Analysis of Mammalian Peroxin Interactions Using a Non-transcription-based Bacterial Two-hybrid Assay*

Marc Fransen‡§, Chantal Brees‡, Karen Ghys‡, Leen Amery‡¶, Guy P. Mannaerts‡, Daniel Ladant‖, and Paul P. Van Veldhoven‡**

In recent years, substantial progress has been made in the identification of proteins involved in peroxisome biogenesis. However, with the exception of the peroxisome-targeting signal receptors and the receptor docking proteins, the function of most of these proteins, called peroxins, remains largely unknown. One step toward elucidating the function of a protein is to identify its interacting partners. We have used a non-transcription-based bacterial two-hybrid system to analyze the interactions among a set of 12 mammalian peroxins and a yeast protein three-hybrid system to investigate whether proteins that interact with the same peroxin and have overlapping binding sites cooperate or compete for this site. Here we report a detailed interaction map of these peroxins and demonstrate that (i) farnesylation, and not the *CAAX* motif, of Pex19p strongly enhances its affinity for Pex13p; (ii) the *CAAX* motif, and not farnesylation, of Pex19p strongly enhances its affinity for Pex11pβ; and (iii) the C_3HC_4 RING (really interesting new gene) finger domain of Pex12p does not alter the binding properties of Pex5p for the C-terminal peroxisome-targeting signal PTS1. Finally, we show that the Pex5p-Pex13p interaction is bridged by Pex14p and that the latter molecule exists predominantly as a dimer *in vivo*. Collectively, as demonstrated in this work with peroxins, these results indicate that the bacterial two-hybrid system is an attractive complementary approach to the conventional transcription-based yeast two-hybrid system for studying protein-protein interactions. ***Molecular & Cellular Proteomics 1:243–252, 2002.***

The biogenesis of peroxisomes requires the concerted action of at least 23 peroxins (1) and one cytosolic, peroxisome-specific DnaJ-like protein (2). The best characterized peroxin is Pex5p, which functions as a cycling signal recognition factor for newly synthesized peroxisomal matrix proteins containing a C-terminal peroxisome-targeting signal (PTS1)[1] (3, 4). Peroxisomal matrix proteins containing an N-terminal peroxisome-targeting signal (PTS2) are recognized by Pex7p, another soluble PTS receptor (5). The PTS receptors dock at the peroxisome membrane via the peroxisomal membrane proteins Pex13p and Pex14p, which, together with Pex17p, constitute the receptor docking complex (6). In mammals, docking of Pex7p at the peroxisome membrane is mediated by the long isoform of Pex5p, Pex5pL (7). In *Saccharomyces cerevisiae*, the targeting of Pex7p to the peroxisome membrane requires two functionally related proteins, Pex18p and Pex21p (8). In *Yarrowia lipolytica*, Pex20p is also required for PTS2 protein import (9). Interestingly, Einwächter *et al*. (10) reported recently that this *Y. lipolytica* Pex20p can substitute functionally for *S. cerevisiae* Pex18p and Pex21p. Other peroxins directly implicated in the import pathway for peroxisomal matrix proteins are Pex1p, Pex2p, Pex4p, Pex6p, Pex8p, Pex9p, Pex10p, Pex12p, Pex15p, Pex22p, and Pex23p. Epistasis analyses suggest that Pex2p, Pex8p, Pex10p, and Pex12p act downstream of the receptor docking event and that Pex1p, Pex4p, Pex6p, and Pex22p function downstream of the matrix protein translocation process (11, 12). An alternative view proposes that Pex1p and Pex6p are involved in the fusion of small peroxisomal precursors (13, 14). Pex9p, Pex15p, and Pex23p have not yet been placed in any particular step of the matrix protein import pathway (12). Pex3p, Pex16p, and Pex19p are implicated in peroxisome membrane biogenesis. Cell lines deficient in these peroxins mislocalize peroxisomal membrane proteins and have no peroxisomal remnants (12). Another peroxin, Pex11p, promotes peroxisomal proliferation (15). However, van Roermund *et al.* (16)

From the ‡Departement Moleculaire Celbiologie, Afdeling Farmacologie, Katholieke Universiteit Leuven, Campus Gasthuisberg, Herestraat 49 (O/N), B-3000 Leuven, Belgium and ‖Département de Biochimie et Génétique Moleculaire, Institut Pasteur, Unité de Biochemie Cellulaire (CNRS URA 2185), 28 rue du Dr. Roux, 75724 Paris CEDEX 15, France

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[1] The abbreviations used are: PTS1, C-terminal peroxisomal targeting signal; PTS2, N-terminal peroxisomal targeting signal; AD, Gal4p activation domain; b-2HS, non-transcription-based bacterial two-hybrid system; BD, Gal4p DNA-binding domain; CHO, Chinese hamster ovary; DSP, dithiobis(succinimidyl propionate); HA, influenza virus hemagglutinin epitope tag; Pexp, peroxin, including a number corresponding to the order of discovery; SH3, Src homology 3; TBST, Tris-buffered saline containing 0.05% (v/v) Tween 20; T18, amino acids 225 to 399 of *B. pertussis* adenylate cyclase; T25, amino acids 1 to 224 of *B. pertussis* adenylate cyclase; y-2HS, transcription-based yeast two-hybrid system; y-3HS, transcription-based yeast protein three-hybrid system.

reported recently that in *S. cerevisiae* Pex11p plays a primary role in the medium-chain fatty acid oxidation pathway and suggested the alternative view that this pathway regulates the level of a signaling molecule that actually modulates peroxisome number and size.

Defining the physical interactions between peroxins is an essential step toward the understanding of how these proteins function and how proteins are translocated through the peroxisomal membrane. Currently, the best established strategy to find connections between peroxins is the transcription-based yeast two-hybrid system (y-2HS) (17). Strategies thus far used to identify or to confirm physical interactions between peroxins are co(immuno)precipitation assays, far western and surface plasmon resonance analyses, blue native polyacrylamide gel electrophoresis, a transcription-based mammalian two-hybrid assay, and the use of cleavable cross-linkers (5, 18–21).

Here we report a detailed interaction map of 12 mammalian peroxins that are available currently in our laboratory. This peroxin linkage map was obtained by using the classical y-2HS, as well as a non-transcription-based bacterial two-hybrid system (b-2HS). The latter system, developed by Ladant and co-workers (22), exploits the functional modularity of the catalytic domain of *Bordetella pertussis* adenylate cyclase. That is, proteins of interest are fused genetically to T18 and T25, two complementary fragments that are both essential for adenylate cyclase activity. If the corresponding fusion proteins interact, cAMP will be produced. In an *Escherichia coli* strain deficient in endogenous adenylate cyclase, this functional complementation can be monitored easily as several resident genes, such as the *LacZ* or *Mal* genes, are activated transcriptionally and give rise to a selectable phenotype. We show that the non-transcription-based b-2HS and the transcription-based y-2HS are complementary approaches and compare our data with existing published interactions. In addition, our results support the observation that farnesylation of Pex19p enhances strongly its affinity for Pex13p (23) and uncover the potential of human Pex14p to homodimerize *in vivo* and to bridge the Pex5p-Pex13p interaction *in vitro*. Finally, we have delineated the different protein interaction domains of Pex5p and Pex14p and show that Pex5p has partially overlapping but distinct binding sites for the C_3HC_4 RING finger domain of Pex12p and PTS1 molecules.

EXPERIMENTAL PROCEDURES

Strains and Cell Lines—The *E. coli* strain *Top10F′* (Invitrogen) was used for all DNA manipulations, as well as for the expression of recombinant proteins. The adenylate cyclase-deficient *E. coli* strain *BTH101* (Hybrigenics) was used for the bacterial two-hybrid assay. Yeast two-hybrid and protein three-hybrid assays were performed using the *S. cerevisiae* strain SFY526 (CLONTECH). Chinese hamster ovary (CHO) cells were cultured in MEMα medium (BioWhittaker) supplemented with 10% (v/v) fetal calf serum (Invitrogen), 2 mM GlutaMAX-I (Invitrogen), and antibiotic/antimycotic (100 μg/ml penicillin G, 100 μg/ml streptomycin sulfate, and 0.25 μg/ml amphotericin B) solution (Invitrogen) in a humidified 37 °C, 5% CO_2 incubator.

