Pex15p of *Saccharomyces cerevisiae* Provides a Molecular Basis for Recruitment of the AAA Peroxin Pex6p to Peroxisomal Membranes

Ingvild Birschmann,^{*†} An K. Stroobants,^{†‡} Marlene van den Berg,[‡]
Antje Schäfer,^{*} Katja Rosenkranz,^{*} Wolf-H. Kunau,^{*§} and Henk F. Tabak[‡]

^{*}Abteilung für Zellbiochemie, Medizinische Fakultät der Ruhr-Universität Bochum, D-44780 Bochum, Germany; and [‡]Department of Biochemistry, Academic Medical Centre, 1105 AZ Amsterdam, The Netherlands

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The gene products (peroxins) of at least 29 PEX genes are known to be necessary for peroxisome biogenesis but for most of them their precise function remains to be established. Here we show that Pex15p, an integral peroxisomal membrane protein, in vivo and in vitro binds the AAA peroxin Pex6p. This interaction functionally interconnects these two hitherto unrelated peroxins. Pex15p provides the mechanistic basis for the reversible targeting of Pex6p to peroxisomal membranes. We could demonstrate that the N-terminal part of Pex6p contains the binding site for Pex15p and that the two AAA cassettes D1 and D2 of Pex6p have opposite effects on this interaction. A point mutation in the Walker A motif of D1 (K489A) decreased the binding of Pex6p to Pex15p indicating that the interaction of Pex6p with Pex15p required binding of ATP. Mutations in Walker A (K778A) and B (D831Q) motifs of D2 abolished growth on oleate and led to a considerable larger fraction of peroxisome bound Pex6p. The nature of these mutations suggested that ATP-hydrolysis is required to disconnect Pex6p from Pex15p. On the basis of these results, we propose that Pex6p exerts at least part of its function by an ATP-dependent cycle of recruitment and release to and from Pex15p.

INTRODUCTION

Eukaryotic cells sequester different cellular functions within distinct membranes and membrane-bound organelles. This highly compartmentalized nature of the eukaryotic cell requires specific mechanisms to sort and deliver proteins vectorially and efficiently from their common site of synthesis to their diverse destinations.

One of these compartments is the peroxisome, which catalyzes a large number of different metabolic reactions (van den Bosch *et al.*, 1992). The functional importance of peroxisomes in human cells is underscored by the existence of peroxisomal disorders (Moser, 1993; Braverman *et al.*, 1995; Wanders *et al.*, 1995; Fujiki, 1997). Those which are caused by defects in peroxisome biogenesis are also referred to as peroxisome biogenesis disorders (Gould and Valle, 2000).

The maintenance of peroxisomes appears to be a complex process involving a relatively large number of distinct proteins. Genetic analysis in different yeast species (Lazarow, 1993), CHO cells, and cells of patients (Fujiki, 2000) has led to the identification of 29 PEX genes that are required for the assembly and maintenance of this organelle (http://www.mips.biochem.mpg.de/proj/yeast/reviews/pex_table.html). Some of the encoded proteins contribute to targeting of newly synthesized proteins to peroxisomes and the transport of these folded proteins across the membrane (Hettema *et al.*, 1999; Subramani *et al.*, 2000). For other proteins their precise role is less well characterized and clues must be derived from conserved structural amino acid motifs, such as SH3 and RING finger domains (Erdmann *et al.*, 1997).

Two peroxins, Pex1p and Pex6p, are members of the large AAA protein family (Patel and Latterich, 1998; Vale, 2000), which has been defined on the basis of an ~220-amino acid region, termed the AAA cassette (Beyer, 1997), which stands for ATPases associated with a wide range of cellular activities (Kunau *et al.*, 1993). This cassette contains Walker A and B motifs typical of P-loop-containing nucleoside triphosphatases. There are two types of AAA proteins known. The members of type I contain one AAA cassette; those of type II

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^{*} Corresponding author. E-mail address: Wolf-H.Kunau@ruhr-uni-bochum.de.

[†] Both authors contributed equally to this work.

are characterized by two of them. In type II AAA proteins the two AAA cassettes, denoted as D1 and D2, are conserved to a different degree and apparently have different functions (Matveeva *et al.*, 1997; Hattendorf and Lindquist, 2002; Zakalskiy *et al.*, 2002). An especially well-characterized example is NSF/Sec18p, which participates in heterotypic membrane fusion events (Whiteheart and Kubalek, 1995; Vale, 2000).

The function of Pex1p and Pex6p, both type II AAA proteins, within peroxisomal biogenesis is still unclear. It has been reported that in the yeast *Yarrowia lipolytica* both AAA peroxins have distinct roles in priming and docking of early peroxisomal vesicle populations before fusion (Titorenko and Rachubinski, 2000). However, it is also possible that Pex1p and/or Pex6p provide the molecular basis for the reported ATP-dependence of matrix protein import into peroxisomes (Bellion and Goodman, 1987; Imanaka *et al.*, 1987; Rapp *et al.*, 1993; Wendland and Subramani, 1993; Dodt *et al.*, 1995), because these are the only two peroxins thus far known to bind ATP. This notion has recently been supported by an epistasis analysis in *Pichia pastoris*, which suggested that Pex1p and Pex6p act in the terminal steps of peroxisomal matrix protein import (Collins *et al.*, 2000).

In our research to define the composition and mode of action of the protein machinery required for peroxisome biogenesis, we identified the membrane-bound peroxin Pex15p (Elgersma *et al.*, 1997; Stroobants *et al.*, 1999) as a membrane anchor of Pex6p in *Saccharomyces cerevisiae*. Pex15p is a peroxisomal integral membrane protein with most of its sequence including the N-terminus facing the cytosol. The intrinsic binding capacity between Pex15p and Pex6p is modulated via the action of the AAA cassettes of Pex6p. We propose that their opposite effect on the interaction leads to a process of binding and release of Pex6p to and from the peroxisomal membrane.

