Pex10p links the ubiquitin conjugating enzyme Pex4p to the protein import machinery of the peroxisome

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Summary

The protein import machinery of the peroxisome consists of many proteins, collectively called the peroxins. By applying the split-ubiquitin technique we systematically tested the pair-wise interactions between the N_{ub}- and C_{ub}-labeled peroxins for the first time in the living cells of the yeast *Saccharomyces cerevisiae*. We found that Pex10p plays a central role in the protein interaction network by connecting the ubiquitin conjugation enzyme Pex4p to the other members of the protein import machinery. A yeast strain harboring a deletion of *PEX3* enabled us to estimate the influence of the peroxisomal membrane on the formation of a subset of the investigated protein-protein interactions.

Key words: Membrane proteins, Protein interaction, Peroxins, Peroxisome, Protein import, Split-ubiquitin

Introduction

Peroxisomes are small, single membrane-bound organelles that participate in many physiological reactions in most eukaryotes (Wanders, 2000; Wanders and Tager, 1998). Although the metabolic functions of the peroxisomes vary considerably between species and tissues (Lazarow and Fujiki, 1985; Reumann, 2000; Titorenko and Rachubinski, 2001), the biogenesis of the peroxisomes relies on a common class of evolutionary conserved genes referred to as *PEX* genes (Eckert and Erdmann, 2003) (Gould and Valle, 2000; Kunau, 1998; Sacksteder and Gould, 2000; Tabak et al., 1999). The corresponding proteins, the peroxins, are involved in different aspects of organelle biogenesis and maintenance (Erdmann and Blobel, 1995; Fujiki, 2000; Ghaedi et al., 2000; Gotte et al., 1998; Hettema et al., 2000; Holroyd and Erdmann, 2001; Just and Distelkötter, 1996; Kiel and Veenhuis, 2000; Sacksteder et al., 2000; Snyder et al., 2000; Subramani et al., 2000). Most of the identified peroxins play a direct role in the import of peroxisomal matrix proteins. The import into the peroxisome is achieved post-translationally and, as shown for some proteins, it occurs without an accompanying cycle of unfolding and refolding of the protein cargo (Titorenko et al., 2002). The underlying transport process must therefore differ from the related activities at the membrane of the endoplasmic reticulum or the mitochondrion (for a review, see Purdue and Lazarow, 2001). Genetic and biochemical studies, as well as two-hybrid analysis, suggest a network of interacting proteins that is dedicated to transporting the peroxisomal matrix proteins efficiently into the peroxisome (Baker et al., 2000; Hettema et al., 1999). Pex5p is the cytosolic receptor for the peroxisomal targeting sequence PTS1, which consists of the C-terminal sequence SKL or conserved variants thereof (Lametschwandtner et al., 1998), whereas Pex7p serves as the receptor for the N-terminal PTS2 (for a review, see Sackstedter and Gould, 2000). Pex5p and Pex7p dock to the cytosolic face of the peroxisomal membrane by binding to Pex14p and Pex13p, two membrane proteins of the peroxisome (Albertini et al., 1997; Elgersma et al., 1996; Erdman and Blobel, 1996; Gould et al., 1996; Girzalsky et al., 1999). Different models have been proposed to explain the ensuing transfer of the protein cargo across the membrane (for a review, see Eckert and Erdmann, 2003; Gould and Collins, 2002). Genetic and biochemical analyses hint at an active role for the peroxins Pex2p, Pex10p and Pex12p in the steps that follow the docking of the Pex5p/Pex7p-bound cargo to the membrane (Chang et al., 1997; Chang et al., 1999; Huang et al., 2000; Kalish et al., 1995; Okumoto et al., 1997; Okumoto et al., 1998a; Okumoto et al., 1998b; Okumoto et al., 2000; Patarca and Fletcher, 1992; Tan et al., 1995; Tsukamoto et al., 1991). Because deleting the genes of any one of these three proteins results in the mislocalization of peroxisomal matrix proteins, and at least for Δ*pex10* and Δ*pex12* cells it also results in the accumulation of Pex5p at the cytosolic face of the peroxisomal membrane, a participation in the transfer of the cargo across the membrane seems plausible (Dodt and Gould, 1996). Interestingly, all three proteins contain a cysteine- and histidine-rich region at their C-terminus. Although the spacing between the histidines and cysteines does vary among the three proteins and does not strictly match the consensus sequence of a typical RING-finger, the motif is often referred to as the RING-finger in the literature. In some ubiquitin E3 ligases, the RING-finger motifs are responsible for bringing the ubiquitin conjugating enzyme (Ubc) and the substrate of their ubiquitylation reaction into close apposition (Pickart, 2001; Xie and Varshavsky, 1999). The role of the RING-finger- containing peroxins and their common motif are, however, poorly understood. Some interactions among RING-finger peroxins and between these and other peroxins have been reported (Chang et al., 1999; Okumoto et al., 2000; Reguenga et al., 2001; Snyder et al., 1999). In this work we used the split-ubiquitin (split-Ub)

technique to identify shared and specific binding partners for the RING-finger peroxins Pex2p, Pex10p and Pex12p, and thereby extended our approach of the in vivo characterization of protein interactions for the first time towards the membrane-associated proteins of the peroxisome (Johnsson and Varshavsky, 1994; Stagljar et al., 1998; Wittke et al., 1999).

Materials and Methods

Construction of test proteins

The *C_{ub}-RURA3* constructs of *PEX2*, *PEX4*, *PEX5*, *PEX10*, *PEX11*, *PEX12*, *PEX14*, *PEX17* and *PEX22* were derived from *STE14-C_{ub}-RURA3* by replacing *STE14* with the *Eco*RI, *Sal*I cut PCR products of the corresponding *PEX* open reading frames (ORFs) (Wittke et al., 1999). *PEX12ΔC-C_{UB}-RURA3* was created analogously but using a 3′ primer for the PCR amplification that led to the deletion of the last 80 C-terminal residues of Pex12p. The ORFs of *PEX3* and *PEX13* were inserted in front of *C_{ub}-RURA3* by cutting the corresponding PCR products with *Cla*I/*Sal*I or *Eco*RI/*Xho*I, respectively. All *PEX-C_{UB}-RURA3* fusions were expressed from the P_{MET17} promoter and resided on a pRS313 centromeric plasmid (Sikorski and Hieter, 1989).

N_{UI}-PEX3, *-PEX4*, *-PEX15*, *-PEX14*, *-PEX19*, *-PEX11* and *-PEX10* were obtained by fusing a PCR product covering the respective ORF and approximately 200 bp of 3′ untranslated sequence in frame behind the *P_{CUP1}-N_{UI}* module using the *Bam*HI and a second restriction site. *P_{CUP1}-N_{UI}-PEX1* and *-PEX5* contained only the first 442 and 480 bp of the respective ORFs. Full-length *N_{UI}-PEX1* and *N_{UI}-PEX5* were created via homologous recombination by transforming the yeast with the *Eco*RI (*PEX1*) or Acc651 (*PEX5*) cut plasmids (Dünnwald et al., 1999). *N_{UI}-PEX12* and *N_{UI}-PEX13* contained an additional sequence at their 3′ end encoding the HA epitope. The linker sequence connecting *N_{UI}* and the *PEX* gene reads: GGG ATC CCT GGG GAT XXX, with the *Bam*HI site underlined and XXX denoting the second codon of the attached *PEX* gene. *PEX12-N_{UI}* and *PEX22-N_{UI}* were obtained by inserting the respective ORF between the sequences of the *P_{CUP1}*-promoter and of *N_{UI}* using the *Eco*RI and *Sal*I restriction sites. The sequence between *PEX22* and *N_{UI}* reads: XXX GGG TCG ACC GGC GGT ATG. XXX denotes the last codons of *PEX22/PEX12* and ATG the first codon of *NUI*. The *Sal*I is underlined. *PEX4-6HA* and *PEX10-6HA* were constructed by cutting the respective ORF after PCR amplification with *Eco*RI and *Sal*I and inserting the fragment between the P_{CUP1}-promoter and a sequence encoding six consecutive HA-epitopes on a pRS313 vector (N. Lewke, Köln, Germany). The sequences encoding SKL and SSS were attached via a PCR in frame behind the *P_{CUPI}-N_{UI}-HA* module (Wittke et al., 1999). *N_{UI}-PEX1* and *N_{UI}-PEX5* resided on the PRS304 vector. All other *N_{UI}* constructs resided on a PRS314 vector. *PEX4-*, *PEX10*, *PEX11-*, *PEX12-* and *PEX22-9MYC* were obtained by integrative insertion according to Knop et al. (Knop et al., 1999). All oligonucleotides were obtained from Metabion (Martinsried, Germany). Additional information on the generation of *N_{ub}-* and *C_{ub}-* constructs can be obtained on request.