Plasmid Constructions—The cDNA fragments encoding the analyzed proteins were amplified by PCR with the appropriate primers (Invitrogen) and cloned into the yeast two-hybrid vectors pGBT9 or pGAD424 (CLONTECH), the yeast protein three-hybrid vector pBridge (CLONTECH), or the bacterial two-hybrid vectors pKT25 or pUT18C (Hybrigenics) using standard molecular biology procedures (24). PCRs were performed routinely using *Pfx* DNA polymerase (Invitrogen). To facilitate the cloning of cDNAs into the bacterial two-hybrid vectors, the multiple cloning sites of pKT25 and pUT18C were modified. For pKT25, the *Pst*I-*Bam*HI fragment, which contains restriction sites for *Sal*I and *Xba*I, was replaced by a DNA cassette (primer pair 5′-GGCTCGAGGCGGCCGCGG-3′/5′-GATCCCGCGGCCGCCTCGAGCCTGCA-3′) containing unique restriction sites for *Xho*I and *Not*I. For pUT18C, the *Bam*HI-*EcoRI fragment*, which contains restriction sites for *Sma*I, *Kpn*I, and *Sac*I, was replaced by a DNA cassette (primer pair 5′-GATCCGAATCCGGTACCGCGGCCGC-3′/5′-AATTGCGGCCGCGGTACCGGATTCG-3′) containing unique restriction sites for *Kpn*I and *Not*I. The corresponding derivatives of pKT25 and pUT18C were termed pMF413 and pMF424, respectively. The detailed cloning procedures (including the list of oligonucleotides) of the extensive number of other constructs can be obtained from the corresponding authors. The identities of essential constructs were confirmed by DNA sequencing.

Two-hybrid and Protein Three-hybrid Analyses—The cotransformation of plasmids in competent SFY526 yeast cells and the colony lift β-galactosidase filter assay were performed as described by the manufacturer (CLONTECH). The interactions were indicated as strong (+) or weak (±), depending on the colony staining times (<5 and <24 h, respectively). The liquid culture β-galactosidase assay with *o*-nitrophenyl-β-D-galactopyranoside as substrate was also performed as described by the manufacturer (CLONTECH). However, for this assay the yeast cells were grown for 72 h in minimal dropout medium without leucine and tryptophan (y-2HS) or in minimal dropout medium without leucine and tryptophan in the absence or presence of 1 mM methionine (y-3HS). Bacterial two-hybrid assays were performed as described (25) using the *E. coli* strain *BTH101* (Hybrigenics). Transformed bacterial cells were plated on indicator plates (either on MacConkey medium (Difco Laboratories) supplemented with maltose (Acros Organics) or on Luria Bertani medium supplemented with 40 μg/ml X-gal (MBI Fermentas) and 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (MBI Fermentas)) containing 50 μg/ml ampicillin (Roche Molecular Biochemicals) and 25 μg/ml kanamycin (Fluka). The interactions were indicated as strong (+) or weak (±) depending on the colony staining times (<28 and <52 h, respectively). Quantification of the functional complementation mediated by interaction between two proteins was done by measuring β-galactosidase activities in liquid cultures (25).

Cross-linking Experiments—90% confluent CHO cells, grown in 100-mm tissue culture dishes (Sarstedt), were washed twice with 10 ml of phosphate-buffered saline. One ml of phosphate-buffered saline, supplemented with 50 μl of Me_2SO containing different concentrations of dithiobis(succinimidyl propionate (DSP; Perbio)) (0 to 2.5 mM final concentration) was added to each plate. After incubation for 60 min at room temperature, the cells were harvested by scraping firmly with a stiff rubber policeman. The cell suspensions were transferred to a microfuge tube, and the proteins were precipitated by the addition of 100 μl of 0.15% (w/v) deoxycholate and 100 μl of 72% (v/v) trichloroacetic acid. After washing the pellets once with 1 ml of acetone, pellets were resuspended in 300 μl of sample buffer (3% (w/v) SDS, 8.7% (v/v) glycerol, 0.004% (w/v) bromphenol blue, and 60 mM Tris/HCl, pH 6.8) in the absence or presence of 5% (v/v) 2-mercaptoethanol. The samples were boiled for 5 min, subjected to SDS-

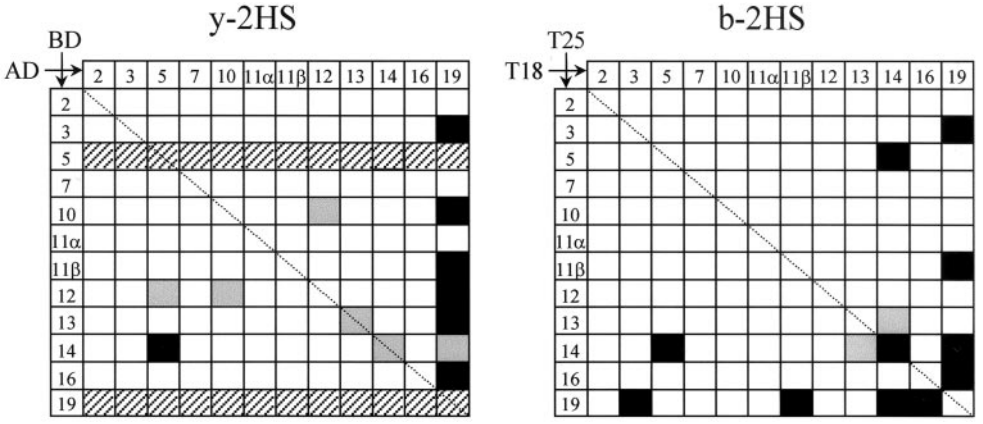

FIG. 1. **Peroxin interaction matrix.** Peroxins, indicated by their corresponding Pexp numbers, were fused to the Gal4p DNA-BD (y-2HS) or T25 (b-2HS) and the Gal4p transcription AD (y-2HS) or T18 (b-2HS) and tested pairwise for interactions in *S. cerevisiae* (strain SFY526) (y-2HS) or *E. coli* (strain *BTH101*) (b-2HS). Double transformants expressing one of the bait (BD and T25, respectively) and one of the prey (AD and T18, respectively) fusion proteins were selected, and β-galactosidase activity was monitored (using the colony staining assay) as described under "Experimental Procedures." The identified interactions between full-length peroxins are *shaded* in *black*. Interactions observed with the peroxin fragments $Pex12p_{(275-359)}$, $Pex13p_{(236-403)}$, $Pex14p_{(1-81)}$ (Pex19p interaction), and $Pex14p_{(138-200)}$ (Pex14p interaction), but not with the corresponding full-length peroxins, are *colored* in *gray*. Conditions that, because of self-activation of the bait protein, could not be tested are *hatched*. Notice that the Pex5p interactions represent the results obtained with both the small (Pex5pS) and long (Pex5pL) isoforms.

PAGE (ExcelGel SDS (gradient 8–18); Amersham Biosciences), transferred to nitrocellulose, and analyzed with the appropriate antisera.

In Vitro Binding Assay—For the microtiter plate assay, 1 μg of human serum albumin cross-linked to the peptide CSYHKHLKPLQSKL (PTS1) (26) was coated (50 mM sodium carbonate buffer, pH 9.6) overnight onto a microtiter plate. After washing the wells five times with TBST, the wells were blocked with 5% (w/v) Protifar (Nutricia) in TBST for 1 h. Subsequently, each well was incubated with 200 μl of bacterial lysate, prepared as described before (27), containing no recombinant protein, $(His)_6$-Pex5p, $(His)_6$-Pex14p, or $(His)_6$-Pex13p/SH3 (18), as schematically indicated in Table II. After extensive washings with TBST, bound Pex5p, Pex14p, and Pex13p were detected photometrically using peroxin-specific rabbit antisera, anti-rabbit IgGs coupled to alkaline phosphatase, and *p*-nitrophenyl phosphate. The optical densities were measured at 405 nm (filter: 690 nm). Data were corrected by performing simultaneously control experiments using 1 μg of human serum albumin cross-linked to the peptide CVHESYHKHLKPLQ (26) as immobilized ligand.