MATERIALS AND METHODS

Strains and Culture Conditions

The yeast strains used in this study were *S. cerevisiae* wild-type BJ1991 (MATa; *leu2*, *trp1*, *ura3-251*, *prb1-1122*, *pep4-3*, *gal2*), *pex6Δ* and *pex15Δ* (same as BJ1991 except *pex6/15::Leu2* (Hettema *et al.*, 1999; Elgersma *et al.*, 1997). Yeast strains used for two-hybrid experiments were PCY2 (MATα, *gal4Δ*, *gal80Δ*, URA3::GAL1-*lacZ*, *lys2-801^{amber}*, *his3-Δ200*, *trp1-Δ63*, *leu2 ade2-101^{ochre}*; Chevray and Nathans, 1992), *pex1Δ* (same as PCY2 except *pex1::kanMX4*; this work), and HF7c (MATa, *ura3-52*, *his3-200*, *lys2-801*, *ade2-101*, *trp1-901*, *leu2-3/112*, *gal4-542*, *gal80-538*, LYS2::GAL1-HIS3, URA3::GAL4 17mers)₃-CYC1-*lacZ*). Complete and minimal media used for yeast culturing have been described elsewhere (Erdmann *et al.*, 1989). YNO medium contained 0.1% oleic acid, 0.05% Tween 40, 0.1% yeast extract, and 0.67% yeast nitrogen base without amino acids, adjusted to pH 6.0. *Escherichia coli* used for cloning were DH5α (*recA*, *hsdR*, *supE*, *endA*, *gyrA96*, *thi-1*, *relA1*, *lacZ*) and for protein expression strain BL21(DE3) (F⁻, ompTr_B m_B⁻ [*hsdS gal(lclI)857 ind1 Sam7 nin5 lacUV5-T7 gene1*])).

Construction of a PEX1 Null Allele

The kanMX4 gene of *PEX1* was amplified by PCR using primers KU681 and KU682 (Wach *et al.*, 1994) and transformed into *S. cerevisiae* wild-type PCY2. Correct replacement of the genomic *PEX1* open reading frame (ORF) by the kanMX4 gene was confirmed by PCR using primers KU484 and KU711.

Plasmids

The primers used are listed in Table 1. The *PEX15* orf including 5'- and 3'-flanking regions was amplified by PCR using primers KU217 and KU218 and genomic DNA as template resulting into plasmid pWG15/1. The *PEX6* ORF and additional 5' (471 bp)- and 3' (243 bp)-flanking regions were amplified from PAS8-YCplac33 (Voorn-Brouwer *et al.*, 1993) by PCR using the primers KU146 and KU145. The resulting product was subcloned into *Bam*HI/*Kpn*I-digested pRS416, resulting in plasmid pBM34.

Further expression constructs were based on YEplac33 (Gietz and Sugino, 1988), with the *PEX6* or the *PEX5* promoter cloned in *Eco*RI/*Sac*I, the GFP fusion protein in *Sac*I/*Bam*HI, and the *PEX6* (wild-type or mutated) in *Bam*HI/*Sal*I. The NH-Pex15p construct was based on YCplac22 (Gietz and Sugino, 1988), with the catalase promoter between the *Eco*RI and *Sac*I site, the NH-tag in *Sac*I/*Bam*HI, and the *PEX15* ORF in *Bam*HI/*Hind*III. Mutations were introduced using the Stratagene QuickChange site-directed mutagenesis kit, primers were AS1 and AS2 introducing the K778A mutation (named pIB6/39), AS3 and AS4 introducing the E832Q mutation (pIB6/40), and KU1353 and KU1354 introducing the *PEX6K489A* mutation (pIB6/38). ORFs and promoter sequences were amplified by PCR using specific primers introducing restriction sites (Elgersma *et al.*, 1997). Cloning of *pex15.1* and *pex15.3* in an expression vector was performed by digestion with *Bam*HI and *Pst*I and by cloning of the resulting insert in a YCplac22-based vector that contained the wild-type *PEX15* (*Bam*HI/*Hind*III) preceded by the catalase promoter (in *Eco*RI site). An expression construct of *pex15.2* was generated by transforming both a *Bam*HI/*Hind*III *pex15.2* ORF and the *Pst*I-linearized vector (same as for *pex15.1* and *pex15.3*) to *pex15Δ* cells. Homologous recombination of the two fragments resulted in an expression plasmid, which was rescued from yeast and analyzed for the presence of the *pex15.2* mutations by sequencing.

For two-hybrid studies the *PEX15* and *PEX6* ORFs and fragments of both were cloned into the DNA-binding domain containing plasmid pPC86 and the transcription activation domain containing pPC97 (Chevray and Nathans, 1992). *PEX15* fragment encoding aa1–315 was amplified by PCR using primers KU296/KU234 and pWG15/1 as template and subcloned *Eco*RI/*Sac*I into pPC86. The *PEX6* ORF was amplified by PCR using primers KU144 and KU145 (template pBM34) and cloned into pPC97 by a *Sal*I site derived from the primer and a genomic *Spe*I site downstream of the stop codon (pBM21). For *PEX6A1* the *Bgl*II/*Spe*I fragment from plasmid pIB6/38 was used to replace the corresponding fragment of plasmid pIB6/4, resulting in plasmid pIB6/18. The *PEX6A2* was cloned in pPC97 replacing *Spe*I/*Bgl*II fragment of plasmid pIB6/39 by the corresponding fragment of plasmid pIB6/4, resulting in the plasmid pIB6/11. For *PEX6B2* the *Nco*I/*Spe*I fragment of plasmid pIB6/40 was used to replace the corresponding fragment of plasmid pBM21, resulting in plasmid pIB6/4.