Deletion of ORFs

The ORFs of the *PEX* genes were deleted from the strain JD53 according to Güldener et al. (Güldener et al., 1996). Transformed yeast cells were selected for kan^r integration by Geneticin (Life Technologies, Paisley, Scotland). The deletions were verified by diagnostic PCR and the inability of the cells to grow on media containing oleate as the sole carbon source. See Table 1 for a list of yeast strains used.

Growth and interaction assays

The assays used yeast-rich (YPD) and synthetic minimal media with 2% dextrose (SD) and followed standard protocols. Transformed JD53 cells were grown at 30°C on selective media containing uracil. Cells were suspended and diluted in sterile water to an OD_{600} of 1 μl and 4 μl, and 4 μl of tenfold serial dilutions were spotted on agar plates, selecting for the presence of the fusion constructs but lacking uracil (SD-Ura) or containing both 1 mg/ml 5′-Fluororotic acid and 50 μg/ml uracil (FOA; WAK-Chemie, Bad-Soden, Germany). The same dilutions were spotted on plates containing uracil to check for cell numbers. The plates were incubated at 30°C for 2-4 days unless otherwise stated.

For testing the growth on oleate, cells were first incubated on SD medium containing reduced concentrations of glucose (0.3%). After 2-3 days at 30°C, cells were transferred onto oleate media containing 1.26% oleic acid and 5% Tween® 40 (palmitate) as sole carbon sources. The plates were incubated at 30°C for 7 days or longer. The ability of the cells to grow and the appearance of the characteristic clearances around the colonies were used to determine the functionality of the fusion proteins.

GFP-SKL import assay

Wild-type and mutant JD53 cells were transformed with pEW88, a plasmid coding for GFP-SKL (courtesy of Ben Distel and Ewald Hettema, Amsterdam, The Netherlands). Cells were assayed for GFP-SKL import into peroxisomes by the appearance of the characteristic punctuated, intracellular fluorescence under a Leica DMRB Microscope equipped with a Xenon-75W-GFP/XBO75 lamp and an L4 filter with a cut-off at 500 nm (Leica, Solms, Germany).

Co-immunoprecipitation of peroxisomal membrane proteins

A 300 ml culture of yeast cells was grown to OD_{600} 0.8-1.0 under aeration at 30°C. Cells were collected at 3000 ***g*** and washed twice with distilled water at room temperature. The cells were resuspended in 3 ml ice-cold IP-buffer (50 mM Tris-HCl, pH 7.5; 50 mM NaCl; 0.2% (V/V) Triton X-100; 0.25 mM PMSF; 15 μg/ml Antipain; 1.5 μg/ml Pepstatin A; 1.0 μg/ml Leupeptin; 0.1 μg/ml Chymostatin) and were broken up in glass vials by the addition of 3 g acid-washed glass beads (425-600 μm) and by vigorous vortexing (15×30 seconds with 30-second intervals on ice). The suspension was centrifuged for 15 minutes at 5000 ***g*** at 4°C and the supernatant was centrifuged again for 30 minutes at 4°C and 17,500 ***g***. Five microlitres of the primary antibody were added to the supernatant and incubated at 4°C for 60 minutes. Twenty-five microlitres of protein A-sepharose beads (Roche Diagnostics, Mannheim, Germany) were added to each vial and incubated for at least 2 hours at 4°C. The beads were washed three times with 2 ml IP-buffer and finally boiled in 80 μl SDS-sample buffer.

Chemical cross-linking

A 500 ml yeast culture was incubated under aeration at 30°C to OD_{600} 1.5-2.0. Cells were collected by centrifugation at 1500 ***g*** and 4°C for 5 minutes. One gram of cells was defined as one volume (Vol) for all subsequent steps. The cell pellet was washed in 2-4 Vol ice-cold distilled water and resuspended in 1 Vol Lyticase-buffer (50 mM KH_2PO_4, pH 7.5; 10 mM $MgCl_2$; 1 M Sorbitol; 1 mM dithiothreitol (DTT)) containing 30 mM DTT. After incubation at room temperature for 15 minutes the cells were pelleted by centrifugation for 5 minutes at 1500 ***g*** and 4°C and resuspended in 3 Vol Lyticase-buffer. 200 U Lyticase (Sigma, Deisenhofen, Germany) per Vol were added and the cell suspension was incubated for 40 minutes at 30°C under slow shaking (~50 rpm). Spheroblasting was confirmed by the lysis-in-water test of small samples. Cells were centrifuged for 5 minutes at 1500 ***g*** and 4°C and resuspended in 10 Vol ice-cold cross-linking buffer (100 mM KH_2PO_4, pH 7.5; 1 mM EDTA; 0.25 mM PMSF; 15 μg/ml Antipain; 1.5 μg/ml Pepstatin A; 1.0 μg/ml Leupeptin; 0.1 μg/ml Chymostatin), and washed twice. The cells were resuspended in 1 Vol ice-cold cross-linking buffer and were lysed by ten strokes with a glas

Table 1. Yeast strains used in this study

Name	Genotype	Origin	Source
JD53	Matα ura3-52 leu2-3,-112 his3Δ200 lys2-801 trp1Δ63	–	Dohmen et al., 1995
JD53-Δpex2	Matα ura3-52 leu2-3,-112 his3Δ200 lys2-801 trp1Δ63 PEX2::KAN^r	JD53	This study
JD53-Δpex3	Matα ura3-52 leu2-3,-112 his3Δ200 lys2-801 trp1Δ63 Δpex3	JD53	This study
JD53-Δpex4	Matα ura3-52 leu2-3,-112 his3Δ200 lys2-801 trp1Δ63 PEX4::KAN^r	JD53	This study
JD53-Δpex5	Matα ura3-52 leu2-3,-112 his3Δ200 lys2-801 trp1Δ63 PEX5::KAN^r	JD53	This study
JD53-Δpex10	Matα ura3-52 leu2-3,-112 his3Δ200 lys2-801 trp1Δ63 PEX10::KAN^r	JD53	This study
JD53-Δpex11	Matα ura3-52 leu2-3,-112 his3Δ200 lys2-801 trp1Δ63 PEX11::KAN^r	JD53	This study
JD53-Δpex12	Matα ura3-52 leu2-3,-112 his3Δ200 lys2-801 trp1Δ63 PEX12::KAN^r	JD53	This study
JD53-Δpex13	Matα ura3-52 leu2-3,-112 his3Δ200 lys2-801 trp1Δ63 PEX13::KAN^r	JD53	This study
JD53-Δpex14	Matα ura3-52 leu2-3,-112 his3Δ200 lys2-801 trp1Δ63 PEX14::KAN^r	JD53	This study
JD53-Δpex15	Matα ura3-52 leu2-3,-112 his3Δ200 lys2-801 trp1Δ63 PEX15::KAN^r	JD53	This study
JD53-Δpex17	Matα ura3-52 leu2-3,-112 his3Δ200 lys2-801 trp1Δ63 PEX17::KAN^r	JD53	This study
JD53-Δpex19	Matα ura3-52 leu2-3,-112 his3Δ200 lys2-801 trp1Δ63 PEX19::KAN^r	JD53	This study
JD53-Δpex22	Matα ura3-52 leu2-3,-112 his3Δ200 lys2-801 trp1Δ63 PEX22::KAN^r	JD53	This study
NUI-PEX1-JD53	Matα ura3-52 leu2-3,-112 his3Δ200 lys2-801 trp1Δ63 PEX1::CUP-NUI-PEX1 TRP1	JD53	This study
NUI-PEX5-JD53	Matα ura3-52 leu2-3,-112 his3Δ200 lys2-801 trp1Δ63 PEX5::CUP-NUI-PEX1 TRP1	JD53	This study

douncer. DSP (Pierce, USA) in DMSO was added to a final concentration of 200 μg/ml and the extracts were incubated for 30 minutes at 23°C. After quenching by the addition of glycine to 20 mM the cross-linked proteins were coprecipitated as decribed above.

SDS-PAGE (polyacrylamide gel electrophoresis), transfer onto nitrocellulose and immunodetection of the transferred proteins was performed as described previously (Wittke et al., 2002). For sequential detection of proteins, membranes were stripped with 2% SDS and 100 mM β-mercaptoethanol for 30 minutes at 55°C.