Antibodies—The rabbit polyclonal antibodies raised against $(His)_6$-Pex13p/SH3, $(His)_6$-Pex14p, and $(His)_6$-Pex19p are described elsewhere (23). The mouse monoclonal anti-HA antibody was obtained from Sigma. The rabbit polyclonal anti-mouse Pex5p antiserum was kindly provided by Dr. M. Baes (Leuven, Belgium). Animal experiments were approved by the local institutional ethics committee.

RESULTS

Analysis of Interactions between Full-length Peroxins Using the Gal4p-based Yeast Two-hybrid System—As a first step toward the development of a reliable peroxin network, we performed a matrix experiment (28) in which 12 full-length mammalian peroxins were tested systematically for pairwise interactions in the Gal4p-based yeast two-hybrid system (Fig. 1). Because the BD-Pex5p and BD-Pex19p fusion proteins self-activated the *LacZ* reporter gene, 24 pairwise combinations were not tested. Of the remaining 120 combinations, seven protein pairs were found to interact; the PTS1 receptor, Pex5p, interacts with its docking protein, Pex14p, whereas Pex19p, a predominantly cytosolic protein that contains a C*AAX* farnesylation consensus sequence, binds the peroxisomal integral membrane proteins Pex3p, Pex10p, Pex11pβ, Pex12p, Pex13p, and Pex16p. These results confirm previous observations that these protein pairs interact in the y-2HS (23, 29–31). However, six other interactions (Pex5p-Pex5p, Pex7p-Pex5pL, Pex12-Pex5p, Pex12p-Pex10p, Pex14p-Pex14p, and Pex14p-Pex19p) were not found. These peroxin pairs were demonstrated previously to interact using other techniques including blot overlay assays, sizing chromatography, electron microscopy, and immunoprecipitation assays (7, 20, 29, 32, 33).

Analysis of Interactions between Full-length Peroxins Using a Non-transcription-based Bacterial Two-hybrid System—Because two peroxins self-activated the *LacZ* reporter gene, and more than 40% of the previously reported interactions between full-length peroxins were not detected in the y-2HS, we changed our strategy and performed a new matrix experiment using a non-transcription-based b-2HS (22). In this system, which exploits a cAMP signaling cascade in *E. coli cya* cells (*i.e.* deficient in endogenous adenylate cyclase), none of the 12 peroxins autoactivated the *lacZ* or *Mal* reporter genes, and six peroxin pairs interacted efficiently and specifically in reciprocal combinations of the two-hybrid moieties (Fig. 1). Four of these interactions (Pex14p-Pex5p, Pex3p-Pex19p, Pe11pβ-Pex19p, and Pex16p-Pex19p) were also detected in the y-2HS. The two remaining interactions, Pex14p-Pex14p and Pex14p-Pex19p, were not found in the y-2HS. The fact that Pex14p and Pex19p bind other peroxins in the y-2HS (Fig. 1) largely eliminates the possibility that these peroxins are expressed poorly or fail to be targeted to the yeast nucleus. However, it may be that, because of a different folding

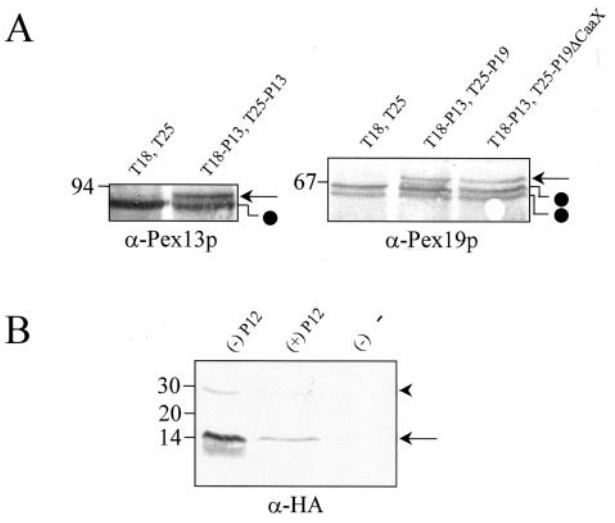

FIG. 2. **Expression levels of recombinant peroxins in b-2HS and y-3HS.** Equal amounts of bacterial cells (strain *BTH101*) (*panel A*) expressing T18 or T18-Pex13p (*T18-P13*) and T25, T25-Pex13p (*T25-P13*), T25-Pex19p (*T25-P19*), or T25-Pex19pΔC*AAX* (*T25-P19ΔCaaX*) or yeast cells (strain SFY526) (*panel B*) expressing a Gal4p DNA-binding domain-PTS1 fusion protein, a Gal4p activation domain-Pex5p fusion protein, and an HA-tagged $Pex12p_{(275–359)}$ fusion protein (*P12*) or no HA-tagged-fusion protein (−), grown in the absence (−) or presence (+) of 1 mM methionine, were subjected to SDS-PAGE and transferred to nitrocellulose. These membranes were then probed with an antiserum raised against $(His)_6$-$HsPex13p_{(236–403)}$, $(His)_6$-HsPex19p, or the HA-tag. Only the relevant portions of the blots are shown. The *arrows* indicate the full-length recombinant expressed fusion proteins. The *dots* indicate the migration of nonspecific, immunoreactive bacterial proteins. The *arrowhead* marks a putative (unreduced) dimer of the HA-tagged $Pex12p_{(275–359)}$ fusion protein. The migration of the molecular mass markers (in kilodaltons) is shown at the *left*.

environment, distinct domains of human Pex14p are exposed in *S. cerevisiae versus E. coli*. Three interactions (Pex10p-Pex19p, Pex12p-Pex19p, and Pex13p-Pex19p) found in the y-2HS were not detected in the b-2HS. Again, this may be related to a different folding environment or to expression problems. Unfortunately, as we currently lack specific antibodies to T18, T25, Pex10p, and Pex12p, we were unable to verify the expression levels of Pex10p and Pex12p. Consequently, we have no conclusive evidence whether the Pex19p-Pex10p and the Pex19p-Pex12p interactions occur in the b-THS. However, using an antiserum raised against $(His)_6$-HsPex13p/SH3 (23), we were able to demonstrate that Pex13p was expressed correctly (Fig. 2). Therefore, in combination with the observation that the C*AAX* prenylation motif of Pex19p strongly enhances its affinity for Pex13p (23), we hypothesize that the observed lack of interaction between these two peroxins is caused by the fact that Pex19p is not prenylated in *E. coli*. Interestingly, $Pex13p_{V164E}$, a mutant displaying a 20-fold enhanced affinity for Pex19p over the wild-type molecule in the y-2HS (23), does display a detectable binding to Pex19p in the b-2HS (Table I). Concerning the

TABLE I
Pex19p and Pex19pΔCaaX have different binding properties in the b-2HS

Exponentially growing bacterial cells (strain HB101), transformed with plasmids encoding one of the indicated T18 fusion proteins (T18-hybrid) and T25 fused to either Pex19p or Pex19pΔCaaX, were assayed for β-galactosidase using *o*-nitrophenyl-β-D-galactopyranoside as substrate (46). Formed *o*-nitrophenol was measured at 420 nm and normalized for culture densities (optical density at 600 nm = 1) and time (1 h). The values given are the mean (± the S.D.) of at least three measurements performed on independent single colonies and corrected for the blank (T18 and T25 only, 3.3 ± 0.2). When the corresponding double transformants where grown on MacConkey/maltose agar plates, they appeared as white (not underlined) or red (underlined) colonies. $Pex13p_{V164E}$ represents a Pex13p mutant in which the valine amino acid residue at position 164 is altered to glutamate (23).