Truncated versions of *PEX6* were created as follows: The N-terminus (aa1–428), the first domain (aa421–716), and the second domain (aa704–1030) of the *PEX6* ORF were amplified from pBM34 using the primers KU144 and KU662, KU663 and KU626, KU664 and KU665, respectively. The PCR fragments, which were used for expression of the N-terminus and the second domain, were digested at the primer derived *Sal*I/*Spe*I sites. *Sal*I and *Not*I were used for subcloning the first domain. All fragments were inserted into the corresponding sites of the plasmid pIB6/4. The resulting plasmids were designated pIB6/21, pIB6/22, and pIB6/23, respectively. Three further truncated versions of *PEX6* were generated by combining the N-terminus and first domain (pIB6/33), the N-terminus and second domain (pIB6/28), and the first and second domain (pIB6/29). Plasmid pIB6/33 was obtained by *Nde*I/*Not*I digestion of plasmid pIB6/22 and ligation with *Nde*I/*Not*I-digested plasmid pIB6/4. For plasmid pIB6/28 the N-terminal part of Pex6p was amplified using primers KU144 and KU727. The coding sequence for the second domain was amplified using primers KU728 and KU698. Subcloning of the two parts into the *Sal*I/*Spe*I-digested

Table 1. Primers used for different Pex1p, Pex6p, and Pex15p constructs

KU142	5'GGAGGATCCGTCGACAATGACGACGACCAAGA3'
KU143	5'TCTAAGCTTGAGCTCTTTCACATAAGGGAGAGTCG3'
KU144	5'TTAGGATCCGTCGACTATGAAGGCATCGCTTACG3'
KU145	5'CCGGGTACCTCTAGAATCTGCACGGATATCGAG3'
KU146	5'GCAGGATCCGTCGACAAAGCTCACTGATAGACGAAGA3'
KU234	5'GCGGATCCCCAGGACTCGAAACAGTAATATCATTGTT3'
KU296	5'ACCCCGGGTTGAATTCAGATGGCTGCAAGTGAGATA3'
KU484	5'CTGTACATAGGTGGCTGTC3'
KU542	5'GAAAGATACGCAGTCTCTGTGC3'
KU544	5'GTGTGGATGGCTTCCGC3'
KU549	5'CCCGCATGCGTCGACAGCACCTTCAAATTAGCTC3'
KU569	5'CGGTCTCGCATGAAGGCATCGCTTACG3'
KU626	5'CCGCATGCGGCCGCTCATTTCGTTTCTAGCTTTTGAAG3'
KU654	5'GCGAATTCGATCCGGAATGGCTGCAAGTGAG3'
KU660	5'CCTTTAACAAAATCAATACCATCGATATCATCCCAAGTTACG3'
KU661	5'CAGGAACATACATTTGTGGCTCCG3'
KU662	5'AGTACTAGTTAGGGTAATGGTCTTCTGTAG3'
KU663	5'CAGCTGTCGACGATGATCACTAACAGAAGACCATTACCC3'
KU664	5'CAGCTGTCGACGATGGATTATCAAAGCTACTTTCG3'
KU665	5'GCATGCGGCCGCTTAAGCACCTTCAAATTAGCTC3'
KU670	5'GCATGCGGCCGCGCTAAAGGGAACAAAAGCTGG3'
KU681	5'GGACGGCAGTAACAAGAAACACCTGAGGAAGTCTTTTCAACAGCTGAAGCTTCGTACGCT3'
KU682	5'CAGCCGCATTTTTTGCCCTTTAAAGGGAAACGCGCTTTGTTTCATAGGCCAAGTGATCTG3'
KU698	5'CGTGTTAACTAGTTTAAGCACCTTCAAATT3'
KU707	5'AGGGARCCGACGTGTAACAGTGTTAAACCTAATTTGG3'
KU708	5'TACCCGGGTAGAATTCGCATGCTGCAGAGTGAGATTTTG3'
KU709	5'AGGGATCCGAGCTCTAAGGGTTTTTATGATGCAG3'
KU710	5'TACCCGGGTAGAATTCGCATGAAACCCTCACCGAATATG3'
KU711	5'GTCCTGCGTGCTCGCAC3'
KU727	5'ATCCGCGGATCCGGTAATGGTCTTCTGTAG3'
KU728	5'CCGAATTCGATCCGATTTATCAAAGCTACTTTCG3'
KU746	5'TACCCGGGTAGAATTCGCATGGTGAACCTTTTCATAAAAAG3'
KU747	5'AGGGATCCGAGCTCTAAAGTTAAGTTAACTGAATAACC3'
KU1353	5'CTACAAACAATGTGGGCGCCGTACAATGGTGAGATTGTC3'
KU1354	5'GCAATCTCACCATTTGTAGCGGCGCCACATTTGTTGTAG3'
AS1	5'CCACCGGGTACAGGTGCAACTCTAATGGCTAAGG3'
AS2	5'CCTTAGCCATTAGAGTTGCACCTGTACCCGGTGG3'
AS3	5'CCTTGTCATATTTTTCGATCAAATCGATTAGTAGCACCC3'
AS4	5'GGGTGCTACTGAATCGATTTGATCGAAAAATATGACACAAGG3'
AS5	5'GGATCCATGGCTGCAAGTGAGATAATGAAC3'
AS6	5'AAAGCTCCACCGCGGTGG3'

pPC97 was performed using *Sall* and *Bam*HI (N-terminus) and *Bam*HI and *Spe*I (D2). For plasmid pIB6/29 the first domain of Pex6p was amplified using primers KU663 and KU660 and the second domain using KU544 and KU698. Subcloning of the two parts into the *Sall*/*Spe*I-digested pPC97 was performed using *Sall* and *Eco*RI (D1) and *Eco*RI and *Spe*I (D2).