Results

A systematic interaction assay between the RING-finger peroxins and the other known peroxins was performed by testing the C_{ub}-RUra3p fusion proteins of Pex2p, Pex10p and Pex12p against a panel of N_{ub}-labeled fusion proteins in an in vivo split-Ub assay. The RUra3p module monitors the interaction-induced reassociation between N_{ub} and C_{ub} by a simple growth assay. Once cleaved off from the C_{ub}, the RUra3p is rapidly degraded by the enzymes of the N-end rule pathway of protein degradation (Varshavsky et al., 2000). Interactions are therefore detected by the growth characteristics of the N_{ub}/C_{ub} cotransformants on SD-ura or 5-FOA-containing media (Fig. 1A). Close proximity between the correspondingly labeled N_{ub} and C_{ub} fusion proteins results in non-growth on SD-ura and growth on medium containing 5-FOA (Wittke et al., 1999; Laser et al., 2000).

The localization and assumed topologies of the N_{ub}- and C_{ub}-labeled fusion proteins that were used in this study are shown in Fig. 1B. Excluded from our study were those peroxins that are known to reside in the lumen of the peroxisome or that are exclusively involved in the import of proteins carrying the PTS2 import signal. Also not considered as N_{ub} fusions were those peroxins whose N-termini had been shown or strongly suspected to point into the matrix of the organelle. To control the specificity of the assay, we measured the reactions between N_{ub}-Pex11p and the C_{ub}-RUra3p-labeled Pex2p, Pex10p and Pex12p. Pex11p is a peroxisomal membrane protein that does not participate in the import of matrix proteins (Erdmann and Blobel, 1995; Li and Gould, 2002). We therefore assumed that Pex11p is not permanently integrated into any of the peroxin import complexes and should therefore serve as a valid control. To verify the correct localization of the C_{ub}-modified peroxins, we measured their interactions with a set of N_{ub}-labeled membrane proteins known to reside in other compartments of the cell (Wittke et al., 1999).

RING-finger-dependent interaction between Pex12p and Pex10p, Pex13p and Pex15p

We attached the C_{ub}-RUra3p module to the C-terminus of Pex12p to construct Pex12-C_{ub}-RUra3p. Yeast cells bearing

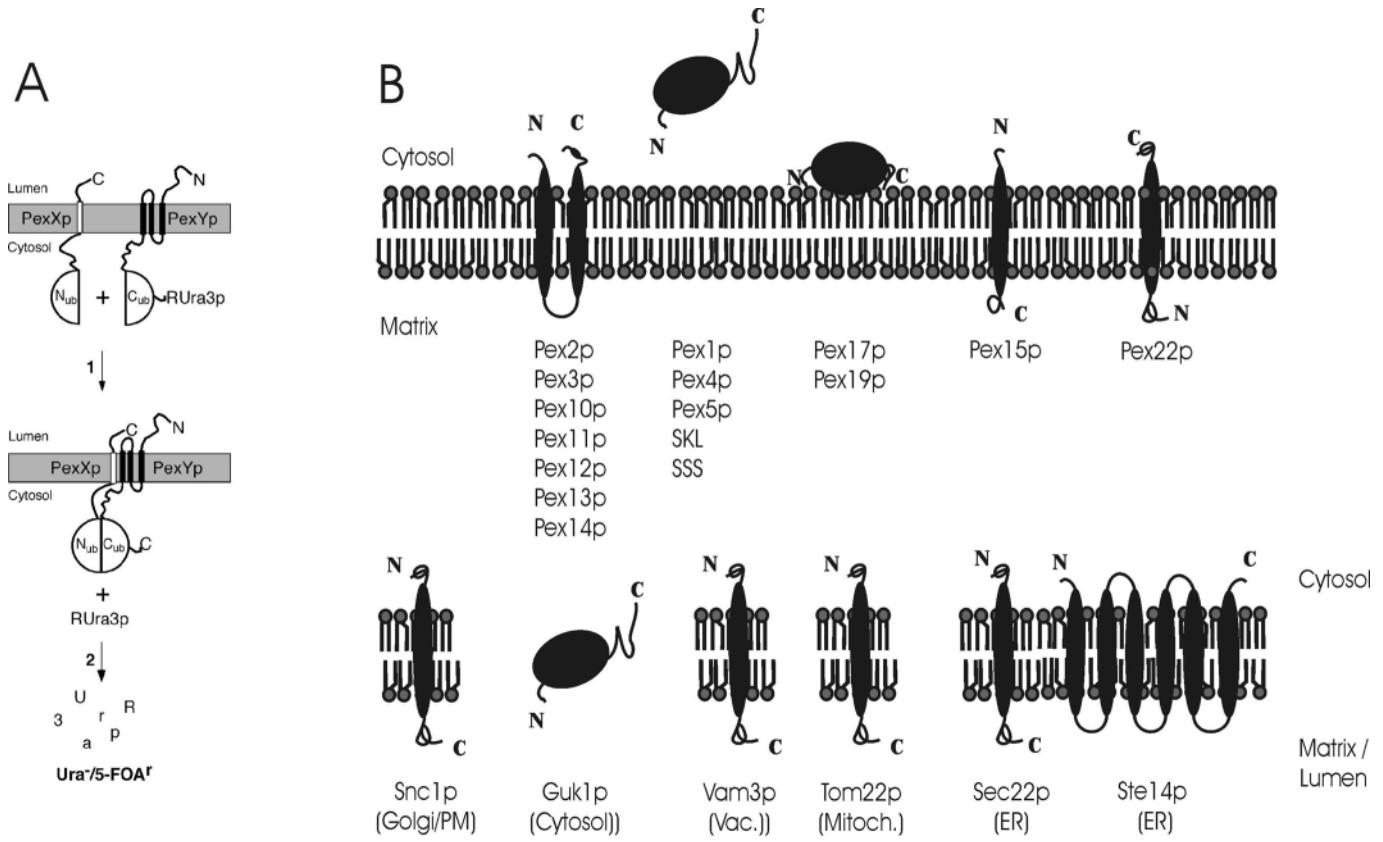

Fig. 1. (A) The split-ubiquitin technique and its application to the analysis of the membrane-associated peroxins. C_{ub}-RUra3p was linked to the C-terminus of peroxin Y and N_{ub} was linked to Peroxin X. Provided that Pex X and Y interact, the complex brings N_{ub} and C_{ub} into close proximity and the Ub-halves will reconstitute the native-like Ub (1). The Ub-specific proteases will recognize the reconstituted Ub and cleave off the attached RUra3p. The released RUra3p is targeted for rapid destruction by the enzymes of the N-end rule (2) to yield cells that are uracil auxotrophs and 5-FOA resistant. (B) N_{ub} and C_{ub} fusions. Upper panel: N_{ub} (residues 1-36 of Ub) was fused to the N-terminus of Pex1p, Pex3p, Pex4p, Pex5p, Pex10p, Pex11p, Pex13p, Pex14p, Pex15p, Pex19p, the peroxisomal targeting sequence SKL and the control sequence SSS, to the N- and C-terminus of Pex12p and to the C-terminus of Pex22p. C_{ub} (residues 35-76 of Ub) was attached to the C-terminus of Pex2p, Pex3p, Pex4p, Pex5p, Pex10p, Pex11p, Pex12p, Pex13p, Pex14p, Pex17p, and Pex22p. Shown are the localization and assumed topologies of the different peroxins. Note that some of the shown topologies are not yet conclusively proven. During the course of the work and in accordance with the results of our assay, the N-terminus of Pex12p was shown to point into the peroxisomal matrix (Albertini et al., 2001). The assay requires that both halves of Ub had to point into the cytosol of the cell. Lower panel: N_{ub} was fused to the N-terminus of Snc1p, Guk1p, Vam3p, Tom22p, Sec22p, Vam3p, and Ste14p (Wittke et al., 1999). These N_{ub} fusions were used as controls for the specificity of the assay and to verify the correct localization of the C_{ub} fusions of the peroxins. ER, endoplasmic reticulum; Mitoch., mitochondrium; PM, plasma membrane; Vac, vacuole.

