Identification of PEX5p-related novel peroxisome-targeting signal 1 (PTS1)-binding proteins in mammals

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Based on peroxin protein 5 (Pex5p) homology searches in the expressed sequence tag database and sequencing of large full-length cDNA inserts, three novel and related human cDNAs were identified. The brain-derived cDNAs coded for two related proteins that differ only slightly at their N-terminus, and exhibit 39.8% identity to human PEX5p. The shorter liver-derived cDNA coded for the C-terminal tetratricopeptide repeat-containing domain of the brain cDNA-encoded proteins. Since these three proteins specifically bind to various C-terminal peroxisome-targeting signals in a manner indistinguishable from Pex5p and effectively compete with Pex5p in an *in vitro* peroxisome-targeting signal 1 (PTS1)-binding assay, we refer to them as 'Pex5p-related proteins' (Pex5Rp). In contrast to Pex5p, however, human PEX5Rp did not bind to Pex14p or to the RING finger motif of Pex12p, and could not restore PTS1 protein import in *Pex5*$^{-/-}$ mouse fibroblasts. Immunofluorescence analysis of epitope-tagged PEX5Rp in Chinese hamster ovary cells suggested an exclusively cytosolic localization. Northern-blot analysis showed that the *PEX5R* gene, which is localized to chromosome 3q26.2–3q27, is expressed preferentially in brain. Mouse PEX5Rp was also delineated. In addition, experimental evidence established that the closest-related yeast homologue, YMR018wp, did not bind PTS1. Based on its subcellular localization and binding properties, Pex5Rp may function as a regulator in an early step of the PTS1 protein import process.

Key words: biogenesis, peroxin, protein import, tetratricopeptide repeat.

INTRODUCTION

Proteins containing a tetratricopeptide repeat (TPR) motif are involved in many cellular functions including regulation of the cell cycle, transcription, splicing, and organellar protein import [1]. The TPR motif consists of loosely conserved 34 amino acid sequences arranged in tandem repeats of three to sixteen copies, and has been proposed to mediate protein–protein interactions. Presently, the TPR protein family contains more than 500 members (Prodom PD00069; [2]) and experimental evidence has confirmed such protein–protein interactions in numerous instances [1]. In the field of protein import, examples include yeast Tom20p (Mas20p) [3] and human TOM34p [4] which are mitochondrial integral-outer-membrane proteins that interact with Tom70p (Mas70p) and HSP90 respectively.

A well-studied TPR molecule, which is involved in peroxisomal protein import, is peroxin protein 5 (Pex5p). The Pex5p proteins of fungi [5–8], protozoa [9,10], plants [11–13], worms (L. Amery and P. P. Van Veldhoven, unpublished work), and mammals [14–18] have been cloned. These proteins have a molecular mass of approximately 65–70 kDa and contain seven to eight TPRs in their C-terminal half. In the mammalian proteins, an additional TPR domain is present in the N-terminal region [14]. Pex5p binds in the cytosol to newly synthesized proteins ending in a type-1 peroxisome-targeting signal (PTS1). This signal is a tripeptide, SKL or a conserved variant thereof, and is found at the C-terminus of most peroxisomal matrix proteins [19]. The C-terminal TPR-containing domain of Pex5p is responsible for PTS1 recognition [19]. Once bound to Pex5p, the PTS1-cargo is directed to the peroxisomal compartment through a complex set of not yet fully characterized interactions of Pex5p and other peroxins. In the N-terminal half of Pex5p, a second motif is found that consists of different copies of a pentapeptide, WXXXF/Y. This sequence is necessary for the binding to a peroxisomal membrane protein, Pex14p [20]. Pex14p is thought to be a docking site for the Pex5p/PTS1-cargo [21,22] and Pex14p, in turn, can interact with a number of other peroxins including Pex7p, Pex13p, Pex17p ([21]; for a review on Pexp interactions see [23]). In man, the absence of Pex5p causes a severe lethal disorder, Zellweger syndrome [24]. In tissues of these patients, no normal peroxisomes can be found and most peroxisomal functions are impaired. Recently, a Zellweger mouse model was generated by knocking out the *Pex5* gene [17].

Whether Pex5p is translocated through the peroxisomal membrane together with the PTS1 protein or is released before translocation of the PTS1 protein, is not known at present.

Abbreviations used: CHO, Chinese hamster ovary; DsRed, red fluorescent protein of a *Discosoma* species; EGFP, enhanced green fluorescent protein; EST, expressed sequence tag; GST, glutathione S-transferase; HSA, human serum albumin; Gal4ad, Gal4 activation domain; Gal4bd, Gal4 DNA-binding domain; Pex5p, peroxin protein 5; Pex5Rp, Pex5p-related proteins; PTS1, peroxisome-targeting signal 1; TPR, tetratricopeptide repeat.

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The nucleotide sequence data will appear in the Genbank® and EMBL Nucleotide Sequence Databases under the accession numbers AJ245503 (short human PEX5R isoform, HsPEX5RS), AB032592 (long human PEX5R isoform A, HsPXR2A; renamed as HsPEX5RA), AB032593 (long human PEX5R isoform B, HsPXR2B; renamed as HsPEX5RB), and AB032591 (mouse PEX5R, MmPXR2; renamed as MmPEX5R).