Truncated versions of *PEX15* were created after restriction with *Eco*RI/*Sac*I of PCR products made with the following primers using pWG15/1 as template: *PEX15*(aa1–103) KU296-KU707, *PEX15*(aa103–207) KU708-KU709, *PEX15*(aa206–315) KU710-KU234, *PEX15*(aa103–315) KU708-KU234, *PEX15*(aa1–207) KU296-KU709, *PEX15*(aa49–151) KU746-KU747, *PEX15*(aa49–207) KU746-KU709, *PEX15*(aa1–151) KU296-KU747, and *PEX15*(aa49–103) KU746-KU707. All fragments were inserted in the corresponding sites of the two-hybrid vector pPC86. A library of randomly mutagenized *PEX15*(aa1–329) was created by gap repair (Bottger *et al.*, 2000). PCR was performed with primers AS5 and AS6, and pPC86-*pex15*ΔC55 (Elgersma *et al.*, 1997) was used as a template. The PCR product was transformed into *HF7c* cells together with a linearized plasmid pPC86 with overlapping regions of the *pex15*ΔC55 (Elgersma *et al.*, 1997) resulting in circular plasmids produced by homologous recombination. The colonies that failed to grow in the

absence of histidine were selected, and *PEX15* mutant plasmids were rescued from these colonies for further analysis.

The *PEX1* ORF was amplified from pRC123 (Erdmann *et al.*, 1991) by PCR using the primers Ku142 and Ku143 and was cloned by primer derived *Sall* and *Sac*I sites into pPC86 (pIB1/31).

To construct *PEX6*-His₆ (pIB6/9) two *PEX6*-fragments were amplified by PCR using KU569 and KU542 and KU549 and Ku661 and pBM34 as template. After restriction with *Bsa*I and *Nde*I and *Sall* and *Nde*I, respectively, the fragments were ligated with the *Nco*I/*Sall* fragment of pET21d. GST-Pex15p(aa1–315) (pIB15/1) was constructed using the primer derived *Bam*HI and *Not*I restriction sites (KU654 and KU670 and pWG15/1 as template) and replacing the corresponding *Bam*HI/*Not*I fragment of pGEX-4T-3 (Amersham Biosciences, Freiburg, Germany).

For overexpression of GFP-GST fusion protein, the GFP ORF was subcloned *Sma*I/*Eco*RI from pGFP (BD Biosciences, Erembodegem, Belgium) into pbluescript (Stratagene, La Jolla, CA), resulting into plasmids pSkG3, and from there finally cloned *Sma*I/*Xho*I into pGEX-4T-3 (Amersham Biosciences), resulting in plasmid pGEXGFP. The fusion protein was overexpressed in *E. coli* TG1 and purified as soluble fusion-protein according to the manufacturer's instructions (Amersham Biosciences).

All point mutations used were confirmed by DNA sequencing. Recombinant DNA techniques, including enzymatic modification of DNA, fragment purification, bacterial transformation, and plasmid isolation were performed as described elsewhere (Maniatis *et al.*, 1982; Ausubel *et al.*, 1992).

Antibodies

Rabbit polyclonal anti-Pex6p antibodies were raised against a synthetic peptide (KLLYLGIPTDTKQLN) corresponding to amino acids 895–910 generated by Eurogentec (Seraing, Belgium).

Anti-GFP was kindly provided by J. Fransen (Nijmegen, the Netherlands) and anti-NH by P. van der Sluijs (Utrecht, the Netherlands). Rabbit polyclonal antibodies to GST-GFP were produced by Eurogentec according to standard methods (Harlow and Lane, 1988). A further antibody used was anti-Pex15p (Elgersma *et al.*, 1997). The secondary antibody used was anti-rabbit IgG-coupled HRP (Sigma, Taufkirchen, Germany) for Pex6p and GST detection.

Two-hybrid Analysis

The two-hybrid assay was based on the described method (Fields and Song, 1989). Cotransformation of two-hybrid vectors into the strain PCY2 was performed according to Gietz and Woods (1994). Transformed yeast cells were plated onto SD synthetic medium without tryptophane and leucine. β -Galactosidase filter assays were performed as described elsewhere (Rehling *et al.*, 1996).

The library of randomly mutagenized PEX15(aa1–329) in pPC86 and pBM21 (PEX6 in pPC97) was transformed in HF7c. The presence of both plasmids was selected on glucose plates lacking leucine and tryptophane. The colonies that grew well on these plates were replica plated to plates lacking histidine. The growth on histidine-lacking plates was tested at 34°C to include possible temperature-sensitive mutants. The colonies that did not grow on these latter plates contained a mutated PEX15, which was unable to interact with Pex6p. To select for full-length PEX15 mutants, TCA lysates of these cells were made and analyzed by SDS-PAGE and Western blotting with antibodies against Pex15p. Three full-length PEX15 mutants in pPC86 were rescued from these yeast cells, and their sequences were analyzed.