Pex12-C_{ub}-RUra3p were transformed with the indicated N_{ub}-fusions and the transformants were spotted onto plates lacking uracil or containing 5-FOA (Fig. 2A,B). In contrast to the other cotransformants, the cells containing N_{ub}-Pex19p, N_{ub}-Pex15p, N_{ub}-Pex13p or N_{ub}-Pex10p do not grow on SD-ura, but grow on 5-FOA instead. As we expected from their different roles in peroxisome biogenesis, Pex12-C_{ub}-RUra3p did not interact with N_{ub}-Pex11p in our assay. To confirm that the non-growth/growth of the cells on SD-ura/5-FOA indeed reflects the interaction-depended reassociation of the attached N_{ub} and C_{ub}, we performed the same assay in cells lacking a functional N-end rule pathway. Cells lacking the recognition component of the N-end-rule pathway (*UBRI*) will not degrade the cleaved RUra3p (Varshavsky et al., 2000). Consequently, the growth of the cells on media containing 5-FOA or lacking uracil should not be influenced by the efficiency of the N_{ub}-C_{ub} reassociation of the co-expressed fusion proteins. Accordingly, and in contrast to the isogenic wild-type cells, we observed good growth on SD-ura of the cells lacking *UBR1*, irrespective of the identity of their expressed N_{ub} and C_{ub} fusion proteins (Fig. 2D). We conclude that Pex12p interacts with Pex10p, Pex15p, Pex19p and Pex13p. To estimate the contribution of the RING-finger to these interactions, we tested a C_{ub}-RUra3p fusion of Pex12p in which the entire C-terminal sequence (residues 320-399) was deleted (Pex12ΔC-C_{ub}-RUra3p). This deletion abolishes almost all interaction signals, leaving N_{ub}-Pex19p as the only interaction partner of Pex12ΔC-C_{ub}-RUra3p (Fig. 3). Besides pointing to the crucial role of the C-terminal domain of Pex12p for its interaction with Pex10p, Pex13p and Pex15p, this experiment again confirms that the measured interactions of the full-length Pex12p are specific.

Pex10p is in close proximity to Pex4p

The interaction profile of Pex10-C_{ub}-RUra3p differs in two

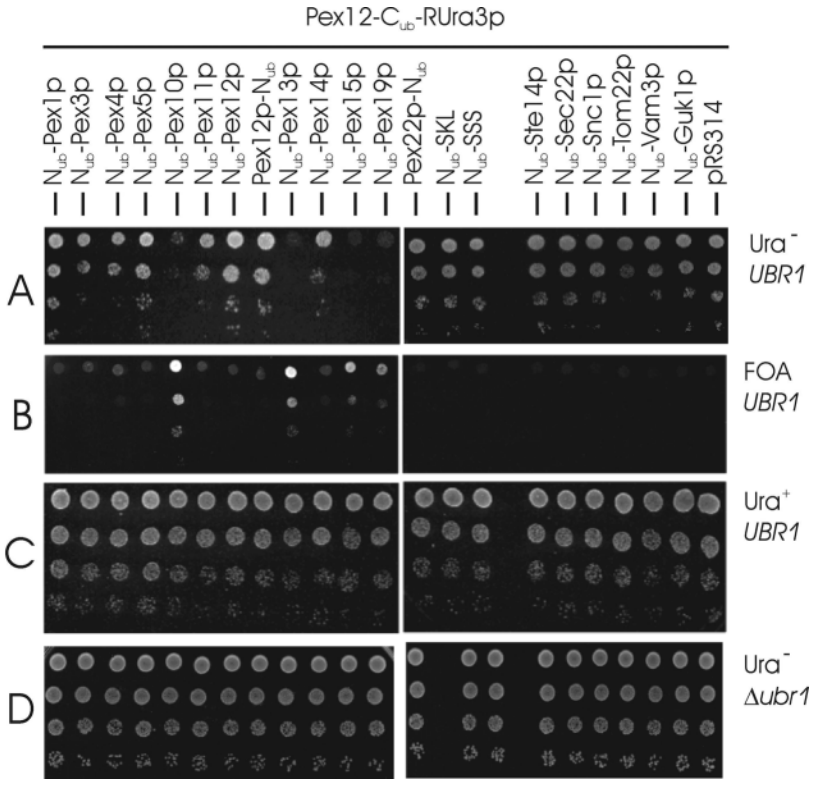

Fig. 2. Binding partners of Pex12p. Pex12-C_{ub}-RUra3p containing cells expressing the indicated N_{ub} fusion proteins were grown on SD-media and diluted in water. 4 μl of an OD_{600} of 1, and of tenfold serial dilutions were spotted onto media either lacking uracil (A) or containing both uracil and 5′-FOA (B) or containing only uracil (C). (D) as in (A) but with cells lacking the gene encoding the recognition component of the N-end rule *UBR1*. All plates contained 100 μM $CuSO_4$ to induce the P_{CUP1}-promotor and were lacking tryptophan and histidine to select for the plasmids. Cells were incubated at 30°C for 48 hours. Interactions between the N_{ub} and C_{ub} fusions are indicated in the *UBR1* cells by the growth of the construct containing cells on 5-FOA and by the non-growth on SD-ura.

aspects from the interaction profile of Pex12-C_{ub}-RUra3p (compare Fig. 4A,B with Fig. 2A,B). Apart from the interactions that were already detected for Pex12p, we observed an additional close proximity between Pex10p and Pex4p, as well as a Pex10p-Pex10p interaction. The weak interaction signal that is observed between N_{ub}-Pex3p and Pex10-C_{ub}-RUra3p on 5-FOA is not above the background that is defined in this experiment by the weak interaction signal of the cells expressing Pex10-C_{ub}-RUra3p and N_{ub}-Tom22p. We conclude that Pex10p and Pex12p share most of their interaction partners, except for Pex4p, which forms a unique interaction with Pex10p. Pex4p is a ubiquitin conjugating enzyme and its measured proximity to Pex10p points to a functional involvement of the RING-finger of Pex10p in a Pex4p-mediated ubiquitylation reaction. Unfortunately, the influence of the role of the RING-finger of Pex10p on the observed interactions could not be assessed. The cellular amount of the corresponding Pex10ΔC-C_{ub}-RUra3p was already so low that the growth of the cells on SD-ura or 5-FOA could no longer serve as an indicator of interaction. To independently confirm some of the newly identified protein interactions of Pex10p (see Fig. 4A,B and Table 2), we performed co-immunoprecipitations between the HA-tagged Pex10p and the MYC-tagged versions of Pex10p, Pex12p, Pex4p and Pex22p. A MYC-tagged Pex11p was introduced as a control for evaluating the specificity of the immunoprecipitations. Pex22p was included because of its measured interaction with Pex10p in a strain expressing Pex22-C_{ub}-RUra3p/N_{ub}-Pex10p (Table 2). Fig. 4C shows the co-precipitation of the two differently tagged Pex10p fusions and the precipitation of Pex12p by Pex10p. MYC- tagged Pex4p and Pex22p co-precipitated with Pex10p only when the precipitation of Pex4p and Pex22p by the MYC antibody was followed by detection of the labeled Pex10p with the HA antibody (Fig. 4D). Changing the order of the employed antibodies in the experiment did not result in measurable co-precipitation. Our interpretation is that the binding of Pex10p to Pex4p and Pex22p is only temporal or so labile that the complexes only partially survive our immunoprecipitation protocol. The binding of the HA antibody to the C-terminus of the labeled Pex10p might further weaken an already unstable interaction. We gained additional confidence in the presence of a Pex4p-Pex10p complex through its independent detection in a genome-wide two-hybrid experiment (P. Uetz, personal communication) and a systematic

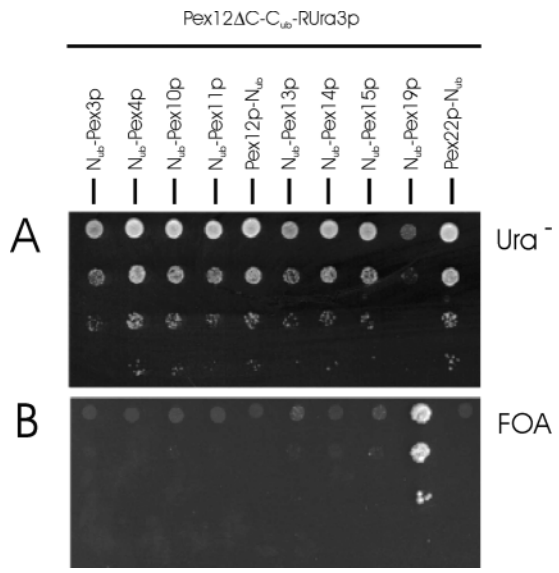

Fig. 3. Removal of the C-terminal domain abolishes most of the interactions of Pex12p. Cells containing Pex12ΔC-C_{ub}-RUra3p and expressing the N_{ub} constructs of Pex3p, Pex4p, Pex10p, Pex11p, Pex12p, Pex13p, Pex14p, Pex15p, Pex19p and Pex22p were diluted to an OD_{600} of 1, and 4 μl of this and of tenfold serial dilutions were spotted onto media containing 100 μM $CuSO_4$ and either lacking uracil (A) or containing both uracil and 5-FOA (B). Both media were lacking tryptophan and histidine to select for the plasmids. Cells were incubated at 30°C for 48 hours.