Furthermore, a number of species-specific mechanistic differences appear to exist with respect to Pex5p trafficking. For example, in *Hansenula polymorpha*, Pex5p is present both in the cytosol and inside peroxisomes [7], whereas in *Yarrowia lipolytica* this protein (Pay32p) is mainly intraperoxisomal [8]. In mammals, however, Pex5p is predominantly cytosolic [15,16], and a small fraction is bound, presumably through Pex14p, to the peroxisomal membrane [22]. On the other hand, in human cell lines severely mutated in the RING finger peroxins PEX2p, PEX10p or PEX12p, PEX5p accumulates on the outer surface of the peroxisomes [25,26]. Mild mutations in Pex10p can result in an intraperoxisomal localization of Pex5p [25]. Also in watermelon, Pex5p appears to be cytosolic, but when expressed in the yeast *H. polymorpha*, some protein is present inside peroxisomes [12]. Despite this discrepancy in localization, most models for peroxisome biogenesis favour a cycling of Pex5p between cytosol and peroxisomes in a process that is ATP-, Pex2p-, Pex10p- and Pex12p-dependent. Factors contributing to the release of PTS1 protein, either occurring at the cytosolic face of the peroxisomal membrane or within the organelle, are ill defined. Recent studies in *Pichia pastoris* suggest that Pex13p may function as such a releasing factor [27]. An alternative mechanism is that another protein exists which displays affinity for PTS1 and plays a regulatory role in the release of Pex5p from PTS1 and/or recycling of Pex5p.

Whereas initially PTS1 was defined as the tripeptide SKL, the consensus for mammals has been broadened in recent years to the consensus **S**/A/C/K/N-**K**/R/H/Q/N/S-**L**/I. Furthermore, it appears that residues upstream of the PTS1 tripeptide modulate the strength of the interaction with Pex5p, and that recognition of some less common PTS1 variants by Pex5p is species-specific [28,29]. Despite this higher degree of degeneracy, cloning of several *bona fide* peroxisomal proteins revealed C-termini that do not fit the proposed consensus. One example is rat trihydroxycoprostanoyl-CoA oxidase ending in HKM [30]. Although fitting the consensus as defined in trypanosomes, HKM does not seem to bind to human PEX5p [30]. In order to explain the peroxisomal localization of such a protein, some have suggested that these molecules bind to a PTS1-containing protein, and are co-imported (piggy backing). Alternatively, perhaps there exist Pex5p or Pex5p-like proteins with distinct affinity profiles. Indeed, in mammals two Pex5p isoforms exist that are formed by differential splicing [15]. However, the C-terminal halves of these isoforms are identical, and consequently also most likely their PTS1 binding affinities.

As a first step to explore the possible existence of additional PTS1-binding proteins, the databases were searched for Pex5p-related proteins. In the yeast database, a hypothetical 59.1 kDa TPR-containing protein (YMR018wp) is present which displays 27 % overall identity to yeast Pex5p. In addition, in the mouse and human expressed sequence tag (EST) databases, a few ESTs were noticed that code for a protein which is similar to the C-terminal region of mammalian Pex5ps. By *in silico* cloning and EST sequencing the cDNA coding for the TPR-containing domain of the human protein was assembled in Leuven (EMBL accession number AJ245503). Independently, through the analysis of full-length brain cDNA clones coding for large proteins that might be involved in important biological functions [31], human cDNAs coding for an identical Pex5p-related protein, but having an extended N-terminus, were cloned in Tsukuba (Genbank® accession numbers AB032592 and AB032593). Expression of these new cDNAs in bacteria indeed resulted in PTS1-binding proteins, the properties of which we report here and to which we refer to as Pex5p-related proteins (Pex5Rp).

EXPERIMENTAL

Animals, cell lines and strains

Male Wistar rats, Swiss mice and white New Zealand rabbits were maintained on a standard laboratory chow diet. Animal treatments were approved by the local Institutional Ethics Committee. Mouse fibroblasts and Chinese hamster ovary (CHO) cells were cultured in Dulbecco's modified Eagle's medium ('DMEM') and minimum essential medium Eagle alpha ('MEMα') respectively, supplemented with 10 % (v/v) foetal calf serum, 100 μg/ml penicillin G, 100 μg/ml streptomycin sulphate and 0.25 μg/ml amphotericin B in a humidified 37 °C, 5 % CO_2 incubator. The *Pex5*$^{-/-}$ mouse fibroblasts have been described previously [17]. The *Escherichia coli* strains *Top10F'* (Invitrogen, Carlsbad, CA, U.S.A.) and *JM110* (A.T.C.C.) were used for all transformations, plasmid isolations and expression of recombinant proteins. The *Saccharomyces cerevisiae* strain *CB80* (*MAT***a**, *leu2-1*, *ura3-52*, *his3-200*, *trp1-63*) [32] was used to express recombinant proteins in yeast as well as to generate a *YMR018w* deletion strain. For protein expression, *CB80* was transformed with plasmids derived from pJR233 [28] by using the lithium acetate method [33].