T18 hybrid	Optical density	
	T25-Pex19p	T25-Pex19pΔCaaX
Pex3p	111 ± 18	117 ± 29
Pex10p	0.8 ± 0.6	0.6 ± 0.2
Pex11pβ	59 ± 10	8.0 ± 1.7
Pex12p	1.1 ± 0.8	1.4 ± 0.4
Pex13p	1.1 ± 0.3	0.6 ± 0.6
$Pex13p_{V164E}$	18 ± 5	15 ± 8
Pex14p	31 ± 9	39 ± 5
Pex16p	33 ± 0.3	35 ± 6

Pex19p-Pex11pβ interaction, we found that the C*AAX* motif, and not prenylation, is an important determinant in the affinity of Pex19p for Pex11pβ. Indeed, Pex19p and Pex11pβ were found to interact in the y-2HS and in the b-2HS, and deleting the prenylation motif from Pex19p (Pex19pΔC*AAX*) resulted, in both systems, in a strongly reduced binding affinity for Pex11pβ (see Ref. 23 and Table I). In this context, it is important to mention that Pex19p and Pex19pΔC*AAX* are expressed similarly (Fig. 2) and display equivalent affinities for Pex3p, Pex14p, and Pex16p (Table I).

Two-hybrid Analysis of Interactions between Peroxin Domains—Although the systematic two-hybrid analysis of 12 full-length mammalian peroxins recapitulated ~70% of the interactions known from previous studies, a substantial number of reported interactions between full-length peroxins (Pex5p-Pex5p, Pex5pL-Pex7p, Pex5p-Pex12p, and Pex10p-Pex12p) could not be detected. However, as the expression of full-length mammalian peroxins in yeast and bacteria may result in variable misfolding, we investigated whether additional interactions could be detected using peroxin domains instead of full-length open reading frames. Using this approach, we were able to confirm that the C_3HC_4 domain of Pex12p, but not the full-length molecule, interacts with Pex5p and Pex10p in the y-2HS (29, 31, 34) (Fig. 1). Evidence for the Pex5p-Pex5p (20, 35) and Pex5pL-Pex7p (7) interactions could not be found. However, using the domain approach, we could confirm that the SH3 domain of Pex13p binds to Pex14p (Fig. 1), an interaction observed previously in a blot overlay assay (18). Interestingly, this domain of Pex13p was

also found to interact weakly with itself, at least in the y-2HS (Fig. 1). Finally, after using the b-2HS to delineate the domains of Pex14p responsible for homodimerization and Pex19p binding (see below), we could show that the domains comprising amino acids 138–200 and 1–81, but not the full-length Pex14p molecule, also interact with Pex14p and Pex19p, respectively, in the y-2HS (Fig. 1).

Pex14p Exists Predominantly as a Dimer in Vivo—Based on (i) our two-hybrid results that suggest that Pex14p, but not Pex5p, may homodimerize *in vivo*; and (ii) other reports suggesting that Pex5p functions as a monomeric (36), a homodimeric (35) or a homotetrameric protein (20), we further investigated the actual oligomeric state of these peroxins in mammalian cells. To stabilize protein-protein interactions, we performed chemical cross-linking experiments on CHO cells using the thiol-cleavable, homobifunctional, and amine-reactive cross-linker DSP. Immunoblot analysis of the cross-linked products after SDS-PAGE of the samples revealed that Pex14p, but not Pex5p, exists mainly as a multimeric complex *in vivo* (Fig. 3). Several lines of evidence suggest that this complex is in fact a homodimer of Pex14p. (i) Pex14p can homodimerize when it is expressed in *E. coli* (Fig. 1); (ii) the cross-linked Pex14p-containing complex of CHO cells migrates during SDS-PAGE to a similar distance as the dimerized fraction of bacterially expressed $(His)_6$-Pex14p (data not shown); and (iii) the cross-linked Pex14p-containing complex is not recognized by antisera raised against the Pex14p-interacting peroxins Pex5p, Pex13p, and Pex19p (data not shown). Interestingly, even in the absence of cross-linker a small but significant fraction of Pex14p migrated as a dimer on SDS-PAGE under non-reducing conditions (Fig. 3). However, deletion analysis studies revealed that the dimeric Pex14p structure is not maintained by disulfide bonding between the subunits but by its coiled-coil structure (see below). The ease of disulfide bridge formation and its specificity support further the view of a very close, non-covalent homotypic interaction *in vivo* (37). Whether dimerization mutants of Pex14p (i) have an altered affinity for the other interacting peroxins, and (ii) are able to complement peroxisome biogenesis in a cell line deficient in Pex14p remains to be investigated.

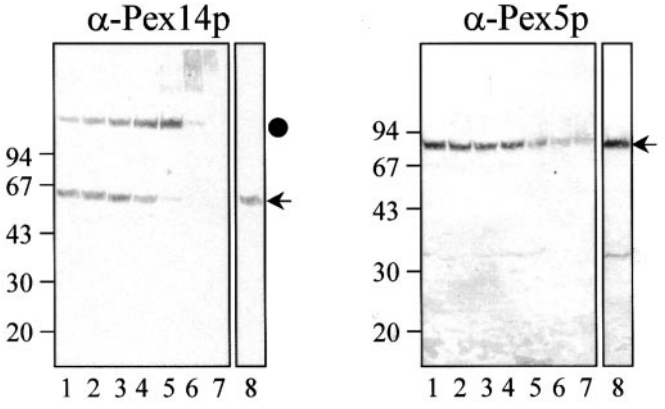

FIG. 3. **Cross-linking and immunoblot analysis of Pex5p and Pex14p in CHO cells.** CHO cells were incubated with various concentrations of DSP as described under "Experimental Procedures." After 60 min, the incubations were terminated with trichloroacetic acid, and the precipitated proteins were separated by non-reducing (*lanes 1–7*) or reducing (*lane 8*) SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antisera raised against Pex14p or Pex5p. *Lanes 1* and *8*, 0 μM DSP; *lane 2*, 10 μM DSP; *lane 3*, 31 μM DSP; *lane 4*, 92 μM DSP; *lane 5*, 277 μM DSP; *lane 6*, 833 μM DSP; and *lane 7*, 2.5 mM DSP. *Arrows* point to the position of the non-cross-linked peroxins. The Pex14p-containing multimeric complex is marked by a *dot*. The migration of the molecular mass markers (expressed in kDa) is indicated.

The Pex5p-Pex13p Interaction Is Bridged by Pex14p—In view of published reports that the SH3 domain of yeast Pex13p functions as a peroxisomal membrane receptor for Pex5p (38–40), our inability to detect an interaction between the corresponding human orthologues in the bacterial and yeast two-hybrid systems may seem surprising (Fig. 1). However, *in vitro* reconstitution experiments of a putative peroxisomal protein import complex consisting of PTS1 ligand, Pex5p, Pex14p, and the SH3 domain of Pex13p (amino acids 236 to 403) demonstrated that Pex14p, but not the SH3 domain of Pex13p, interacts with Pex5p loaded with PTS1 cargo (Table II). In addition, these experiments show that Pex14p can bind simultaneously to Pex5p and Pex13p/SH3

TABLE II

In vitro reconstitution of a Pex5p-containing peroxisomal protein import subcomplex

Microtiter plate wells, coated with 1 μg of human serum albumin cross-linked to the peptide CSYHKHLKPLQSKL (PTS1), were exposed in three subsequent incubations to bacterial lysates containing no recombinant protein (−), $(His)_6$-Pex5p, $(His)_6$-Pex14p, and $(His)_6$-Pex13p/SH3 as indicated schematically. After extensive washings, bound Pex5p, Pex14p, and Pex13p were detected photometrically using peroxin-specific rabbit antisera and anti-rabbit IgGs coupled to alkaline phosphatase and *p*-nitrophenyl phosphate. To substract the background of the anti-Pex13p antiserum due to a weak cross-reactivity with human serum albumin, data were corrected by performing simultaneously control experiments using one microgram of human serum albumin cross-linked to the peptide CVHESYHKHLKPLQ as immobilized ligand. The experiments were performed in triplicate. The positive interactions are underlined.