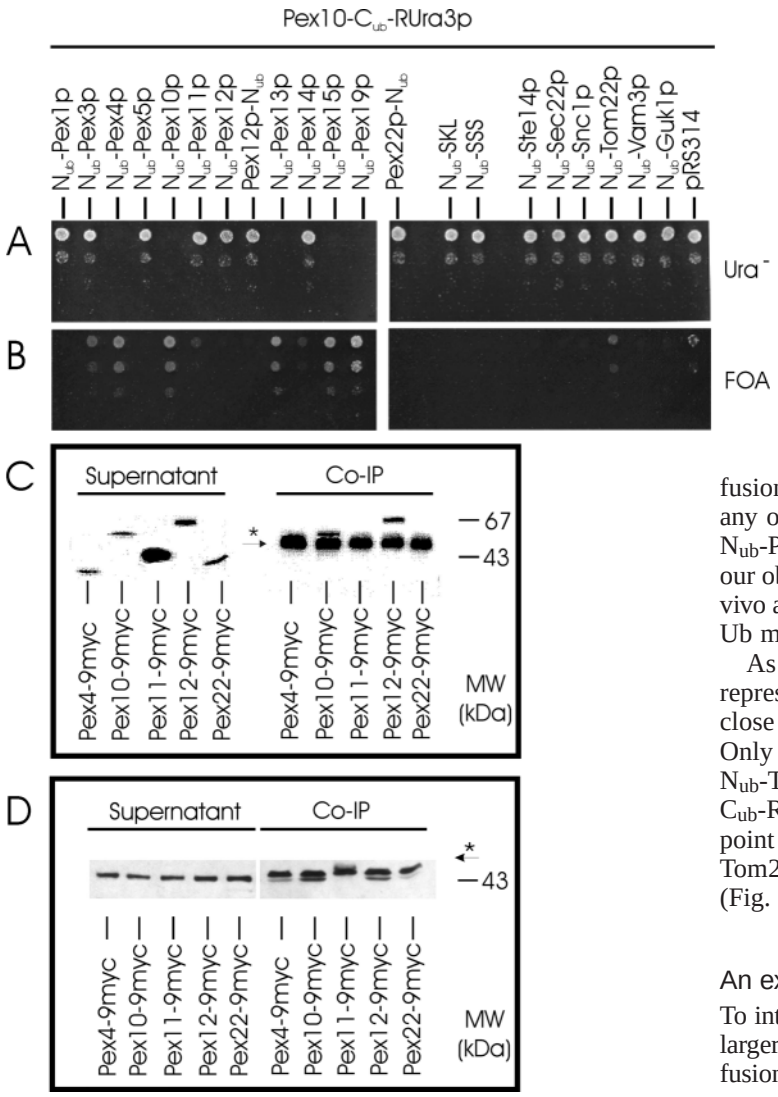

Fig. 4. Binding partners of Pex10p. (A,B) Cells containing Pex10-C_{ub}-RUra3p and expressing the indicated N_{ub} fusion proteins were diluted to an OD_{600} of 1, and 4 μl of this and of tenfold serial dilutions were spotted onto media containing 100 μM $CuSO_4$ and either lacking uracil (A) or containing both uracil and 5-FOA (B). Both media were lacking tryptophan and histidine to select for the plasmids. Cells were incubated at 30°C for 48 hours. (C) Yeast cells co-expressing Pex10p tagged with a six-fold repeated ha-epitope (Pex10-6ha) together with either Pex4p, Pex10p, Pex12p, Pex11p or Pex22p, all bearing a ninefold repeated myc-epitope at their C-termini, were subjected to a co-precipitation using the anti-ha-antibody to precipitate and the anti-MYC antibody to detect the co-precipitated protein after SDS-PAGE and transfer to nitrocellulose (C). (D) as in (C) but using anti-myc antibody for precipitation and anti-ha antibody for the detection of the co-precipitated Pex10-6ha. The asterisk indicates the heavy chain of the antibody used for the precipitation.

two-hybrid experiment on peroxins (K. Schulz and R. Erdmann, personal communication).

Pex2p interacts only with Pex19p in the split-Ub assay

Pex2-C_{ub}-RUra3p as the third RING-finger peroxin displayed a completely different interaction profile when co-expressed with the same set of N_{ub}-fusions (Fig. 5A,B). We only detected an interaction between Pex2p and Pex19p. N_{ub}-Pex13p, -Pex10p and -Pex15p, although interacting with both the C_{ub}-RUra3p-labeled Pex12p and Pex10p, are not in close proximity to Pex2-C_{ub}-RUra3p.

As already observed for the correspondingly modified Pex12p and Pex10p, Pex2-C_{ub}-RUra3p also does not interact with N_{ub}-Pex11p (Figs 2-5). These results are in agreement with the assumed absence of Pex11p in any of the peroxin complexes. However, to be considered as a valuable control, we had to confirm that the lack of N_{ub}-Pex11p interactions are not caused by either the mislocalization or a low activity of N_{ub}-Pex11p. We therefore constructed Pex11-C_{ub}-RUra3p and determined its proximity to the same set of N_{ub}-fusions (Fig. 5C,D). Pex11-C_{ub}-RUra3p does not interact with any of the other N_{ub}-labeled peroxins except N_{ub}-Pex19p and N_{ub}-Pex11p. Because Pex11p is known to self-multimerize, our observation confirms this behavior for the fusion protein in vivo and validates N_{ub}-Pex11p as a proper control for the split-Ub measured peroxin interactions (Marshall et al., 1996).

As expected, N_{ub}-Sec22, N_{ub}-Vam3p or N_{ub}-Guk1p as representatives of other cellular compartments did not show close proximity to any of the RING-finger peroxins (Figs 2-5). Only cells co-expressing the mitochondrial marker protein N_{ub}-Tom22p together with Pex10-C_{ub}-RUra3p or Pex12ΔC-C_{ub}-RUra3p displayed a weak interaction signal that might point to a certain amount of mislocalization of either N_{ub}-Tom22p or the C_{ub}-RUra3p-labeled Pex10p and Pex12ΔCp (Fig. 4 and authors' unpublished observation).

An extended network of peroxin interactions

To integrate the interactions of the RING-finger peroxins into a larger context, we tested an extended subset of C_{ub}-RUra3p fusions against the same set of N_{ub} fusion proteins. The measured interactions between the different N_{ub}- and C_{ub}-labeled peroxins are summarized in Table 2 and Fig. 8. We only interpreted growth or non-growth on 5-FOA or SD-ura as an indication of interaction if the signals were significantly stronger than those derived from the co-expression of N_{ub}-Pex11p or other N_{ub}-fusion proteins that were introduced as controls. To assist the interpretation of the interaction data, we measured the functionality of all fusion constructs by complementation tests of the corresponding deletion strains. Successful complementation was determined by two criteria: (1) peroxisomal localization of a green fluorescent protein (GFP) carrying a peroxisome import signal and (2) growth on oleic acid as the sole carbon source. The results of the complementation assays are documented in Table 3. We regard the interaction signal between N_{ub}-Tom22p and Pex13-C_{ub}-RUra3 on 5-FOA plates as a false positive (Table 2). Because Pex13-C_{ub}-RUra3p is not functional, the observed interaction might indicate a high percentage of mislocalization of Pex13-C_{ub}-RUra3p to the mitochondrion (Tables 2, 3). Pex10-C_{ub}-RUra3p displays a similar, albeit less extreme, behaviour (Fig. 4A,B).

Pex22p is required for the Pex4p-Pex10p interaction

The split-Ub assay measures a close proximity rather than a

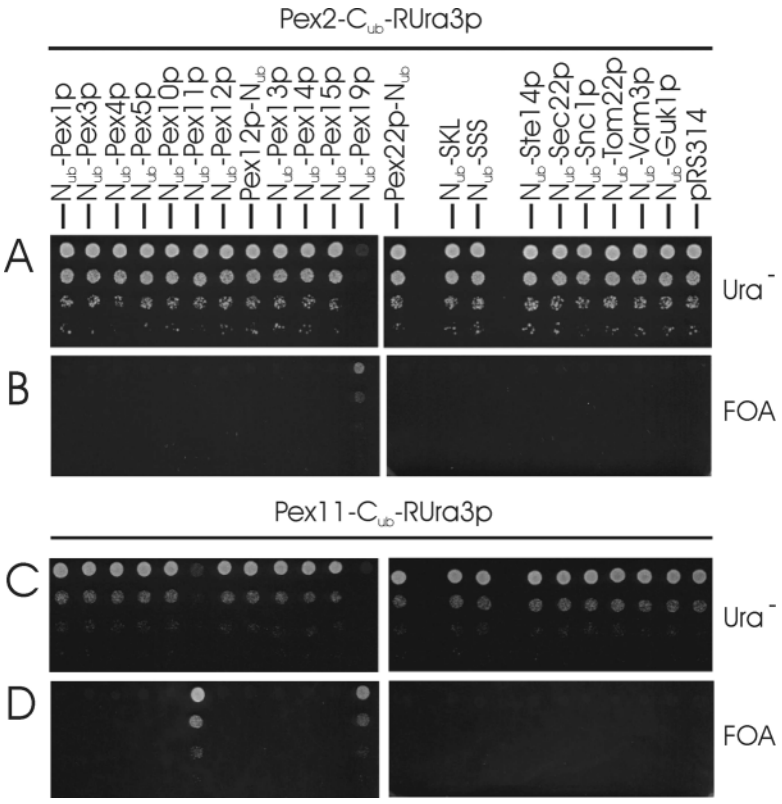

Fig. 5. Binding partners of Pex2p and Pex11p. Yeast cells containing Pex2-C_{ub}-RUra3p (A,B) or Pex11-C_{ub}-RUra3p (C,D) and expressing the indicated N_{ub}-fusion proteins were diluted to an OD_{600} of 1, and 4 μl of this and of tenfold serial dilutions were spotted onto media containing 100 μM $CuSO_4$ and either lacking uracil (A,C) or containing both uracil and 5-FOA (B,D). Both media were lacking tryptophan and histidine to select for the plasmids. Cells were incubated at 30°C for 48 hours.