Cloning of the full-length human PEX5R cDNA

By probing the human EST database with the sequence for human PEX5p, five overlapping ESTs were found that coded for a protein highly similar (50–60 % identity) to the bait. I.M.A.G.E. clones (where I.M.A.G.E. stands for Integrated Molecular Analysis of Genomes and their Expression) [34] with accession numbers W39509 (5′), N67381 (3′), W38416 (3′), AI014896 (3′), AI073945 (3′) and AI090876 (3′) were obtained from UK-HGMP (Hinxton, U.K.) and the cDNA inserts were sequenced. Based on the obtained sequence, a 1055 bp fragment was amplified from a human liver cDNA library using two sequential PCRs [first reaction: 1 μl of a human liver cDNA library (TaKaRa Biomedicals Europe S.A., Gennevilliers, France) as the template, gene-specific primers PEX5H1/PEX5H2 (Table 1), *Pfx* DNA polymerase (Life Technologies, Rockville, MD, U.S.A.); second reaction: 1 μl of the first PCR as the template, same set of primers, *Pfx* DNA polymerase]. Direct cloning of the obtained PCR product into the TopoTA cloning vector (Invitrogen) was performed by the addition of a 3′-A overhang to the gel-purified PCR product as described by the manufacturer. The cDNA insert of the resulting construct, pLA86, was sequenced by using the vector primers pBADTOPO-F and pBADTOPO-R (Table 1). To obtain additional 5′-sequence information, a similar procedure as described above was employed [first reaction: 1 μl of a human liver cDNA library (TaKaRa Biomedicals) as the template, gene-specific primer PEX5HomR1 and vector primer T7PAP (Table 1), *Pfx* DNA polymerase; second reaction: 1 μl of the first PCR reaction as the template, gene-specific nested primer PEX5HomR2 and vector primer T7PAP (Table 1), *Pfx* DNA polymerase]. The resultant PCR product was cloned into the TopoTA cloning vector (pLA109) and sequenced in both directions.

A mouse brain cDNA library was constructed by using the biotinylated cap trapper method [35] and the Lambda ZAP II vector (Stratagene, La Jolla, CA, U.S.A.) as a cloning vector. The inserts of randomly picked lambda plaques were amplified (InsertCheck; Toyobo, Katata, Japan) and their length determined by electrophoresis. The inserts of clones with a length of more than 2 kb were excised with helper phage according to the manufacturer's instructions to generate subclones in the

Table 1 Alphabetical list of oligonucleotides

Restriction sites are underlined.

FusiePEX5.1	5′-GAGAACCCCTTGCGTGATCACCCTGGAGCATTTGAAGAAGGC-3′
FusiePEX5.2	5′-GCCTTCTTCAAATGCTCCAGGGTGATCACGCAAGGGGTTCTC-3′
HsPex2.1	5′-CGGAATTCATGGCTTCCAGAAAAGAGAATGC-3′
HsPex2.2	5′-CGGGATCCTAAAGAGCATTTACTTCTGAC-3′
HsPex3.1	5′-CCTAGATCTTAAAGATGCTGAGGTCTGTATGG-3′
HsPex3.2	5′-GAAGGTCGACTCATTTCTCCAGTTGCTGAG-3′
HsPex7.fwd	5′-CGGAATTCGGGATGAGTGCGGTGTGC-3′
HsPex7.rev	5′-CCACTCGAGTGTATCTCAAGCAGGAATAG-3′
HsPex10.3	5′-CAAGAATTCATGGCCCCGGCCGCCGCCAG-3′
HsPex10.4	5′-GGCGGATCCTCAGCGGTAGTGCCGAAGGTA-3′
HsPex11α.1	5′-CGGAATTCATGGACGCCTTCACCCGC-3′
HsPex11α.2	5′-GAAGATCTCTAACGGGTCTTCAGCTTCATC-3′
HsPex11β.1	5′-GGGGATCCAAATGGACGCCTGGGTCCGCT-3′
HsPex11β.2	5′-CTTCGTCGACCCCTAAATCTCTGAATTTC-3′
HsPex12.1	5′-CGGAATTCATGGCTGAGCACGGGGCTC-3′
HsPex12.2	5′-CGGGATCCTCAGTTCTCAGGGGAGTAGAG-3′
HsPex13.1	5′-CGGGATCCGTATGGCGTCCCAGCCGCCAC-3′
HsPex13.2	5′-GTTCAACTGCAGGCAAACATG-3′
HsPex14.1	5′-GGAAAGATCTTGATGGCGTCCTCGGAGCAG-3′
HsPex14.2	5′-AAACTGCAGCTACCTGGGCTCCAAGCTG-3′
HsPex19.1	5′-AGGGATCCAGATGGCCGCCGCTGAGGAAGG-3′
HsPex19.2	5′-TGTCTCGAGTCACATGATCAGACACTGTTC-3′
K2	5′-GTCAAGACTGTCAAGGAGGG-3′
K3	5′-CATCATCTGCCCAGATGCGAAG-3′
MmPex16.1	5′-GTGAATTCAGGATGGAGAAGCTACGGCTCC-3′
MmPex16.2	5′-TCAAGATCTTCAGCCCCAACTGTAGAAAT-3′
pBADTOPO-F	5′-GCTATGCCATAGCATTTTTATCC-3′
pBADTOPO-R	5′-GATTTAATCTGTATCAGGCTG-3′
pDsRED-fw1	5′-CACCCCATTGACGTCAATG-3′
pDsRedKSKL-rv1	5′-GCTCTAGATCACAGCTTGGACTTCAGGAACAGGTGGTGGCGGC-3′
PEX5.2	5′-GCCCGTCGACCTGTCACTGGGGAGGCCAAAC-3′
PEX5.31	5′-GGGAGATCTATGGCAATGCGGGAGCTGGTG-3′
PEX5.32	5′-CCTTCGTCGACTGGGGCAGGCCAAACATAG-3′
PEX5H1	5′-GGGAATTCAAGATGCAAGCAGAATGGG-3′
PEX5H2	5′-GGGAGATCTTCAAGGATCCAAGTTGAAAGC-3′
PEX5H.F7	5′-GGGAATTCCAGAATGTACCAGGGACACATG-3′
PEX5H.F8	5′-GGGGAATTCATGTACCAGGGACACATGCAG-3′
PEX5HomR1	5′-GGGTTATCCCGAGGAACTGCCATGCCTCTG-3′
PEX5HomR2	5′-GAAGAATTGCTGCTTCCATGAACAGGATGG-3′
PEX5H.R1	5′-GGGGGTCGACTCAAGGATCCAAGTTGAAAGCTC-3′
PEX5H.R3	5′-GGGGGGTCGACGGATCCAAGTTGAAAGCTC-3′
YMR018w-A1	5′-GAGGCCTTGGTAGTACCTTACTAAC-3′
YMR018w-A2	5′-GCGCTATCGTTAGTCTGAAAATCAG-3′
YMR018w-A3	5′-GCGATCGCCTACATGAATAAGGGTG-3′
YMR018w-A4	5′-GCTTGCTGTGTTTAGTGTTCCTTTAC-3′
YMR018w-S1	5′-GAGTGGAGAATCATTAAGAATAAAGGGTACCTTTTGTAAGCACGATGC GTACGCTGCAGGTCGAC-3′
YMR018w-S2	5′-GCTTTGTTGTGTTCATATCCTTTACTTCGTAACAGTATAACTTCGAATCG ATGAATTCGAGCTCG-3′
YPEX5H.F2	5′-GAAAGGGAAGCTTTTGTAAGCACGATGGAC-3′
YPEX5H.R2	5′-GGGGCTGCAGATCCATTTTGTTATAAATAG-3′
T7PAP	5′-GTAATACGACTCACTATAGGGAATTCG-3′