In Vitro Protein Binding Assays

GST, GST-Pex15p(aa1–315), and Pex6p-His₆ were expressed in *E. coli* BL21(DE3) and isolated as follows: Cells were harvested and diluted in PBS buffer (phosphate-buffered saline: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, pH 7.3), containing protease inhibitors (240 μ g/ml PMSF, 2 μ g/ml aprotinin, 5 μ g/ml antipain, 1 μ g/ml pepstatin, 2.5 μ g/ml leupeptin, 6 μ g/ml chymostatin, 0.21 mg/ml NaF), 0.5 mM DTT, 1 mM ATP, and 25 mM MgCl₂). Cells were broken using a french press, and cell debris was removed after centrifugation (100,000 $\times$ g, 45 min). The supernatant containing the soluble proteins including GST-Pex15p or GST was loaded directly on a glutathione-Sepharose 4B (Amersham Biosciences) column equilibrated with PBS buffer. After intensive washing (PBS buffer), supernatant containing Pex6p-His₆ was loaded on the column and after further intensive washing steps, the proteins were eluted from the column with 10 mM glutathione.

Microscopy

For immuno-electron microscopy (immuno-EM) experiments, oleate-induced cells were fixed with 2% paraformaldehyde and 0.5% glutaraldehyde. Immunolabeling of ultrathin cryosections with antibodies against GFP and NH was performed as described elsewhere (Gould *et al.*, 1990).

Fractionations

Oleate-induced cells were subcellularly fractionated as described previously (Hettema *et al.*, 1999). The sorbitol/phosphate and MES buffers used contained usually no EDTA. Fractionations were performed by centrifugation of lysed spheroplasted cells at 30,000 $\times$ g, at 4°C for 30 min.

RESULTS

Interaction between Pex6p and Pex15p

Over the last couple of years 29 proteins have been described to contribute to the biogenesis or maintenance of peroxisomes (http://www.mips.biochem.mpg.de/proj/yeast/reviews/pex_table.html). In a search to find possible interactions between these proteins (peroxins) in *S. cerevisiae* using the two-hybrid technique, we observed that Pex6p interacted with the cytoplasmic part (aa1–315) of Pex15p. Activation of the reporter gene lacZ, indicated by blue colonies on X-Gal medium, was observed when PEX6-GAL4-BD was coexpressed with PEX15-GAL4-AD in *S. cerevisiae* PCY2 cells (Figure 1A). The controls showed that coexpression of either of the fusion proteins, together with the respective Gal4p domains encoded by pPC86 and pPC97, did not support transcription activation of the reporter genes. The interaction between Pex6p and Pex1p, which was described for *Pichia pastoris* (Faber *et al.*, 1998), *Homo sapiens* (Tamura *et al.*, 1998), and *Hansenula polymorpha* (Kiel *et al.*, 1999), could be detected in *S. cerevisiae* as well (our unpublished results). Furthermore in the absence of Pex1p (in PCY2 *pex1* Δ) Pex15p and Pex6p still showed interaction in the two-hybrid assay (Figure 1A).

Because the interaction between Pex6p and Pex15p was detected in a homologous context, it was important to prove a direct interaction and exclude the possibility of a third partner acting as a bridge between Pex6p and Pex15p. We therefore synthesized a His-tagged PEX6 gene and expressed it in *E. coli* using the expression vector pET9d. The cytosolic part of Pex15p(aa1–315) was produced in *E. coli* as a fusion protein to glutathione-S-transferase (GST). GST-Pex15p(aa1–315) was bound to glutathione-agarose and was incubated with an extract containing overproduced Pex6p-His₆. After washing with buffer, protein complexes were eluted from the column with glutathione and analyzed by Western blotting. The results presented in Figure 1B indicated that Pex6p-His₆ exclusively bound to the GST-Pex15p(aa1–315) fusion protein. No binding was observed in control experiments, using GST bound to glutathione-Sepharose. These data demonstrate that the Pex6p/Pex15p interaction is a direct one and that it does not require an intermediary yeast protein.

Regions of Pex6p and Pex15p Involved in the Interaction

To find regions in Pex6p and Pex15p that are responsible for interaction, a number of different mutations were made and the effects on the mutual interaction were studied.

Various truncated parts of Pex6p (Figure 2A) were tested for their ability to bind the cytoplasmic part of Pex15p(aa1–315) in the two-hybrid system: the N-terminus (N, aa1–428), the first AAA cassette (D1, aa421–716) and the second AAA cassette (D2, aa704–1030). As judged by a low but significant

Figure 1. Pex6p and Pex15p(1–315) interaction. (A) Two-hybrid interaction of Pex6p and Pex15p(aa1–315). *PCY2* (rows 1, 3, and 4) and *PCY2 pex1Δ* (row 2) transformants expressing the indicated fusion protein combination of Pex6p or Pex15p were analyzed for β-galactosidase activity by a filter assay using X-gal as substrate. Three representative independent double transformants are shown (lanes 1–3). (B) In vitro binding studies using bacterially expressed Pex15p(aa1–315) and Pex6p fusion proteins. GST-Pex15p(aa1–315), Pex6p-His₆, and GST were expressed in *E. coli*. GST and GST-Pex15p(aa1–315) were bound to glutathione-Sepharose and then the columns were incubated with *E. coli* extracts containing Pex6p-His₆. Whole cell extracts (lanes 1 and 2) as well as proteins bound to and eluted from the column (lanes 3 and 4) were separated on SDS-PAGE and subjected to immunoblot analysis with antibodies against GST and Pex6p. Glutathione, 10 mM, was used for elution.

lacZ gene expression, the N-terminus of Pex6p can mediate binding to Pex15p(aa1–315) (Figure 2A). The D1 or D2 domains of Pex6p showed no interaction (Figure 2B). Additionally, a Pex6p fragment consisting of the N-terminus and the first AAA cassette (N-D1) gave rise to a *lacZ* gene activation even stronger to that obtained with wild-type Pex6p (Figure 2A). In contrast a Pex6p fragment consisting of the N-terminus and the second AAA cassette (N-D2) showed the same weak *lacZ* gene activation as obtained with the N-terminus of Pex6p alone (Figure 2A).