PTS1	1	2	3	$A_{405\ nm}$		
	Pex5p	Pex14p	Pex13p/SH3	α-Pex5p	α-Pex14p	α-Pex13p
+	−	+	+	0.000 ± 0.000	0.026 ± 0.015	0.035 ± 0.030
+	+	−	+	0.698 ± 0.032	0.053 ± 0.005	0.014 ± 0.012
+	+	+	−	0.471 ± 0.008	0.584 ± 0.031	0.010 ± 0.014
+	+	+	+	0.440 ± 0.015	0.537 ± 0.015	0.122 ± 0.055

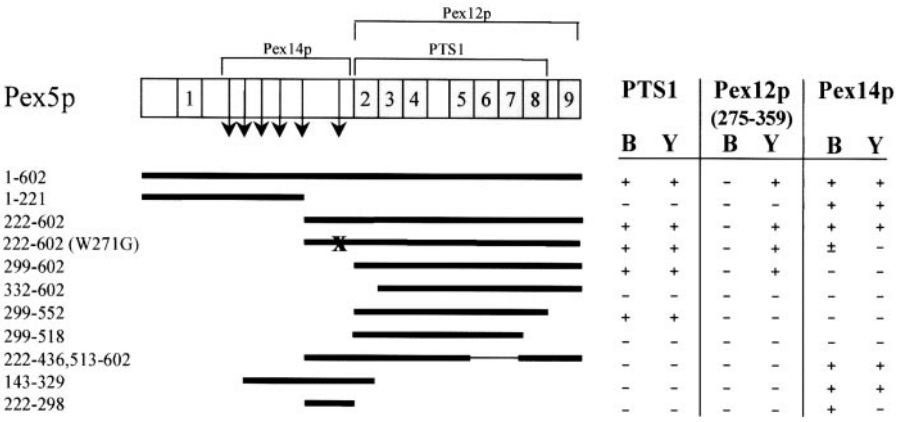

FIG. 4. **Mapping of the interaction domains of Pex5p.** Pex5p deletion mutants, fused to the Gal4p AD (y-2HS) or T18 (b-2HS), were tested for interaction with PTS1, Pex12p$_{(275–359)}$, and Pex14p, fused to the Gal4p DNA-BD (y-2HS) or T25 (b-2HS), in the yeast (y) or bacterial (b) two-hybrid system. Double transformants expressing one of the bait (BD and T25, respectively) and one of the prey (AD and T18, respectively) fusion proteins were selected, and β-galactosidase activity was monitored as described under "Experimental Procedures." The binding affinities of the tested protein pairs are indicated as follows: strong interaction (+), weak interaction (±), or no interaction (−). The *numbers* on the *left* indicate the amino acid residues present in the corresponding Pex5p deletion mutants, and *X* represents the alteration of the tryptophan residue to glycine at position 271. The tetratricopeptide repeat motifs are *shaded* in *gray*, whereas the W*XXX*(F/Y) repeats are indicated by *vertical arrows*. The delineated interaction domains are indicated *above* the schematic representation of the full-length Pex5p molecule.

(Table II). Summarized, these results suggest that in mammals (i) Pex14p, and not Pex13p, functions as the docking protein for cargo-loaded Pex5p, and (ii) the Pex5p-Pex13p interaction is bridged by Pex14p (Table II).

Mapping the Interaction Domains of Pex5p and Pex14p—Pex5p and Pex14p interact with each other and a number of other proteins. We employed the yeast and bacterial two-hybrid assays to further delineate the specific domains involved in the binding of these peroxins to their respective partners. For Pex5p, we mapped the site required for PTS1 and Pex12p interaction to the tetratricopeptide repeat domains labeled 2–8 and 2–9, respectively (Fig. 4). In addition, we found that Pex5p has multiple binding sites for Pex14p, and these binding sites are localized in the N-terminal portion of the molecule (Fig. 4). Similar observations were published by Schliebs *et al.* (20). In addition, these authors proposed that the di-aromatic W*XXX*(F/Y) pentapeptide repeat motifs in the N-terminal portion of Pex5p are the determinants for the interaction with Pex14p. To verify this hypothesis, we altered in the deletion mutant Pex5p$_{(222–602)}$, which still interacts with Pex14p while containing only one di-aromatic pentapeptide motif, the amino acid residue tryptophan to glycine at position 271. The observation that the binding affinity of Pex5p$_{(222–602)(W271G)}$ for Pex14p, but not for PTS1 and Pex12p$_{(275–359)}$, decreases nearly to background levels (Table III), confirms that the di-aromatic peptide repeats are indeed important for Pex14p binding. With respect to Pex14p, we have conducted a series of experiments to map the Pex5p-binding site, the Pex19p-binding site, and the domain that is capable of self-association (Fig. 5). Surprisingly, the Pex14p-binding sites for Pex5p and Pex19p could not be separated physically and encompass the amino acids 22 to 81 (Fig. 5). The putative coiled-coil region was sufficient and required for homodimerization (Fig. 5).

TABLE III

The WXX(F/Y) peptide repeats of Pex5p are important determinants for Pex14p binding

Exponentially growing cells (yeast strain SFY526), transformed with plasmids encoding one of the bait proteins (PTS1, Pex12p$_{(275–359)}$, or Pex14p, fused to the Gal4p DNA-binding domain) and one of the prey proteins (Pex5p$_{(222–602)}$, Pex5p$_{(222–602)(W271G)}$, or Pex5p$_{(332–602)}$, fused to the Gal4p activation domain), were assayed for β-galactosidase using *o*-nitrophenyl-β-D-galactopyranoside as substrate (see "Experimental Procedures"). The optical densities were measured at 420 nm, corrected for the blank ("empty" plasmids), and normalized for culture densities (optical density at 600 nm = 10) and time (24 h). The values given are the mean (± the S.D.) of at least three measurements performed on cultures derived from independent single yeast colonies.

Bait	Optical density	
	Pex5p$_{(222–602)}$	Pex5p$_{(222–602)(W271G)}$
PTS1	672 ± 261	478 ± 38
Pex12p$_{(275–359)}$	82.4 ± 11.5	79.2 ± 21.4
Pex14p	8.56 ± 2.28	1.73 ± 0.75

The C_3HC_4 RING Finger Domain of Pex12p Has No Regulatory Role in the PTS1 Binding Properties of Pex5p—The fact that (i) the Pex12p$_{(275–359)}$-binding domain and the PTS1-binding domain of Pex5p are overlapping (Fig. 4), and (ii) Pex12p acts downstream of the Pex5p-receptor docking event (11), suggests that Pex12p may have a regulatory role in the PTS1 binding properties of Pex5p. To investigate whether this is indeed the case, we employed y-3HS. In this system, which can be seen as an extension of the y-2HS, the effect of a third protein (in our case HA-tagged Pex12p$_{(275–359)}$ or HA-tagged PTS1) on the binding properties of two other (interacting) proteins (in our case the Gal4p DNA-BD-PTS1 fusion protein and the Gal4p activation domain (AD)-Pex5p

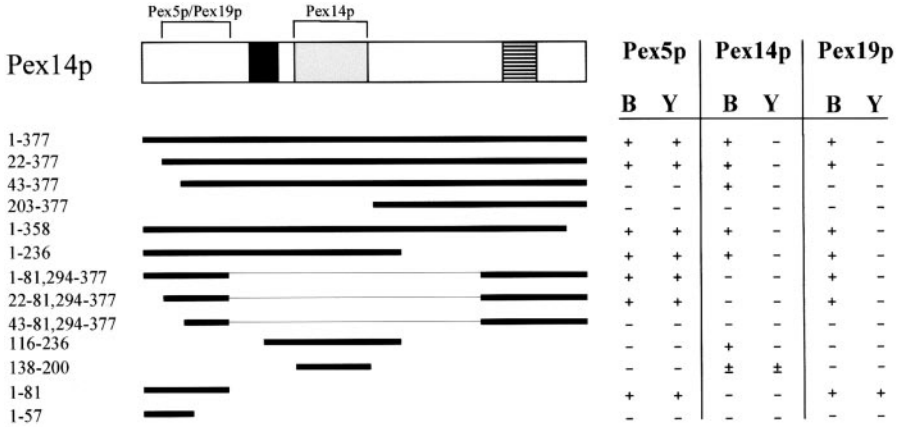

FIG. 5. **Mapping of the interaction domains of Pex14p.** Pex14p deletion mutants, fused to the Gal4p DNA-BD (y-2HS) or T18 (b-2HS), were tested for interaction with Pex5p, Pex14p, and Pex19p, fused to the Gal4p AD (y-2HS) or T25 (b-2HS), in the yeast (*Y*) or bacterial (*B*) two-hybrid system. Double transformants expressing one of the bait (BD and T25, respectively) and one of the prey (AD and T18, respectively) fusion proteins were selected, and β-galactosidase activity was monitored as described under "Experimental Procedures." The binding affinities of the tested protein pairs are indicated as follows: strong interaction (+), weak interaction (±), or no interaction (−). The *numbers* on the *left* indicate the amino acid residues present in the corresponding Pex14p deletion mutants. The hydrophobic region and the putative coiled-coil domain are *shaded* in *black* and in *gray*, respectively. The region rich in glutamate and aspartate residues is *hatched* with *horizontal lines*. The delineated interaction domains are indicated *above* the schematic representation of the full-length Pex14p molecule.