pBluescript SK(−) phagemid vector. One-pass sequencing from both ends of 137 selected clones revealed 22 novel cDNAs. Further sequencing of clone #G68 by the shotgun cloning methodology revealed a transcript of 2188 bases with an open reading frame coding for a protein of 615 amino acids (Genbank® accession number AB032591) displaying 40 % sequence identity to human PEX5p. Using this mouse cDNA sequence as a query, the human EST database was screened. Based on two EST sequences (accession numbers AA382844 and N51156), a pair of primers was designed to amplify a DNA fragment from human fetal brain Marathon-Ready cDNA (Clontech, Heidelberg, Germany) by PCR. Using colony hybridization, one million clones of a human fetal brain cDNA library (Clontech) were screened with the PCR product as a probe. Two positive clones, pPXR2A and pPXR2B, containing an open reading frame that differed at their 5′-ends were isolated and sequenced in both directions. These clones were renamed to pPEX5RA and pPEX5RB respectively.

Cloning of a putative *S. cerevisiae PEX5R* homologue

Using ScPex5p as a query to search the yeast protein database, an open reading frame encoding a 59.1 kDa protein of unknown function was identified. The corresponding gene, *YMR018w* (accession number Q04364), was amplified from genomic yeast DNA by PCR by using the primer set YPEX5H.F2/YPEX5H.R2 (Table 1). The corresponding PCR product was, after digestion with *Hin*dIII and *Pst*I, subcloned into the pEGFP-C1 (pLA101; where EGFP is enhanced green fluorescent protein) and pEGFP-N1 (pLA102) vectors (Clontech) digested with the same restriction enzymes. To construct pLA104, a plasmid coding for biotinylated YMR018wp, the *Bgl*II–*Sma*I restriction fragment of pLA101, was subcloned into the *Bam*HI–*Sma*I-digested PinPoint Xa3 expression vector (Promega Benelux b.v., Leiden, The Netherlands).

Construction and analysis of a *S. cerevisiae YMR018w* null mutant

A *YMR018w* deletion mutant was generated by a PCR-based one-step gene disruption [36]. For this, we transformed the yeast strain *CB80* with the amplification product obtained by performing PCR on the pFA6a-*kanMX4* template DNA [36] with the 65-mer oligonucleotides YMR018w-S1 and YMR018w-S2 (Table 1). To select for yeast transformants carrying the *kanMX4* deletion cassette, the yeasts were plated on YPD (yeast extract/peptone/dextrose) plates containing 200 μg of geneticin per ml. The deletion of *YMR018w* in geneticin-resistant transformants was confirmed by PCR using the primer pairs YMR018w-A1/K2 and K3/YMR018w-A4 (Table 1) [36]. An additional PCR verification was performed by using the primer pairs YMR018w-A1/A2 and YMR018w-A3/A4 (Table 1), which resulted in a 349 bp and a 368 bp PCR product respectively only when wild-type genomic DNA was used as the template.