direct interaction between the N_{ub}- and C_{ub}-labeled membrane proteins. Although the measured interactions can be indirect, the technique requires a relatively close apposition of the two labeled termini of the proteins. Spatially more distant subunits in a large assembly might therefore give no or only a very weak interaction signal as C_{ub}/N_{ub}-modified proteins, whereas a strong interaction signal indicates a close proximity between the two labeled subunits in this complex. A split-Ub measured interaction might therefore reflect aspects of the actual geometry of the complex. This assumption helps to explain why Pex4p interacts with Pex10p, but not with Pex12p or Pex13p, although Pex10p, Pex12p and Pex13p were all found to be connected through interactions (Table 2). As can be seen from Table 2, N_{ub}-Pex4p interacts not only with Pex10-C_{ub}-RUra3p, but also with Pex22-C_{ub}-RUra3p and Pex4-C_{ub}-RUra3p. Pex22-C_{ub}-RUra3p interacts with N_{ub}-Pex4p, N_{ub}-Pex10p, N_{ub}-Pex15p and N_{ub}-Pex19p (Table 2). The interaction between N_{ub}-Pex10p and Pex22-C_{ub}-RUra3p is only seen on 5-FOA, but could be confirmed by a co-precipitation experiment (Fig. 4D). To estimate the importance of Pex22p for the interaction between Pex10p and Pex4p, we measured the proximity between N_{ub}-Pex4p and Pex10-C_{ub}-RUra3p in cells lacking Pex22p (Fig. 6). The absence of Pex22p only impairs the interaction between Pex10p and Pex4p, whereas the interactions between Pex10p and Pex12p, Pex10p, Pex13p, Pex15p and Pex19p persist (Fig. 6A,C). The effect is specific for Pex22p given that the deletion of Pex5p does not affect the interaction between Pex4p and Pex10p nor any of the other measured interactions of Pex10p (Fig. 6A,B).

Table 2. Summary of peroxin interactions

-C_{ub}-RUra3p	N_{ub}-
Pex1p	Not detectable
Pex2p	Pex19p
Pex3p	Pex3p*, Pex13p, Pex15p
Pex4p	Pex4p*
Pex5p	Pex5p, Pex13p*, Pex19p*
Pex10p	Pex4, Pex10p, Pex13p, Pex15p, Pex19p
Pex11p	Pex11p, Pex19p
Pex12p	Pex10p, Pex13p, Pex15p, Pex19p
Pex13p	Pex13p, Pex14p, Pex15p, Pex19p, Tom22p
Pex14p	Pex5p, Pex13p, Pex15p, Pex19p
Pex17p	Pex5p*, Pex13p, Pex15p, Pex19p
Pex22p	Pex4p, Pex10*, Pex15p, Pex19p

Summary of interactions between peroxin-C_{ub}-RUra3-fusion proteins and N_{ub}-peroxins. Unless otherwise indicated, the interactions could be detected in both the SD-ura and 5-FOA assays.
*Detection in the 5-FOA assay only.

It was reported that the concentration of Pex4p is reduced on deletion of *PEX22* in *Pichia pastoris* (Koller et al., 1999). However, our *Saccharomyces cerevisiae pex22Δ* cells express enough Ura3p activity from Pex4-C_{ub}-RUra3p to confer 5-FOA sensitivity and they express sufficient amounts of N_{ub}-Pex4p to enable the interaction between N_{ub}-Pex4p and Pex4-C_{ub}-RUra3p to be measured (Fig. 6D). The Pex4p-Pex4p interaction is also detected in wild-type cells and is specific, given that the endoplasmic reticulum (ER)-based N_{ub}-Ubc6p does not interact with Pex4-C_{ub}-RUra3p (Fig. 6E). Ubc6p and Pex4p belong to the same family of ubiquitin conjugating enzymes (Pickart, 2001). The split-Ub-measured proximity between N_{ub}-and C_{ub}-labeled Pex4p could be confirmed by the co-precipitation of HA and MYC-tagged Pex4p from yeast extracts. Successful co-precipitation required the addition of the chemical crosslink DSP before the incubation with antibodies (Fig. 6F).

Interaction assays in the absence of peroxisomal membranes

Pex3p and Pex19p are both crucial for the formation of peroxisomal membranes. In the absence of either of the two corresponding genes, the cells are free of any recognizable peroxisomal structures (Ghaedi et al., 2000; Matsuzono et al., 1999; South and Gould, 1999). This feature distinguishes Pex19p and Pex3p from all the other known peroxins, although recent work revealed remnant membranous structures in *pex3Δ* cells, which are, however, different from those found in other peroxin deletion strains (Hazra et al., 2002). The importance of Pex19p for the biogenesis of the peroxisome is clearly reflected in our interaction matrix. All peroxin-C_{ub}-RUra3p fusion proteins show interactions with N_{ub}-Pex19p, except for