fusion protein) can be investigated. Notice that in the y-3HS we used, the expression levels of the third protein could be regulated by the conditional *Met25* methionine promoter (41). As shown in Table IV, the PTS1 binding properties of Pex5p were not altered when the HA-tagged Pex12p$_{(275–359)}$ molecule was expressed at different levels (Fig. 2). On the other hand, HA-tagged PTS1 did compete with BD-PTS1 for Pex5p binding (Table IV). These results, combined with the observation that Pex12p$_{(275–359)}$ interacts with Pex5p in the y-2HS (see Refs. 29 and 34, and see Fig. 4), suggest that Pex5p has distinct binding sites for the C_3HC_4 RING finger domain of Pex12p and PTS1 proteins and that Pex5p and Pex5p-Pex12p$_{(275–359)}$ have similar PTS1 binding properties.

TABLE IV

Pex5p has distinct binding sites for the C_3HC_4 RING finger domain of Pex12p and PTS1 proteins.

Yeast cells (strain SFY526), transformed with plasmids coding for a Gal4p DNA-binding domain-PTS1 fusion protein, a Gal4p activation domain-Pex5p fusion protein, and a third (conditionally expressed from the *Met25* promotor) HA-tagged fusion protein (PTS1 or Pex12p$_{(275–359)}$, were grown for 72 h in minimal dropout medium without leucine and tryptophan, in the absence (−) or presence (+) or 1 nM methionine. The cells were assayed for β-galactosidase using *o*-nitrophenyl-β-D-galactopyranoside as the substrate (see "Experimental Procedures"). The optical densities were measured at 420 nm and normalized for culture densities (optical density at 600 nm = 10) and time (24 h). The values given are the mean (± the S.D.) of three measurements performed on each of four separate transformants. Notice that the HA-tagged fusion protein is repressed in the presence of 1 mM methionine and expressed in the absence of methionine.

	Optical density	
	+ Methionine	− Methionine
HA-PTS1	600 ± 280	120 ± 22
HA-Pex12p$_{(275–359)}$	532 ± 165	596 ± 223

DISCUSSION

To decipher the peroxisomal protein import process, it is essential to identify the physical interactions between peroxins. Currently, the y-2HS is the most commonly used tool to dissect these interactions. However, as this assay may yield false positive results (42–44) and false negative results (44, 45), it is important that identified interactions (or non-interactions) are confirmed by at least one alternative, non-yeast-based assay. Techniques used frequently to confirm yeast two-hybrid interactions include blot overlay assays, sizing chromatography, and co(immuno)precipitation assays. However, these *in vitro* assays are often laborious, require optimized experimental conditions, and sometimes require specialized reagents (*e.g.* purified proteins, ^{35}S-labeled proteins, or highly specific antibodies of sufficient titer). As an alternative to these *in vitro* assays, the b-2HS was evaluated (22, 46). This *in vivo* interaction system differs basically from the y-2HS in that (i) a bacterial cell, and not a yeast cell, is used as host organism, and (ii) it is based on the reconstitution of a cAMP-dependent signal transduction pathway and not on the reconstitution of a functional transcriptional activator. Hence, there is no need for nuclear localization signals on the fusion proteins.

As a first step to validate the b-2HS, we performed a matrix experiment in which 12 full-length mammalian peroxins were tested systematically for pairwise interactions and compared these results with data obtained from similar yeast two-hybrid experiments. This validation study yielded 9 different interacting peroxin pairs; four pairs (Pex14p-Pex5p, Pex19p-Pex3p, Pex19p-Pex16p, and Pex19p-Pex11pβ) were found to interact in both two-hybrid systems, three pairs (Pex19p-Pex10p, Pex19p-Pex12p, and Pex19p-Pex13p) were only detected in

the y-2HS, and two pairs (Pex14p-Pex14p and Pex14p-Pex19p) interacted solely in the b-2HS. Presumably, peroxin interactions observed in both two-hybrid systems are likely to have a physiologically relevant function. Indeed, it has been reported that Pex14p functions as a Pex5p-docking protein (18, 19, 30) and that Pex19p, a molecule required for peroxisome membrane synthesis, binds to a broad spectrum of peroxisomal membrane proteins (12). With respect to the interactions that were observed only in the y-2HS, it is essential to notice that it has been reported that the C*AAX* prenylation motif of Pex19p is an important determinant in the affinity of Pex19p for Pex10p, Pex11pβ, Pex12p, and Pex13p (23). In combination with the fact that protein prenylation is a typical eukaryotic post-translational modification process (47), our data suggest that prenylation, and not the C*AAX* motif itself, is an important determinant in the affinity of Pex19p for Pex10p, Pex12p, and Pex13p. However, with respect to the Pex19p-Pex11pβ interaction, we provide evidence that the C*AAX* motif, and not prenylation, of Pex19p seems to be important. Whether the C*AAX* tetrapeptide is required directly for binding to Pex11pβ, or whether the corresponding deletion changes the folding of Pex19p in such a manner that binding to distinct sites is affected, is not currently clear. With respect to the interactions that were only detected in the b-2HS, it has to be mentioned that, depending on the host organism, full-length proteins might prevent the identification of several interactions because of various intrinsic folding problems (45, 48). For example, the finding that the coiled-coil structure, but not the full-length version, of Pex14p interacts with itself in the y-2HS supports this hypothesis.

Protein subdomains may be revealed by conformational changes (*e.g.* caused by post-translational modifications and/or interactions with other molecules) in the holomolecule. Furthermore, these newly exposed domains may mediate interactions not necessarily found with the parent protein. As an example of this phenomenon, we tested systematically Pex12p$_{(275–359)}$ and Pex13p$_{(236–403)}$ to uncover additional interactions. Importantly, this approach yielded one novel (Pex13p$_{(236–403)}$-Pex13p) and three known (Pex5p-Pex12p$_{(275–359)}$, Pex10p-Pex12p$_{(275–359)}$, and Pex13p$_{(236–403)}$-Pex14p) peroxin pairs (18, 29, 34).

Two reported peroxin pairs, Pex5p-Pex5p (20, 35) and Pex5pL-Pex7p (35), could not be detected in the yeast and bacterial two-hybrid systems. However, we have currently indirect evidence that human Pex7p is not properly folded when it is expressed in yeast or bacteria.[2] When expressed in a mammalian expression system, Pex7p does interact with Pex5pL (49).

In another series of experiments, we employed the bacterial and yeast two-hybrid assays to delineate the specific interaction domains of Pex5p and Pex14p that are involved in the binding to their respective partners. With respect to Pex5p, we obtained experimental evidence for the hypothesis that the W*XXX*(F/Y) repeat motifs of this molecule are important determinants for Pex14p binding (20), and showed that the Pex12p-binding domain and the PTS1-binding domain overlap physically. While this work was in preparation, similar conclusions were reached by Dodt *et al*. (49) and Saidowsky *et al.* (50). However, by using a y-3HS, we could also show that PTS1 ligands and Pex12p$_{(275–359)}$ do not compete for the same binding site on Pex5p. With respect to Pex14p, we also found that the Pex14p-binding sites for Pex5p and Pex19p could not be separated physically. Unfortunately, as Pex19p did not interact with Pex14p in the y-2HS, we were not able to perform a yeast protein three-hybrid assay to investigate whether Pex19p is able to alter the Pex5p binding properties of Pex14p.