Plasmid pJR233, encoding EGFP-SKL [28], was transformed into the *CB80* wild-type strain as well as into the *YMR018w* disruption mutant. Transformants were selected and grown on minimal medium containing 0.67 % (w/v) yeast nitrogen base without amino acids (Difco, Detroit, MI, U.S.A.), 2 % (w/v) glucose, and a supplement of bases and amino acids (20–50 μg/ml) as required (synthetic dropout medium without uracil). For fluorescence microscopy, transformed cells were grown at 30 °C with shaking in selective media with 0.5 % (w/v) glucose as the sole carbon source until the glucose concentration was very low ($\sim$ 0.05 %, usually 12–16 h), and were analysed for direct fluorescence as described [32]. Minimal oleate plates containing 0.125 % (w/v) oleic acid as the sole carbon source were made according to Elgersma et al. [37].

Yeast two-hybrid methodology

To generate plasmids encoding $Gal4_{ad}$-HsPex5RpA (pLA129) and $Gal4_{ad}$-HsPex5RpB (pLA130), the cDNA inserts of pPEX5RA and pPEX5RB were amplified by PCR using the primer set PEX5H.F8/PEX5H.R1 (Table 1). The resultant PCR

Table 2 Summary of the cloning procedures used to generate plasmids encoding $Gal4_{bd}$-peroxin fusion proteins

cDNAs encoding full-length peroxins were amplified by PCR using the appropriate templates and primers, digested with the listed restriction enzymes, and subcloned into the pGBT9 or pAS2-1 DNA-binding-domain cloning vectors (Clontech).

cDNA (plasmid)	Digested cloning vector	Template DNA	Primers (see Table 1)	Restriction enzymes
PEX2 (pMF108)	pGBT9 (*Eco*RI, *Bam*HI)	EST AA452747	HsPex2.1, HsPex2.2	*Eco*RI, *Bam*HI
PEX3 (pMF158)	pGBT9 (*Bam*HI, *Sal*I)	EST AA305508	HsPex3.1, HsPex3.2	*Bgl*II, *Sal*I
PEX7 (pKG26)	pAS2-1 (*Eco*RI, *Sal*I)	Human liver cDNA	HsPex7.fwd, HsPex7.rev	*Eco*RI, *Xho*I
PEX10 (pMF198)	pGBT9 (*Eco*RI, *Bam*HI)	Human liver cDNA	HsPex10.3, HsPex10.4	*Eco*RI, *Bam*HI
PEX11α (pMF116)	pGBT9 (*Eco*RI, *Bam*HI)	EST AA476689	HsPex11α.1, HsPex11α.2	*Eco*RI, *Bgl*II
PEX11β (pMF260)	pGBT9 (*Bam*HI, *Sal*I)	Human liver cDNA	HsPex11β.1, HsPex11β.2	*Bam*HI, *Sal*I
PEX12 (pMF304)	pGBT9 (*Eco*RI, *Bam*HI)	Human liver cDNA	HsPex12.1, HsPex12.2	*Eco*RI, *Bam*HI
PEX13 (pMF103)	pGBT9 (*Bam*HI, *Pst*I)	EST AA295033	HsPex13.1, HsPex13.2	*Bam*HI, *Pst*I
PEX14 (pMF101)	pGBT9 (*Bam*HI, *Pst*I)	EST H16035	HsPex14.1, HsPex14.2	*Bgl*II, *Pst*I
PEX16 (pMF187)	pGBT9 (*Eco*RI, *Bam*HI)	EST W34967	MmPex16.1, MmPex16.2	*Eco*RI, *Bgl*II
PEX19 (pMF132)	pGBT9 (*Bam*HI, *Sal*I)	EST AA337880	HsPex19.1, HsPex19.2	*Bam*HI, *Xho*I

Table 3 Specific binding of Pex5Rp to PTS1

Different peptides were coated on a microtiter plate and binding of the bacterially expressed biotinylated C-terminal half (amino acids 259 to 639) of HsPex5p or the biotinylated TPR-containing region (amino acids 293 to 624) of HsPex5Rp was analysed photometrically with streptavidin–alkaline phosphatase. *p*-Nitrophenyl phosphate was used as the streptavidin–alkaline phosphatase substrate [14]. The underlined cysteine residues were introduced for cross-linking studies which were reported elsewhere [50]. PCOX, palmitoyl-CoA oxidase; THCCOX, trihydroxycoprostanoyl-CoA oxidase.

		A_{405nm}	
Peptide	Sequence	Pex5p (259–639)	Pex5Rp (293–624)
pSKL	CSYHKHLKPLQSKL (C-terminal 13 amino acids of rat PCOX)	0.622	0.204
pSKL-$CONH_2$	CSYHKHLKPLQSKL-$CONH_2$ (amidated SKL peptide)	0.164	0.050
pΔSKL	CVHESYHKHLKPLQ (peptide devoid of SKL motif)	0.075	0.040
pHKM	CKYIRPLMLGWRHKM (C-terminal 13 amino acids of rat THCCOX)	0.131	0.048

fragments were gel purified, digested with *Eco*RI and *Sal*I, and subcloned into pGAD424 (Clontech) digested with *Eco*RI and *Sal*I. The fusions between $Gal4_{bd}$ and HsPex2p (pMF108), HsPex3p (pMF158), HsPex7p (pKG26), HsPex10p (pMF198), HsPex11pα (pMF116), HsPex11pβ (pMF260), HsPex12p (pMF304), HsPex13p (pMF103), HsPex14p (pMF101), MmPex16p (pMF187), and HsPex19p (pMF132), were generated as described in Table 2. To subclone the putative yeast homologue of Pex5Rp into pGBT9 (pLA106) and pGAD424 (pLA107), the *Xho*I–*Pst*I restriction fragment of pLA101 was ligated into the corresponding yeast two-hybrid vectors digested with *Sal*I and *Pst*I.