Similar studies with truncated versions of Pex15p showed that the N-terminal part of Pex15p(aa1–151) is sufficient for

Figure 2. Two-hybrid interaction of Pex6p and Pex15p(aa1–315). (A) *PCY2* transformants expressing the indicated fusion protein combination of Pex6p truncations and Pex15p(aa1–315). The interactions were analyzed for β-galactosidase activity by filter assay using X-gal as substrate. Two representative independent double transformants are shown (lanes 1 + 2). (B) Schematic representation of two-hybrid interaction between Pex15p(aa1–315) and truncated versions of Pex6p. The two-hybrid constructs of N-terminal fragment, D1 fragment and D2 fragment overlap by 8 and 13 amino acids, respectively. For simplicity these amino acids are not indicated in the schematic presentation of these fragments.

interacting with Pex6p (Figure 3A). To complement the protein truncation mutants with more subtle point mutations, a mutant library was made by error prone PCR of the first part of Pex15p(aa1–329). *PEX15* mutants defective in interaction with Pex6p in the two-hybrid trap were selected, and mutants were chosen that still gave rise to full-length protein on the basis of Western blot analysis. This screen resulted in two temperature-sensitive mutants (*pex15.1/pex15.3*) that showed a disturbed growth phenotype at 34°C and one that did not grow on oleate at both temperatures (18°C and 34°C) (*pex15.2*; Figure 3B). The plasmids containing a mutated

A

Figure 3. Effects of different Pex15p mutants. (A) Two-hybrid interaction of Pex6p and Pex15p truncations. *PCY2* transformants expressing the indicated fusion protein combination of Pex15p truncations and Pex6p. The interactions were analyzed for β -galactosidase activity by filter assay using X-gal as substrate. Three representative independent double transformants are shown (lanes 1–3). (B) Growth on selective oleate of wild-type (*pex15Δ*) cells with plasmid based expression of *PEX15*, *pex15Δ* cells and *pex15Δ* cells with plasmid based expression of *pex15.1*, *15.2* and *15.3* at 18°C and 34°C. Equal amounts of cells were spotted.

PEX15 gene were rescued from yeast and their sequence was determined. All mutations were found in the smallest fragment (aa1–151) of Pex15p (Table 2), which still interacted with Pex6p in the two-hybrid assay. Moreover, this result indicates that the amino acids at positions 22, 47, and/or 50 of Pex15p are important for the interaction of Pex15p with Pex6p.

Different Effects of the Two AAA Domains of Pex6p on the Interaction with Pex15p

The contribution of Pex6p and Pex15p to peroxisome biogenesis is well established on the basis of the phenotypes of

Table 2. Mutations found in 3 *PEX15* mutants that lost their capacity to interact with Pex6p in a two-hybrid assay

Mutant name	nt Mutation	aa Mutation	Change in aa
Mutant 15.1	nt 66 A→T	aa 22 L→F	Small→big
Mutant 15.2	nt 65 T→C	aa 22 L→S	Apolar→polar
	nt 393 A→G	aa 131 K→K	
Mutant 15.3	nt 140 T→A	aa 47 Q→L	Hydrophilic→hydrophobic
	nt 149 T→C	aa 50 V→A	Small difference

the null mutants. Both *pex6Δ* (Hettema *et al.*, 1999) and *pex15Δ* (Elgersma *et al.*, 1997) mutants show a characteristic *pex* phenotype with only residual, ghost-like peroxisomal structures. Here we analyzed the effects of more subtle alterations in Pex6p and Pex15p.

The AAA peroxin Pex6p contains two AAA cassettes (D1 and D2) and thus two consensus ATP-binding sites. To determine whether these cassettes are essential for peroxisome biogenesis we used site-directed mutagenesis to create mutants with point mutations in Walker A (A mutants) or Walker B (B mutants) motifs of either the first or the second cassette. The resulting mutants were designated: Pex6pA1(K489A), Pex6pA2(K778A), and Pex6pB2(D831Q/E832Q). It was not necessary to create a B1 point mutation because wild-type Pex6p contains an alanine instead of the critical aspartate (aa548) strongly suggesting that D1 can bind but not hydrolyze ATP.

We tested the mutant constructs with (Figure 4A) or without (our unpublished results) a GFP fusion protein using the oleate plate assay (functional test for peroxisome biogenesis) for their ability to complement the *pex* phenotype of the *pex6* null mutant. Addition of this fusion protein does not influence the function of Pex6p because GFP-Pex6p is fully capable of restoring growth on oleate in a strain with a *pex6* deletion. As demonstrated in Figure 4A, the *pex6* null mutant expressing Pex6p mutated at the first ATP-binding site (A1) showed the wild-type phenotype with respect to growth on oleic acid medium. In contrast, the *pex6* null mutants expressing Pex6p mutated at the second ATP-binding or -hydrolysis site (A2 and B2) were not able to grow on oleic acid as sole carbon source (Figure 4A). Although some small colonies can be observed, oleate consumption is completely abolished (halo formation). This was confirmed by growth on oleate in liquid culture (our unpublished results).

To investigate whether ATP-binding and/or -hydrolysis is critical not only for growth on oleate but also for the described Pex6p/Pex15p interaction we introduced the various mutant forms of Pex6p carrying point mutations in the ATP-binding and -hydrolysis sites of both AAA cassettes into two-hybrid vectors and tested the interaction with Pex15p(aa1–315). Compared with wild-type Pex6p, only the A1 mutation of the Walker A motif in the first AAA domain (D1) resulted in a clearly reduced interaction with Pex15p(aa1–315) (Figure 4B). These results demonstrate that the ability of the first AAA cassette of Pex6p to bind ATP, considerably strengthened the interaction of Pex6p and Pex15p.