The experiments described above demonstrate that the bacterial and yeast two-hybrid systems are complementary approaches for the analysis of protein interactions. In addition, the b-2HS has, compared with the y-2HS, a lower false positive rate. That is, acidic proteins with amphipathic α-helices like Pex5p and Pex19p, which in the y-2HS act frequently as transcriptional activators when they are fused to the Gal4p DNA-binding domain (51), do not self-activate the *LacZ* and *Mal* reporter genes in the b-2HS. This finding underscores one of the benefits of the non-transcription-based two-hybrid systems; specifically, the spatial separation of the association between the hybrid proteins and the transcriptional activation readout.

To investigate the actual oligomeric state of Pex5p and Pex14p, we performed an *in vivo* cross-linking approach on CHO cells. These experiments provided physical evidence that Pex14p exists predominantly as a homodimer in its native configuration. In addition, our results also suggest that Pex5p exists predominantly as a monomer in mammalian cells. Although these results are in agreement with our two-hybrid data, they do not exclude the possibility that Pex5p can transiently form oligomers. In this context, it is interesting to note that purified, bacterially expressed $(His)_6$-tagged Pex5p does form homotetramers (20). However, the fact that the same molecule tends to form filamentous aggregates in low salt buffer (20) indicates that these *in vitro* experiments have to be interpreted with care.

Finally, as we did not observe an interaction between Pex5p and Pex13p in both two-hybrid systems, we investigated further the role of the SH3 domain of Pex13p in the Pex5p-receptor docking event. Therefore, we developed an *in vitro* binding assay in which a putative peroxisomal protein import complex consisting of PTS1 ligand, Pex5p, Pex14p, and Pex13p/SH3 was reconstituted. These experiments revealed that Pex14p, and not Pex13p, functions as the initial docking site for cargo-loaded Pex5p. These findings are supported further by the fact that Pex5p accumulates on the peroxiso-

[2] K. Ghys, M. Fransen, G. P. Mannaerts, and P. P. Van Veldhoven, submitted for publication.

mal membrane in mammalian cells that are deficient in Pex13p or overexpress Pex14p and remains cytosolic in cells deficient in Pex14p (35).

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§ Supported by a postdoctoral fellowship from the F.W.O.-Vlaanderen. To whom correspondence may be addressed. Tel.: 32-16-345786; Fax: 32-16-345699; E-mail: marc.fransen@med.kuleuven.ac.be.

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** To whom correspondence may be addressed. Tel.: 32-16-345802; Fax: 32-16-345699; E-mail: paul.vanveldhoven@med.kuleuven.ac.be.

REFERENCES

1. Holroyd, C., and Erdmann, R. (2001) Protein translocation machineries of peroxisomes. *FEBS Lett.* **501,** 6–10
2. Hettema, E. H., Ruigrok, C. C. M., Koerkamp, M. G., van den Berg, M., Tabak, H., Distel, B., and Braakman, I. (1998) The cytosolic DnaJ-like protein Djp 1p is involved specifically in peroxisomal protein import. *J. Cell Biol.* **142,** 421–434
3. Dammai, V., and Subramani, S. (2001) The human peroxisomal targeting signal receptor, Pex 5p, is translocated into the peroxisomal matrix and recycled to the cytosol. *Cell* **105,** 187–196
4. Harano, T., Nose, S., Uezu, R., Shimizu, N., and Fujiki, Y. (2001) Hsp70 regulates the interaction between the peroxisome targeting signal type 1(PTS1)-receptor Pex5p and PTS1. *Biochem. J.* **357,** 157–165
5. Subramani, S., Koller, A., and Snyder, W. B. (2000) Import of peroxisomal matrix and membrane proteins. *Annu. Rev. Biochem.* **69,** 399–418
6. Terlecky, S. R., and Fransen, M. (2000) How peroxisomes arise. *Traffic* **1,** 465–473
7. Matsumura, T., Otera, H., and Fujiki, Y. (2000) Disruption of the interaction of the longer isoform of Pex 5p, Pex5pL, with Pex7p abolishes peroxisome targeting signal type 2 protein import in mammals. *J. Biol. Chem.* **275,** 21715–21721
8. Purdue, P. E., Yang, X., and Lazarow, P. B. (1998) Pex 18p and Pex21p, a novel pair of related peroxins essential for peroxisomal targeting by the PTS2 pathway. *J. Cell Biol.* **143,** 1859–1869
9. Titorenko, V. I., Smith, J. J., Szilard, R. K., and Rachubinski, R. A (1998) Pex20p of the yeast *Yarrowia lipolytica* is required for the oligomerization of thiolase in the cytosol and for its targeting to the peroxisome. *J. Cell Biol.* **142,** 403–420
10. Einwächter, H., Sowinski, S., Kunau, W. H., and Schliebs, W. (2001) *Yarrowia lipolytica* Pex20p, *Saccharomyces cerevisiae* Pex18p/Pex21p and mammalian Pex5pL fulfil a common function in the early steps of the peroxisomal PTS2 import pathway. *EMBO Rep.* **2,** 1035–1039
11. Collins, C. S., Kalish, J. E., Morrell, J. C., McCaffery, J. M., and Gould, S. J. (2000) The peroxisome biogenesis factors Pex 4p, Pex22p, Pex1p, and Pex6p act in the terminal steps of peroxisomal matrix protein import. *Mol. Cell. Biol.* **20,** 7516–7526
12. Sacksteder, K. A., and Gould, S. J. (2000) The genetics of peroxisome biogenesis. *Annu. Rev. Genet.* **34,** 623–652
13. Faber, K. N., Heyman, J. A., and Subramani, S. (1998) Two AAA family proteins, PpPex1p and PpPex6p, interact with each other in an ATP-dependent manner and are associated with different subcellular membranous structures distinct from peroxisomes. *Mol. Cell. Biol.* **18,** 936–943
14. Titorenko, V. I., Chan, H., and Rachubinski, R. A. (2000) Fusion of small peroxisomal vesicles *in vitro* reconstructs an early step in the *in vivo* multistep peroxisome assembly pathway of Yarrowia lipolytica. *J. Cell Biol.* **148,** 29–43
15. Titorenko, V. I., and Rachubinski, R. A. (2001) The life cycle of the peroxisome. *Nature Rev.* **2,** 357–368
16. van Roermund, C. W. T., Tabak, H. F., van den Berg, M., Wanders, R. J. A., and Hettema, E. H. (2000) Pex11p plays a primary role in medium-chain fatty acid oxidation, a process that affects peroxisome number and size in *Saccharomyces cerevisiae*. *J. Cell Biol.* **150,** 489–497
17. Fields, S., and Song, O. (1989) A novel genetic system to detect protein-protein interactions. *Nature* **340,** 245–247
18. Fransen, M., Terlecky, S. R., and Subramani, S. (1998) Identification of a human PTS1 receptor docking protein directly required for peroxisomal protein import. *Proc. Natl. Acad. Sci. U. S. A.* **95,** 8087–8092
19. Will, G. K., Soukupova, M., Hong, X., Erdmann, K. S., Kiel, J. A. K. W., Dodt, G., Kunau, W. H., and Erdmann, R. (1999) Identification and characterization of the human orthologue of yeast Pex14p. *Mol. Cell. Biol.* **19,** 2265–2277
20. Schliebs, W., Saidowsky, J., Agianian, B., Dodt, G., Herberg, F. W., and Kunau, W. H. (1999) Recombinant human peroxisomal targeting signal receptor PEX5, structural basis for interaction of PEX5 with PEX14. *J. Biol. Chem.* **274,** 5666–5673
21. Reguenga, C., Oliveira, M. E. M., Gouveia, A. M. M., Sá-Miranda, C., and Azevedo, J. E. (2001) Characterization of the mammalian peroxisomal import machinery: Pex2p, Pex5p, Pex12p, and Pex14p are subunits of the same protein assembly. *J. Biol. Chem.* **276,** 29935–29942
22. Karimova, G., Pidoux, J., Ullmann, A., and Ladant, D. (1998) A bacterial two-hybrid system based on a reconstituted signal transduction pathway. *Proc. Natl. Acad. Sci. U. S. A.* **95,** 5752–5756
23. Fransen, M., Wylin, T., Brees, C., Mannaerts, G. P., and Van Veldhoven, P. P. (2001) Human Pex19p binds peroxisomal integral membrane proteins at regions distinct from their sorting sequences. *Mol. Cell. Biol.* **21,** 4413–4424
24. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning. A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
25. Karimova, G., Ullmann, A., and Ladant, D. (2000) A bacterial two-hybrid system that exploits a cAMP signaling cascade in *Escherichia coli*. *Methods Enzymol.* **328,** 59–73
26. Fransen, M., Brees, C. A. E., de Béthune, B., Van Veldhoven, P. P., and Mannaerts, G. P. (1994) The 80 kDa cytosolic protein that binds the C-terminal part of rat acyl-CoA oxidase is not a peroxisomal import receptor but a prolyl-endopeptidase. *Biochim. Biophys. Acta* **1201,** 157–164
27. Fransen, M., Brees, C., Van Veldhoven, P. P., and Mannaerts, G. P. (1996) The visualization of peroxisomal proteins containing a C-terminal targeting sequence on Western blot by using the biotinylated PTS 1-receptor. *Anal. Biochem.* **242,** 26–30
28. Walhout, A. J. M., Sordella, R., Lu, X., Hartley, J. L., Temple, G. F., Brasch, M. A., Thierry-Mieg, N., and Vidal, M. (2000) Protein interaction mapping in *C. elegans* using proteins involved in vulval development. *Science* **287,** 116–122
29. Chang, C. C., Warren, D. S., Sacksteder, K. A., and Gould, S. J. (1999) PEX12 interacts with PEX5 and PEX10 and acts downstream of receptor docking in peroxisomal matrix protein import. *J. Cell Biol.* **147,** 761–774
30. Ghaedi, K., Tamura, S., Okumoto, K., Matsuzono, Y., and Fujiki, Y. (2000) The peroxin Pex3p initiates membrane assembly in peroxisome biogenesis. *Mol. Biol. Cell* **11,** 2085–2102
31. Amery, L., Sano, H., Mannaerts, G. P., Snider, J., Van Looy, J., Fransen, M., and Van Veldhoven, P. P. (2001) Identification of Pex5p-related novel peroxisome-targeting signal 1 (PTS1)-binding proteins in mammals. *Biochem. J.* **357,** 635–646
32. Shimizu, N., Itoh, R., Hirono, Y., Otera, H., Ghaedi, K., Tateishi, K., Tamura, S., Okumoto, K., Harano, T., Mukai, S., and Fujiki, Y. (1999) The peroxin Pex14p, cDNA cloning by functional complementation on a Chinese hamster ovary cell mutant, characterization, and functional analysis. *J. Biol. Chem.* **274,** 12593–12604
33. Sacksteder, K. A., Jones, J. M., South, S. T., Li, X., Liu, Y., and Gould, S. J.