In vitro binding assays

For the microtiter plate assay, 1 μg of the peptides pSKL, pSKL-$CONH_2$, pΔSKL or pHKM (see Table 3) was coated (50 mM sodium carbonate buffer, pH 9.6) in the appropriate wells and incubated with the biotinylated C-terminal half (amino acids 259 to 639) of HsPex5p [14] or the biotinylated TPR-containing region (amino acids 293 to 624) of HsPex5Rp. pLA56, the construct encoding the latter biotinylated protein, was generated by subcloning the *Eco*RV–*Not*I restriction fragment of I.M.A.G.E. [34] clone 1640223 (accession number AI090876, here renamed pLA51) into the PinPoint Xa2 vector digested with *Eco*RV and *Not*I. The same biotinylated fusion proteins as well as GST-Pex5Rp (encoded by pLA126; where GST is glutathione S-transferase) and the biotinylated putative yeast homologue of HsPex5Rp (encoded by pLA104) were used in the competitive microtiter plate assay [29], or in blot overlay assays [38]. Detection of the formed complexes was accomplished as described [14,38]. To construct pLA126, the cDNA insert of pPEX5RA was amplified by PCR using the primer set PEX5H.F8/PEX5H.R1, and the corresponding PCR product was digested with *Eco*RI and *Sal*I, and subcloned into the *Eco*RI/*Sal*I-digested pGEX-4T-1 expression vector (Amersham Pharmacia Biotech AB, Uppsala, Sweden).

Transfections and immunofluorescence

To construct plasmids encoding EGFP-HsPex5RpA (pLA118) and EGFP-HsPex5RpB (pLA119), the cDNAs coding for HsPex5RpA and HsPex5RpB were amplified by PCR using the primer pair PEX5H.F7/PEX5H.R1 (Table 1). These PCR products were, after digestion with *Eco*RI and *Sal*I, subcloned into the *Eco*RI/*Sal*I-digested pEGFP-C1 vector. To generate constructs encoding HsPex5RpA-EGFP (pLA120) and HsPex5RpB-EGFP (pLA121), the cDNAs encoding HsPex5RpA and HsPex5RpB were amplified by using the primers PEX5H.F7 and PEX5H.R3 (Table 1). The corresponding PCR products were digested with *Eco*RI and *Sal*I and subcloned into the pEGFP-N1 vector digested with *Eco*RI and *Sal*I. To construct plasmids encoding FLAG-HsPex5RpA (pLA122) and FLAG-HsPex5RpB (pLA123), a similar procedure was used as for pLA118 and pLA119, except that the pCMV-Tag2A (Stratagene) was used for subcloning. Plasmids encoding HsPex5RpA-FLAG (pLA124) and HsPex5RpB-FLAG (pLA125) were constructed as pLA120 and pLA121 by using the pCMV-Tag4B vector. Plasmids encoding EGFP fused to the N-terminus (pLA101) or C-terminus (pLA102) of the putative yeast homologue of HsPex5Rp were constructed as described above. CHO cells were transiently transfected according to the polyethylenimine transfection method [39]. Mouse fibroblasts were transiently transfected by using the LIPOFECTAMINE™ Plus Reagent (Life Technologies). Immunofluorescence studies were performed essentially as described [14]. Fluorescence was ob-

served under a Leica DMR microscope equipped with standard FITC and rhodamine isothiocyanate ('RITC') filters.