A **B**

Gal-DB fused to	Gal-AD fused to	β-galactosidase filter assay		
		1	2	3
Pex6p	Pex15p ₁₋₃₁₅			
Pex6pA1	Pex15p ₁₋₃₁₅			
Pex6pA2	Pex15p ₁₋₃₁₅			
Pex6pB2	Pex15p ₁₋₃₁₅			
Pex6pA1	—			
Pex6pA2	—			
Pex6pB2	—			

Figure 4. Effects of mutated *PEX6*. (A) Growth behavior on oleic acid medium (YNO) of wild-type, *pex6Δ* and *pex6Δ* expressing wild-type or point mutated GFP-Pex6p. Growth on YNO agar plates is indicated by a typical halo reflecting the consumption of oleic acid. (B) Two-hybrid interaction between Pex6p point mutations and Pex15p(aa1–315). *PCY2* transformants expressing the indicated fusion protein combination of Pex6p point mutations and Pex15p(aa1–315) were analyzed for β-galactosidase activity by filter assay using X-gal as substrate. Three representative independent double transformants are shown (lanes 1–3).

Interplay between Pex6p and Pex15p in Relation to Their Cellular Location

Endogenous (full-length) Pex15p can only be detected on peroxisomal membranes (Elgersma *et al.*, 1997). The localization of Pex6p as studied in several organisms by various techniques is controversial. Pex6p contains two putative transmembrane domains and some reports describe it as a membrane or membrane-associated protein; others, however, as a partially cytosolic protein. To distinguish between these alternatives, we used two distinct techniques employing Pex6p fused to GFP at its N-terminus.

First, we fractionated *pex6Δ* cells expressing GFP-Pex6p in a 30,000 × *g* organellar pellet and a cytosolic supernatant. GFP-Pex6p was found to be mainly (~90%) present in the supernatant (Figure 5). This suggests that Pex6p is for the largest part or period of time cytosolic and for a smaller part or period bound to or inside organelles. Fractionation experiments were also carried out with strains carrying mutations in the ATP-binding sites of Pex6p. The membrane-bound fraction of GFP-Pex6pA1 (~1%) was even considerably smaller than that of GFP-Pex6p. After fractionation of GFP-Pex6pA2 or GFP-Pex6pB2 cells, however, larger fractions of Pex6p were pelletable (25 and 32%, respectively; Figure 5). The same was true when Pex15p was overexpressed (Figure 5). Thus, a larger fraction of Pex6p is organelle bound when either the A2 or B2 mutation or an excess of Pex15p is present.

Second, we studied the localization of Pex6p with immuno-EM (Figure 6). With antibodies against the GFP fusion protein we visualized wild-type and mutated GFP-Pex6 proteins (Figure 6, A–D), which were expressed under control of the weak *PEX5* promoter in *pex6Δ* cells (when using the *PEX6* promoter, no labeling could be achieved). GFP-Pex6p (Figure 6A) and GFP-Pex6pB2 (Figure 6B) were located in the area of cells in which peroxisomes and peroxisomal ghosts, respectively, were present; however, no labeling was seen on the peroxisomal membranes themselves. GFP-Pex6p and GFP-Pex6pB2 might be present in the cytosol or in/on membrane structures distinct from peroxisomes or peroxisomal ghosts, which we indicate as “clouds” of GFP-Pex6p. When the amount of Pex15p was increased (by expressing NH-Pex15p under the control of the catalase promoter) in *pex6Δ* cells containing GFP-Pex6p (Figure 6C) or GFP-Pex6pB2 (Figure 6D), the localization of GFP-Pex6 proteins changed: they were mainly on peroxisomal membranes. In addition to this effect on Pex6 protein localization we also noticed that the peroxisomes (Figure 6C) were often surrounded by double membranes. GFP-Pex6p was found on double as well as on single peroxisomal membranes. Overexpressed NH-Pex15p, visualized by anti-NH antibodies, was found on very similar structures as GFP-Pex6p (Figure 6E) and GFP-Pex6pB2 (Figure 6F). These results confirm that in vivo the level of Pex15p has an effect on the cellular distribution of Pex6p and are in line with the conclusion that Pex15p provides a membrane anchor for Pex6p.

Combined, these results support the notion that Pex6p has a dual localization: it is partially peroxisome bound via Pex15p, whereas the largest part is cytosolic. Whether this cytosolic part represents free or structurally bound Pex6p, for instance in the form of very small vesicles, could not be established unambiguously.

Figure 5. Localization of mutated *PEX6*. (A) PNS prepared from oleate-grown *pex6Δ* cells expressing GFP-tagged Pex6p, Pex6pA1, Pex6pA2, Pex6pB2 or both GFP-Pex6p and NH-Pex15p was subjected to centrifugation. Equal volumes of PNS, 30,000 × g pellet (30,000 × g P) or supernatant (30,000 × g S) were loaded on each lane and analyzed by Western blot analysis with antibody to Pex6p. (B) Densitometric analysis of signal intensity for Western blots depicted in A. For each yeast strain pellet and supernatant together were set to 100%. Dark bars represent the 30,000 × g pellet (P) and gray bars the 30,000 × g supernatant (S). The results represent the mean of three independent experiments.