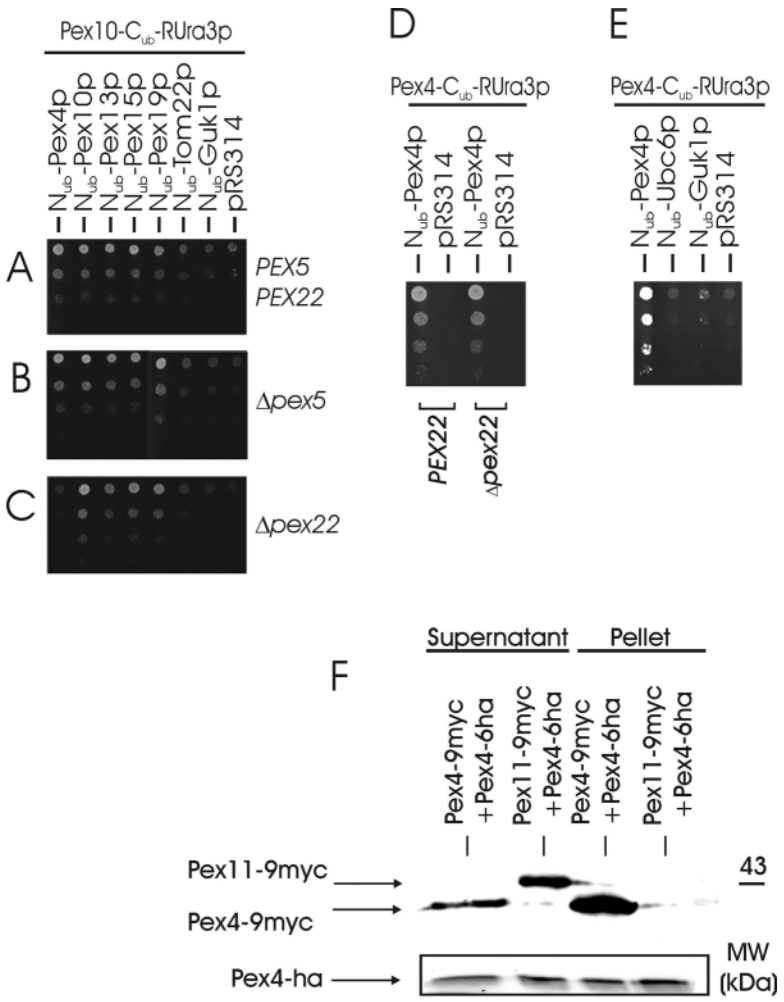

Fig. 6. The interaction between Pex10p and Pex4p depends on the presence of Pex22p. (A) *PEX5/PEX22* cells, (B) Δ*pex5/PEX22* cells and (C) *PEX5/Δpex22* cells containing Pex10-C_{ub}-RUra3p and expressing the N_{ub} constructs of Pex4p, Pex10p, Pex13p, Pex15p, Pex19p, Tom22p, Guk1p and the empty plasmid were diluted to an OD_{600} of 1, and 4 μl of this and of tenfold serial dilutions were spotted onto media containing 100 μM $CuSO_4$ and 5-FOA. The media lacked tryptophan and histidine to select for the plasmids. Cells were incubated at 30°C for 48 hours. (D) as (C) but cells contained Pex4-C_{ub}-RUra3p and expressed N_{ub}-Pex4p or the empty vector in wild-type or *Δpex22* cells. (E) The Pex4p-Pex4p interaction is specific. Cells co-expressing Pex4-C_{ub}-RUra3p and the N_{ub}-constructs of Pex4p, Ubc6p, Guk1p or the empty vector were analyzed as in (A-C). (F) Yeast cells co-expressing Pex4-6ha together with either Pex4-9myc or Pex11-9myc were subjected to anti-ha precipitation after DSP crosslinking. The co-precipitated proteins were detected with anti-myc antibody after reverting the cross-link, 12.5% SDS-PAGE, and transfer onto nitrocellulose. The blot was stripped and re-probed with anti-ha-antibody to ensure that similar quantities of Pex4-6ha had been loaded (boxed).

Pex4-C_{ub}-RUra3p and Pex3-C_{ub}-RUra3p (Table 2, Fig. 8). Pex3p does not share this feature of multiple interactions and only reveals a weak interaction signal with Pex15p and Pex13p in our assay (Table 2). To gauge the importance of Pex3p and thereby the importance of the peroxisomal membrane for the interactions of the RING-finger peroxins Pex10p and Pex12p, we performed the interaction assays in cells lacking Pex3p. N_{ub}-Pex10p and N_{ub}-Pex19p still interacted with Pex12-C_{ub}-RUra3p in *pex3Δ* cells, whereas the split-Ub measured interactions between Pex12p and Pex15p, or Pex13p, disappear (Fig. 7A). The absence of Pex3p had an even more dramatic effect on Pex10-C_{ub}-RUra3p, leaving only its interaction with N_{ub}-Pex19p visible on the FOA plate (Fig. 7B). The effects of the deletion are specific, given that the measured interaction between the N_{ub}- and C_{ub}- labeled Pex11p persisted even in the absence of Pex3p (Fig. 7E). As was already observed for Pex12p-C_{ub}-RUra3p in the *pex3Δ* strain, an interaction signal between N_{ub}-Tom22p and Pex11-C_{ub}-RUra3p became clearly apparent as though Pex11p mislocalizes to the mitochondrial membrane while keeping its oligomerization domain still intact (Fig. 7E). A tendency of some of the non-functional or only partially functional peroxins to localize to the mitochondria was also observed in the wild-type strain (see Fig. 4, Tables 2, 3). Interestingly, we did not observe an increase in proximity between the Peroxin-C_{ub}-RUra3p and N_{ub}-Sec22p on deletion of *PEX3* (Fig. 7A,B,E). Because N_{ub}-Sec22p was shown to interact with all of the ER-membrane localized C_{ub}-RUra3p fusion proteins tested so far (Wittke et al., 1999), we conclude that the deletion of *PEX3* does not cause the membrane-associated peroxins to get trapped in the membrane of the ER.

Table 3. Functionality assays

Construct	Growth assay	Import assay	Construct	Growth assay	Import assay
NuI-Pex1p	–	–	Pex22-NuI	(+)	(+)
NuI-Pex3p	(+)	(+)	Pex2-Cub-RUra3p	(+)	(+)
NuI-Pex4p	+	(+)	Pex3-Cub-RUra3p	+	(+)
NuI-Pex5p	–		Pex4-Cub-RUra3p	(+)	(+)
NuI-Pex10p	+	(+)	Pex5-Cub-RUra3p	–	–
NuI-Pex11p	+	+	Pex10-Cub-RUra3p	(+)	(+)
NuI-Pex12p	(+)	–/(+)	Pex11-Cub-RUra3p	+	+
Pex12-NuIp	–	–	Pex12-Cub-RUra3p	–	–
NuI-Pex13p	(+)	(+)	Pex13-Cub-RUra3p	–	–
NuI-Pex14p	–	–	Pex14-Cub-RUra3p	–	–/(+)
NuI-Pex15p	(+)	+	Pex17-Cub-RUra3p	+	(+)
NuI-Pex19p	+	+	Pex22-Cub-RUra3p	+	+

The results of the functionality assays of the Pex-fusion proteins by growth on oleic acid and GFP-SKL import are indicated. +, full functionality; (+), partial functionality; –, no functionality.

Discussion

Extending the C-termini of peroxisomal proteins with C_{ub}-RUra3p and measuring their interactions with a set of N_{ub} fusion proteins enabled us to construct a limited network of protein interactions at the membrane of the peroxisome. Because the membrane of the peroxisome can be deleted without affecting the growth of the yeast cells on glucose medium (Erdmann et al., 1989), the implementation of the assay in a *Δpex3* strain revealed the specific influence of the peroxisomal membrane onto a subset of those interactions.

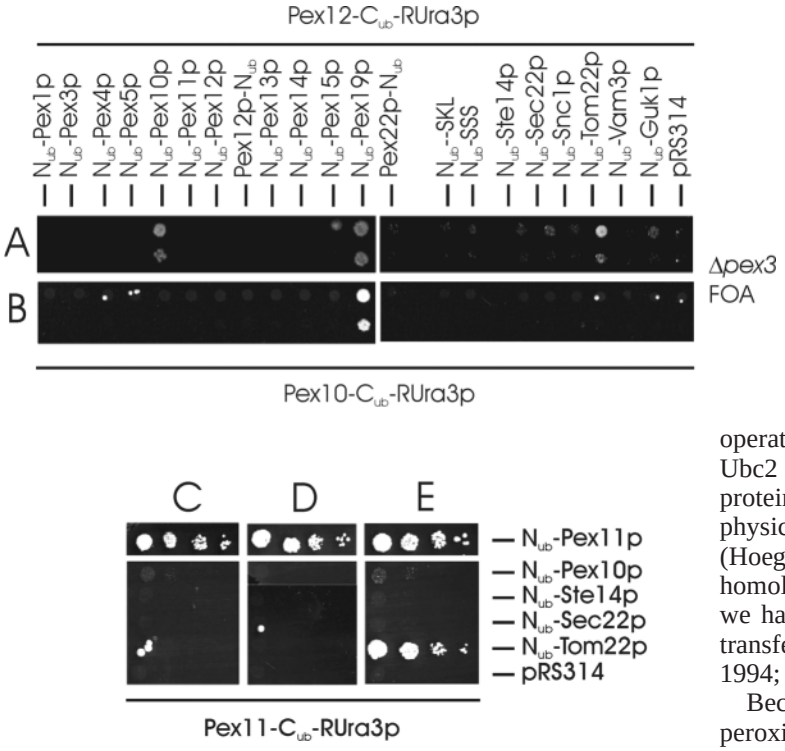

Fig. 7. Analysis of peroxin interactions in the absence of peroxisomal membranes. *Δpex3* cells (A,B,E) containing Pex12-C_{ub}-RUra3p (A), Pex10-C_{ub}-RUra3p (B) or Pex11-C_{ub}-RUra3p (E) and expressing the indicated N_{ub}-fusions or the empty plasmid were diluted to an OD_{600} of 1, and 4 μl of this and of tenfold serial dilution were spotted onto media containing 100 μM $CuSO_4$ and 5-FOA. The media lacked tryptophan and histidine to select for the plasmids. The cells were incubated at 30°C for 48 hours. (C) and (D) as in (E) but with wild-type cells (C) or Δ*pex11* cells (D).

RING-finger peroxins

The measured interactions of the three different RING-finger peroxins Pex2p, Pex10p and Pex12p are summarized in Fig. 8. The split-Ub assay detected more interaction partners for Pex10p than for Pex12p and Pex2p. Among the unique interactions of Pex10p are the measured proximities to both Pex4p and Pex22p (Fig. 4, Table 2). Repeating the split-Ub assay in cells lacking *PEX22* revealed the importance of Pex22p for the stability of the Pex4p-Pex10p interaction (Fig. 6). Together with the measured proximity between N_{ub}-Pex4p and Pex22-C_{ub}-RUra3p and two-hybrid studies by other groups (see above), these data strongly suggest that Pex22p/Pex4p docks to the peroxin import apparatus via a unique binding to Pex10p (Table 2) (Koller et al., 1999). The RING-finger of Pex10p displays a significant sequence similarity to the RING finger of Rad18p. Rad18p operates in the DNA repair pathway and forms a complex with Ubc2 (Rad6p), a Pex4p-like Ub-conjugating enzyme, and the protein Rad5p. Rad5p also contains a RING finger and physically associates with the Ub-conjugating enzyme Ubc13p (Hoege et al., 2002; Ulrich and Jentsch, 2000). Although this homology and the observed interactions are quite suggestive, we have no direct evidence that Pex10p is an Ub-ligase and transfers an Ub-moiety to a protein substrate (Crane et al., 1994; van der Klei et al., 1998; Wiebel and Kunau, 1992).