PTS1 import rescue experiments

To test whether HsPex5RpA or HsPex5RpB could complement *Pex5*$^{-/-}$ mouse fibroblasts, constructs encoding these (fusion) proteins were cotransfected with a plasmid encoding EGFP-KSKL (kindly provided by Dr M. Baes, Campus Gasthuisberg, Labo Klinische Chemie, Leuven, Belgium) or DsRed-KSKL (pMF578; where DsRed is red fluorescent protein of a *Discosoma* species). The plasmids for eukaryotic expression encoding non-tagged, full-length HsPex5RpA (pLA131) and HsPex5RpB (pLA132) were constructed by using the primer pair PEX5H.F7/PEX5H.R1 (Table 1) in combination with a template encoding HsPex5RpA or HsPex5RpB. The two resulting PCR products were, after digestion with *Eco*RI and *Sal*I, subcloned into the *Eco*RI/*Xho*I digested pcDNA3.1/Myc-HisC vector (Invitrogen). To construct the plasmid encoding His_6-HsPex5RpA (pLA150), the corresponding cDNA was amplified by using the primers PEX5H.F7 and PEX5H.R1 (Table 1). The obtained PCR product was digested with *Eco*RI and *Sal*I, and subcloned into the pcDNA4/HisMaxB vector digested with *Eco*RI and *Xho*I. The plasmids encoding EGFP (or FLAG) fused to the N-terminus or C-terminus of HsPex5RpA are already described above. The plasmids pMycPex5p (kindly provided by Dr M. Baes), pFlagPex5p, pPex5pFlag, and pHis_6Pex5p were used as a positive control. To construct the plasmids encoding FLAG-HsPex5p (pLA139) and His_6-HsPex5p (pLA149), the cDNA encoding Pex5p was amplified by PCR using the primer pair PEX5.2/PEX5.31 (Table 1). The resulting PCR product was, after digestion with *Bgl*II and *Sal*I, subcloned into the *Bam*HI/*Sal*I-digested pCMV-Tag2B (Stratagene) and the *Bam*HI/*Xho*I-digested pcDNA4/HisMaxC (Invitrogen) vectors respectively. The plasmid pPex5pFlag (pLA140) was generated by subcloning the *Bgl*II/*Sal*I-digested PCR product (primer pair PEX5.31/PEX5.32) encoding Pex5p into the *Bam*HI/*Sal*I-digested pCMV-Tag4B vector (Stratagene). To construct pMF578, the 741 bp *Eco*RI–*Xba*I fragment of pDsRed-N1 (Clontech) was replaced by a cDNA fragment coding for DsRed-KSKL, which was obtained by PCR (template, pDsRed-N1; primer pair, pDsRed.fw1/pDsRedKSKL.rv1). The construct (pLA142) coding for the N-terminal 298 amino acids of HsPex5p fused to the C-terminal TPR-containing domain of HsPex5Rp (amino acids 325 to 624) was generated by fusion PCR. In a first PCR, two PCR fragments were generated {PCR 1: primer set PEX5.31/FusiePEX5.2, template pMF66 (encodes His_6-HsPex5p; [22]); PCR 2: primer set PEX5H.R1/FusiePEX5.1, cDNA encoding HsPex5RpA} which were used as the templates in a second PCR to generate a cDNA fusion. After digestion with *Bgl*II and *Sal*I, this PCR fragment was subcloned into the *Bam*HI/*Xho*I-digested pcDNA3.1/Myc-HisC vector (Invitrogen). Mouse fibroblasts deficient in PEX5p were transfected as described above.

Antibodies

To raise a polyclonal antiserum against human PEX5Rp, the 519 bp *Pst*I–*Hin*dIII fragment of pLA51 was transferred into the *Pst*I/*Hin*dIII-digested pQE-31 vector (Invitrogen). The resulting plasmid, pLA71, encoded an in-frame fusion between a His_6-tag and the C-terminal 151 amino acids of human PEX5Rp. To express His_6-HsPex5Rp$_{(aa474–aa624)}$, *Top10F′* cells were transformed with pLA71 and induced with 1 mM of isopropyl β-D-thiogalactoside ('IPTG'). At 4 h after induction, the His_6-tagged fusion protein was purified on a Ni-NTA resin (where NTA stands for nitrilotriacetic acid; Qiagen, Hilden, Germany) using the manufacturer's procedure for denatured proteins. After SDS/PAGE and transfer onto nitrocellulose, 50 μg of the ultrapure His_6-HsPex5Rp$_{(aa474–aa624)}$ was cut out and the nitrocellulose membrane was dissolved by adding DMSO. An equal amount of Freund's complete adjuvant (priming dose) or Freund's incomplete adjuvant (successive boosting doses) was added and, after mixing, the emulsified solution was injected subcutaneously into a rabbit. The mouse monoclonal anti-(FLAG M2) antibody was purchased from Stratagene. The anti-GST antiserum is reported elsewhere [40].

Sequencing, computer analysis and alignments

DNA sequencing was done by using an A.L.F. sequencer (AmershamPharmacia) and vector-specific primers (Table 1). Database searches and protein alignments were performed by using the BLAST algorithm [41]. Multiple DNA/protein sequences were aligned with hierarchical clustering [42] or an iterative pairwise method [43]. Searches for protein motifs were conducted with the Expasy ScanProsit tool (Swiss Institute of Bioinformatics, Genève, Switzerland).

Northern-blot analysis

The human multiple tissue Northern blot and the human RNA dot blot were purchased from Clontech. The 757 bp *Eco*RV–*Nco*I (5′-probe) and the 688 bp *Hin*dIII–*Bam*HI (3′-probe) fragments of human PEX5RA cDNA, and glyceraldehyde-3-phosphate dehydrogenase cDNA, were labelled with ^{32}P by the random priming method. The hybridization and washing were performed under standard high-stringency conditions.

RESULTS

Isolation and analysis of human PEX5R cDNAs

By probing the human EST database with human PEX5p, some ESTs were found encoding an unidentified protein highly similar to HsPex5p. The corresponding cDNA, which was obtained by PCR using a human liver cDNA library as the template, encoded a putative protein of 344 amino acids with a calculated molecular mass of 38.5 kDa (accession number AJ245503). Since the protein showed 54 % sequence identity with the C-terminal 385 amino acids of the PTS1 receptor Pex5p (Figure 1), it was called the short Pex5p-related protein (PEX5RpS). The 76 bp of the cloned cDNA (PEX5RS) upstream of the putative start codon contained two in-frame stop codons (results not shown), and were also found in one EST clone (accession number AI090876) and two genomic bacterial artificial chromosome ('BAC') clones (accession numbers AC007653 and AC007687). Because of the near complete identity of fragments of bacterial artificial chromosome clone AC007687 to portions of the obtained PEX5RS cDNA, the *PEX5RS* gene is localized on chromosome 3q26.2–3q.27. In addition, three STS sequences (accession numbers G30540, G23399 and G19649), which matched the non-translated 3′-end, all mapped to chromosome 3 (reference interval D3S1553–D3S1580).