DISCUSSION

Insight into the mode of action of the AAA peroxin Pex6p is an important step toward a molecular understanding of peroxisome biogenesis and especially toward the energy requirements of this process. A central result is that Pex6p is targeted to peroxisomal membranes by binding to the cytoplasmic part of the integral peroxisomal membrane protein Pex15p. This is supported by the following evidence: 1) The N-terminal cytoplasmic part of Pex15p(aa1–315) interacted in the yeast two-hybrid assay with the N-terminal region of Pex6p (N in Figure 7), whereas the D1, D2, and D1+ D2 fragments gave negative results. The binding site for this interaction in Pex15p could be limited to amino acid residues 1–151. This result is supported by the location of three point mutations, which were all located within this area. These temperature-sensitive mutants showed no interaction with Pex6p and exhibited no growth on oleate at the non-permissive temperature. 2) This interaction is direct because it could be reconstituted *in vitro* with proteins expressed in *E. coli*. This central finding functionally interconnects the hitherto unrelated two peroxins Pex6p and Pex15p by showing that they are physical partners in one of the many distinct steps of peroxisome biogenesis.

The binding of Pex15p and Pex6p was analyzed in further depth by a combination of specific mutations in Pex6p and their *in vivo* effect on growth on oleate. From the two-hybrid interaction studies it is clear that although the N-terminal part of Pex6p alone shows weakened but demonstrable intrinsic binding activity to Pex15p, a longer version of Pex6p, containing both the N-terminal part and D1, interacts even stronger than complete Pex6p. In contrast, the mutation A1, replacing the conserved lysine by a glutamate in the D1 Walker A motif of full-length Pex6p, lowers the binding activity of Pex6p to Pex15p to the intrinsic level of the N-terminal region alone. This is apparently good enough for residual function *in vivo* because this mutation does not affect the growth of the mutant cells on oleate. Because wild-type Pex6p does not have the critical active-site residue (D replaced by A) from the Walker B motif of D1, we conclude from our findings that ATP bound to D1 acts as an allosteric activator to increase the binding capacity to Pex15p.

Two different mutations were chosen to inactivate the D2 cassette of Pex6p in analogy to reported mutations of NSF (Whiteheart and Kubalek, 1995): one preventing ATP-binding (A2), and the other preventing ATP-hydrolysis (B2). Functionally, we could not distinguish between them. Both mutations in D2 completely abolish growth on oleate, but did not reduce the binding of Pex6p and Pex15p found in the yeast two-hybrid assay. In addition, we observed that cells carrying a *PEX6* allele with either one of the two mutations (A2 or B2) after fractionation contain more Pex6p in the crude organellar fraction, suggesting that ATP-hydrolysis catalyzed by D2 is required to dissociate Pex6p from Pex15p. Taking together, these findings demonstrate that D1 and D2 have not only different functions in the overall process of peroxisome biogenesis but also in the binding of Pex6p and Pex15p.

Although we have not strictly proven the reversibility of the interaction between Pex15p and Pex6p, the following observations favor this proposal: (a) In cell fractionation experiments the amount of structurally bound Pex6p correlates with the amount of Pex15p in cells. Furthermore, Pex6p

Figure 6. Electron microscopic analysis of the location of Pex6p in oleate-grown *pex6Δ* cells expressing GFP-Pex6p (A, C, and E) and GFP-Pex6pB2 (B, D, and F) without (A and B) and with coexpression of NH-Pex15p behind the catalase promoter (C–F). The localization of NH-Pex15p is shown (E and F). Immuno-EM was performed with anti-GFP (A–D) and anti-NH (E and F). Bar, 0.25 μ m.

with a mutation in the first ATP-binding cassette (D1) behaves almost exclusively as a cytosolic protein. (b) These observations are qualitatively confirmed by morphological observations with immuno-EM. Membrane-bound Pex6p correlates with the amount of Pex15p. Because Pex15p is an integral peroxisomal membrane protein (Elgersma *et al.*, 1997), it is very likely that the membrane-bound Pex6p is associated with peroxisomal membranes.

On the basis of our data and published work, we would like to propose that Pex6p exerts at least part of its function by an ATP-dependent cycle of binding to and release from Pex15p (Figure 7). The three parts of Pex6p have distinct roles in this interaction. Although the N-terminal part provides the binding site for Pex15p, the two AAA cassettes have opposing roles. ATP-binding to D1 is needed to strengthen the interaction of Pex6p with Pex15p, whereas ATP-hydrolysis by D2 is required to disconnect Pex6p from Pex15p. Interestingly, the interaction of the membrane protein Pex15p with Pex6p and its various mutants observed using the two-hybrid technique was independent of Pex1p. However, this does not exclude the possibility that the latter could be involved in vivo.

A comparison of our proposal and the function established for NSF (May *et al.*, 2001), one of the best studied member of the AAA family, suggests the possibility that, in analogy to the dissociation of SNAREs mediated by NSF, Pex6p dissociates Pex15p from a membrane-bound complex in an ATP-dependent manner and that unassembled Pex15p fulfills an essential function. An ATP-dependent binding and release of Pex6p from a peroxisomal vesicle population was recently reported in *Y. lipolytica* (Titorenko and Rachubinski, 2000) and shown to lead to the priming of a distinct peroxisomal vesicle population required for a subsequent fusion event.

As an alternative to involvement in vesicle fusion it is also conceivable that Pex6p alone or via Pex15p is associated with a proposed import complex (Collins *et al.*, 2000) and may thus provide the reported ATP-dependence of peroxisomal import of matrix proteins (Bellion and Goodman, 1987; Rapp *et al.*, 1993; Wendland and Subramani, 1993). These possibilities are fertile ground for future research aimed to understand the processes that are supported by the interaction between Pex6p and Pex15p.

Figure 7. Schematic representation of interaction between Pex15p and Pex6p. The N-terminus of Pex15p interacts with the N-terminal part of Pex6p, an interaction which is stimulated by ATP-binding to the first AAA domain (A1) of Pex6p. On the other side hydrolysis of ATP by the second AAA domain of Pex6p (B2) stimulates release of Pex6p from Pex15p.

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