Because Pex2p was recently found in complex with other peroxins (Reguenga et al., 2001), the absence of any interaction partner for Pex2-C_{ub}-RUra3p except N_{ub}-Pex19p is curious. There are two alternative explanations. Pex2p exerts its function in isolation from most of the other peroxins, or the C-terminal modification interferes with all potential binding partners or is otherwise inept to sensing the interactions of Pex2p. The latter argument is supported by the only partial functionality of the Pex2-C_{ub}-RUra3p (Table 3). In addition, we note that a potential interaction between Pex12p and Pex2p would have escaped the current configuration of the split-Ub assay. The N_{ub} at the N-terminus of Pex12p seems to point into the lumen of the peroxisome, in accordance with Albertini et al. (Albertini et al., 2001), whereas the expected topology of Pex2p discouraged us from constructing the corresponding N_{ub}-Pex2p. The N_{ub} attached to the C-terminus of Pex12p did not produce any of the interactions found for Pex12-C_{ub}-RUra3p (Figs 2, 3). This observation confirms our previous experience that a N-terminal attachment to N_{ub} reduces the sensitivity of the split-Ub assay.

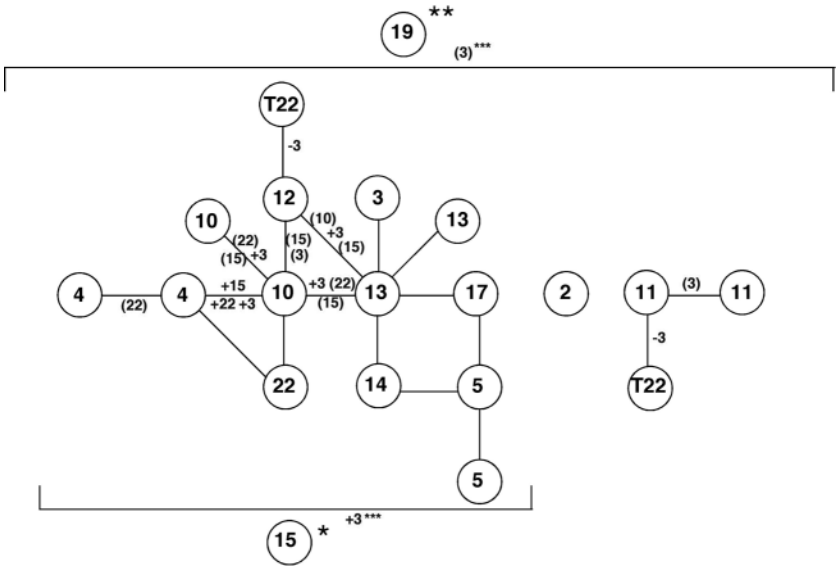

Fig. 8. Summary of the found peroxin interactions. The numbers represent the corresponding peroxins. T22 stands for Tom22p. The bars connecting the circles indicate interactions between the proteins in the split-Ub assay. A plus indicates an interaction that is detected only in the presence of the gene; a minus indicates an interaction that is only detected in the absence of the gene. The bars enclose the multiple interactions of a single peroxin. The numbers accompanying the bars specify the interactions in a certain *pex* deletion strain. Brackets enclosing a number indicate that the interaction occurs independently of the presence of the respective *PEX* gene. *Except Pex4p, Pex5p; **except Pex3p, Pex4p; ***measured only for Pex10p, Pex12p.

Network

Although this study focuses on the interaction of the RING-finger peroxins Pex12p, Pex10p and Pex2p, we constructed a network of interactions around those proteins that comprised most of the other members of the peroxins as well (Table 2, Fig. 8). In Fig. 8, the bars connecting the peroxins (only in numbers) highlight the interactions between the proteins in the split-Ub assay. The numbers accompanying the bars specify the interactions in a certain *PEX* deletion strain. A plus indicates an interaction that is dependent on the presence of a certain gene, whereas a minus indicates an interaction that is only detected in the absence of a certain gene. Brackets enclosing the number indicate that the interaction occurs independently of the presence of the respective *PEX* gene. The interaction of Pex10-C_{ub}-RUra3p and Pex12-C_{ub}-RUra3p with N_{ub}-Pex15p occurs only in the presence of Pex3p, whereas the interactions of Pex19p with Pex10p, Pex12p, Pex2p and Pex11p are not affected by a deletion of *PEX3*. Because the absence of Pex3p in the budding yeast is thought to result in a lack of recognizable peroxisomal membranes (see also Hazra et al., 2002), we argue that the interactions between Pex19p and the other peroxins are independent of the peroxisome and probably occur before the proteins are integrated into the membrane. Our results therefore confirm the proposed role of Pex19p in the recognition and insertion of peroxisomal membrane proteins (Sacksteder et al., 2000).

Pex15p plays an important, albeit enigmatic, role in the biogenesis of the peroxisomes and the import of protein cargo (Elgersma et al., 1997). The many interactions we observe for Pex15p merely underline, yet do not explain, this role (Figs 2, 4; Table 2). Interestingly, Pex11p and Pex2p do not interact with Pex15p in our assay, thereby confirming the isolated status of both proteins in our interaction matrix (Fig. 5). As for Pex19p, it is difficult to imagine how Pex15p can connect to seven proteins at a time without showing a spatial preference to any of them. It is therefore more conceivable to assume that these interactions do not occur in a single complex, but in either a spatially or temporally resolved manner. Pex15p as a peroxin-specific chaperone during disassembly or readjustments of the import-complex is one role that is compatible with the abundance of interactions seen in our assay. Other potential roles of Pex15p, such as serving as a scaffold protein for the members of the import complex, are also in accordance with the observed interaction profile. However, the dependence of the peroxin interactions on the presence of Pex15p is not general but seems to be quite specific for the Pex4p-Pex10p interaction (Fig. 8 and data not shown)

An interesting aspect of the interaction map concerns the increase in proximity between N_{ub}-Tom22p and Pex12-C_{ub}-RUra3p and Pex11-C_{ub}-RUra3p on deletion of *PEX3* (Fig. 7). This observation might hint at an alternative route to the mitochondrion that is taken by some of the peroxins in the absence of functional peroxisomes. This is not a yeast-specific phenomenon and has been shown to occur in mammalian cell lines lacking Pex19p (Sacksteder et al., 2000).

We could show for the first time that the yeast Pex10p and Pex5p multimerize in vivo (Fig. 4, Table 2). These interactions were already detected by biochemical means in other organisms and confirm the general conservation of many of the peroxin interactions (Schliebs et al., 1999). However, some of the interactions that were reported in the literature were not detected in our assay. An obvious explanation for missing some of those interactions is their steric incompatibility with the N- or C-terminally attached N_{ub} or C_{ub}-RUra3p. Other interactions might have gone undetected due to an insufficient sensitivity of the assay or due to their temporal nature. For example, the split-Ub assay does not reveal the interactions between Pex5p and Pex13p or Pex12p (Fig. 2, Table 2). Because Pex5p is thought to travel as a bearer of the cargo, some of its contacts are likely to be very short-lived. This argument might also explain the lack of detectable interactions between the different C_{ub}-RUra3p modified receptors and the PTS1-bearing N_{ub}-fusion (Figs 2-6, Table 2). In addition, some of the missed interactions might be caused by the non- or only partial functionality of the N_{ub}-/C_{ub}- constructs of the peroxins (Table 3). Again, a prominent example is the missed interaction between Pex13p and Pex5p. Both the N_{ub}- and the C_{ub}-constructs of Pex5p are nonfunctional, as is the C_{ub}-construct of Pex13p. As a general rule we therefore recommend the reader to compare the interaction data from Table 2 with the functionality assays in Table 3. Although a protein that is non-functional in the peroxisomal import might still properly intact with its binding partners, the non- or only partial functionality of a protein makes it more likely that some of its interactions might be missed in this assay. The presented network is therefore still rudimentary. Because this shortcoming is shared among the different available technologies to analyze protein complexes, a complete picture will only begin to emerge by integrating the information from the many sources available (Fransen et al., 2002; Gavin et al., 2002; Uetz et al., 2000).

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