(2000) PEX19 binds multiple peroxisomal membrane proteins, is predominantly cytoplasmic, and is required for peroxisome membrane synthesis. *J. Cell Biol.* **148,** 931–944
34. Okumoto, K., Abe, I., and Fujiki, Y. (2000) Molecular anatomy of the peroxin Pex12p, ring finger domain is essential for Pex12p function and interacts with the peroxisome-targeting signal type 1-receptor Pex5p and a ring peroxin, Pex10p. *J. Biol. Chem.* **275,** 25700–25710
35. Otera, H., Harano, T., Honsho, M., Ghaedi, K., Mukai, S., Tanaka, A., Kawai, A., Shimizu, N., and Fujiki, Y. (2000) The mammalian peroxin Pex 5pL, the longer isoform of the mobile peroxisome targeting signal (PTS) type 1 transporter, translocates the Pex7p·PTS2 protein complex into peroxisomes via its initial docking site, Pex14p. *J. Biol. Chem.* **275,** 21703–21714
36. Gouveia, A. M. M., Reguenga, C., Oliveira, M. E. M., Sá-Miranda, C., and Azevedo, J. E. (2000) Characterization of peroxisomal Pex5p from rat liver. *J. Biol. Chem.* **275,** 32444–32451
37. Lorenz, P., Maier, A. G., Baumgart, E., Erdmann, R., and Clayton, C. (1998) Elongation and clustering of glycosomes in *Trypanosoma brucei* overexpressing the glycosomal Pex11p. *EMBO J.* **17,** 3542–3555
38. Elgersma, Y., Kwast, L., Klein, A., Voorn-Brouwer, T., van den Berg, M., Metzig, B., America, T., Tabak, H. F., and Distel, B. (1996) The SH3 domain of the *Saccharomyces cerevisiae* peroxisomal membrane protein Pex13p functions as a docking site for Pex5p, a mobile receptor for the import of PTS1-containing proteins. *J. Cell Biol.* **135,** 97–109
39. Erdmann, R., and Blobel G. (1996) Identification of Pex13p, a peroxisomal membrane receptor for the PTS1 recognition factor. *J. Cell Biol.* **135,** 111–121
40. Gould, S. J., Kalish, J. E., Morrell, J. C., Bjorkman, J., Urquhart, A. J., and Crane, D. I. (1996) Pex13p is an SH3 protein of the peroxisome membrane and a docking site for the predominantly cytoplasmic PTS receptor. *J. Cell Biol.* **135,** 85–95
41. Tirode, F., Malaguti, C., Romero, F., Attar, R., Camonis, J., and Eggly, J. M. (1997) A conditionally expressed third partner stabilizes or prevents the formation of a transcriptional activator in a three-hybrid yeast system. *J. Biol. Chem.* **272,** 22995–22999
42. Bartel, P. L., Chien, C.-T., Sternglanz, R., and Fields, S. (1993) Elimination of false positives that arise in using the two-hybrid system. *Biotechniques* **14,** 920–924
43. Serebriiskii, I., Estojak, J., Berman, M., and Golemis, E. A. (2000) Approaches to detecting false positives in yeast two-hybrid systems. *Biotechniques* 328–336
44. Legrain, P., and Selig, L. (2000) Genome-wide protein interaction maps using two-hybrid systems. *FEBS Lett.* **480,** 32–36
45. Ito, T., Chiba, T., Ozawa, R., Yoshida, M., Hattori, M., and Sakaki, Y. (2001) A comprehensive two-hybrid analysis to explore the yeast protein interactome. *Proc. Natl. Acad. Sci. U. S. A.* **98,** 4569–4574
46. Karimova, G., Ullmann, A., and Ladant, D. (2001) Protein-protein interactions between Bacillus stearothermophilus tyrosyl-tRNA-synthetase subdomains revealed by a bacterial two-hybrid system. *J. Mol. Microbiol. Biotechnol.* **3,** 73–82
47. Gelb, H. M., Farnsworth, C. C., and Glomset, J. A. (1991) in *Lipid Modification of Proteins: a Practical Approach* (Hooper, N. M., and Turner, A. J., eds) pp. 231–259, Oxford University Press, Oxford
48. Uetz, P., and Hughes, R. E. (2000) Systematic and large-scale two-hybrid screens. *Curr. Opin. Microbiol.* **3,** 303–308
49. Dodt, G., Warren, D., Becker, E., Rehling, P., and Gould, S. J. (2001) Domain mapping of human PEX5 reveals functional and structural similarities to *S. cerevisiae* Pex18p and Pex21p. *J. Biol. Chem.* **276,** 41769–41781
50. Saidowsky, J., Dodt, G., Kirchberg, K., Wegner, A., Nastainczyk, W., Kunau, W. H., and Schliebs, W. (2001) The di-aromatic pentapeptide repeats of the human peroxisome import receptor PEX 5 are separate high affinity binding sites for the peroxisomal membrane protein PEX14. *J. Biol. Chem.* **276,** 34524–34529
51. Giniger, E., and Ptashne, M. (1987) Transcription in yeast activated by a putative amphiphatic alpha helix linked to a DNA binding unit. *Nature* **330,** 670–672