., 2004).

Fluorescence microscopy
Human fibroblast cells were cultured as described previously (Stanley et al., 2006). For localization and functional analyses of plasmid-encoded PEX14–TEV–ProteinA, PEX14- deficient fibroblast cells were double-transfected with expression vector pPEX14-TEV–PA alone or together with either EGFP-PTS1 (synonymous EGFP-SCP2) (Stanley et al., 2006) or PTSS–YFP using the Amaza Nucleofector Kit (Lonza, Switzerland). At 48 hours after transfection, cells were subjected to fluorescence and immuno/fluorescence microscopy using antibodies against PEX14 (Will et al., 1999), PMP70 (rabbit, Zymed Invitrogen) and Protein A (Sigma-Aldrich).

Live cell imaging
To detect peroxisomes, cells were transfected with plasmid encoding GFP–PEX26 (Halbach et al., 2006). Fibroblast cells were observed for 48 hours after Amaza transfection in DMEM medium. Cells were observed by confocal laser scanning microscopy (Zeiss LSM510; Meta, Germany). To maintain the incubation settings at 37°C and 5% CO₂ on the microscope stage, a CTI controller 3700 digital, O₂ controller, Tempcontrol 37-2 digital, and the Incubator Soxygen together with the Incubation controller, Tempcontrol 37-2 digital, and the Incubator Soxygen together with the Incubation software (Zeiss LSM image browser version 4.2) (Theiss et al., 2005). For time-lapse imaging, cells were imaged at low magnification (zoom level 1, 1024×1024 pixels) and laser power of 5%. Images were taken every 5 seconds. At each time point, optical sections were captured in 2-μm steps, covering the thickness of the cell, and were reconstructed with ZEN software (Zeiss LSM image browser version 4.2) (Theiss et al., 2005).

For disassembly of the actin and microtubule network, cells were incubated with 10 μg/ml Cytochalasin D for at least 30 minutes or 10 μg/ml colchicine for 1 hour, respectively.

In vitro binding
Recombinant glutathione S-transferase (GST) or GST–PEX14 (1–78) (Schleibs et al., 1999) was expressed in E.coli and bound to glutathione Sepharose 4B (GE Healthcare).
Purification of porcine brain tubulin was carried out as described previously (Waterman-Storer, 2001). Essentially, pig brain was homogenized and the supernatant after centrifugation was enriched by tubulin through repetitive polymerization and depolymerization cycles and ultracentrifugation at 100,000 g. Polymerization occurred in 100 mM PIPES, 2 mM EGTA, 1 mM MgSO4, 2 mM DTE, pH 6.9. For depolymerization, 8 mM glyceraldehyde was added to the mixture. A final carbon exchange chromatography step yielded pure tubulin (supplementary material Fig. S2).

We thank Elisabeth Becker and Christiane Sprenger for technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft (SF8642).

Supplementary material available online at http://jcs.biologists.org/cgi/content/full/124/10/1759/DC1

References


Forskungsgemeinschaft (SFB642).


Pex14-dependent peroxisome motility 1767

Pex14p-dependent peroxisome motility 1767

Pex14p-dependent peroxisome motility 1767

Pex14p-dependent peroxisome motility 1767

Pex14p-dependent peroxisome motility 1767