Independently, by analysing a mouse brain full-length enriched cDNA library, subjected to size selection, a mouse open-reading frame was found that encoded a Pex5p-related protein of 615 amino acids with a calculated molecular mass of 68.5 kDa (accession number AB032591). By hybridization cloning, two isoforms were isolated from a human fetal brain cDNA library. Based on the similarity (90 % identity) of the deduced amino acid

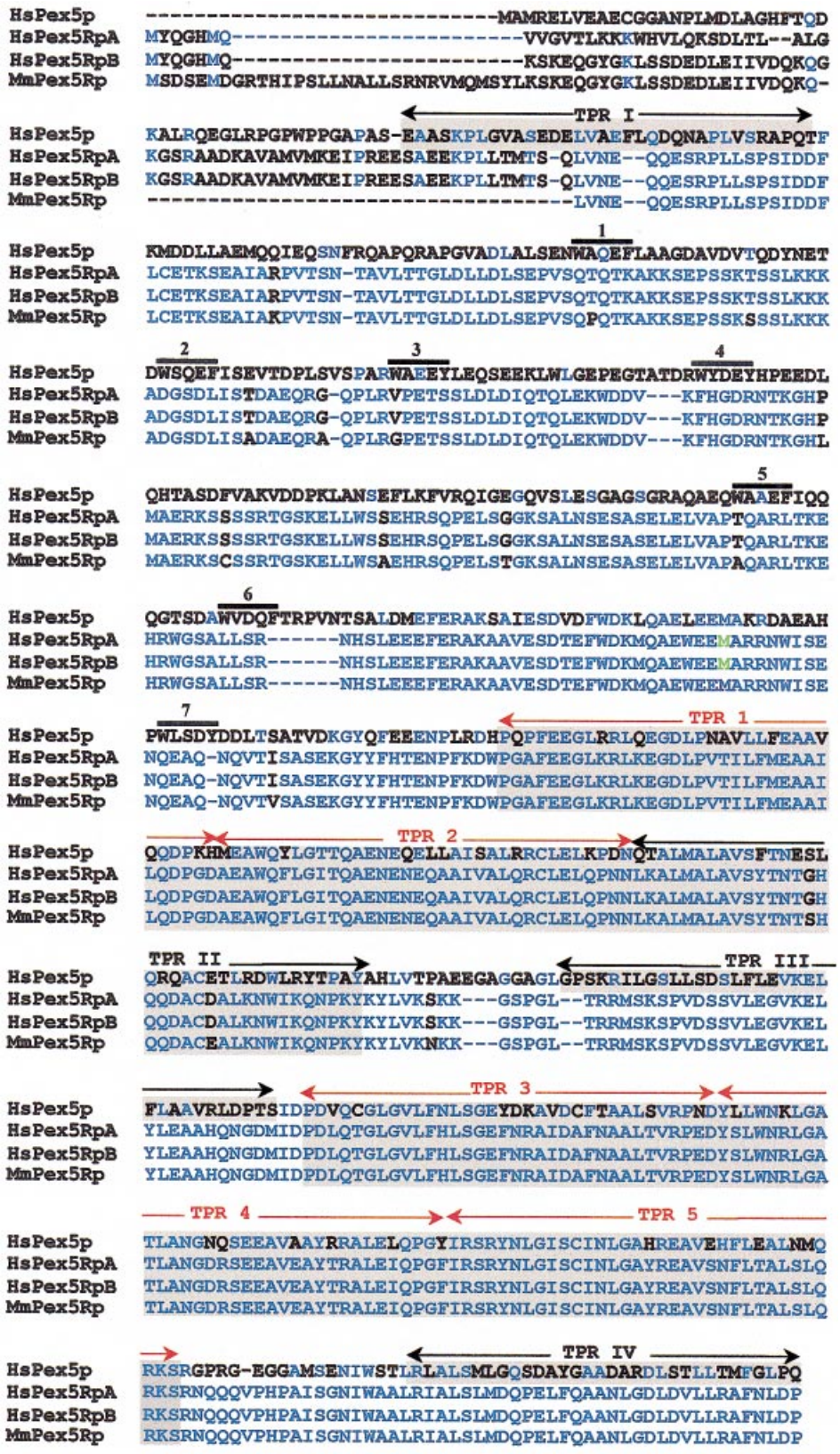


Figure 1 Comparison of mouse and human PEX5Rp isoforms with human PEX5p

The amino acid sequences of mouse PEX5Rp (MmPex5Rp), human PEX5RpA (HsPex5RpA) and PEX5RpB (HsPex5RpB) were aligned to the longer isoform of human PEX5p (HsPex5p) by using MULTALIN [42]. The initiator methionine of the short PEX5Rp isoform is marked in green. Amino acid residues identical in three sequences are blue. The TPR are shaded in grey. TPR domains that could be identified by scanning the PROSITE database of protein domains by using the ISREC ProfileScan server are indicated by horizontal red arrows. The other, visually identified TPR domains [14] are indicated by horizontal black arrows. WXXXF/Y repeats present in human PEX5p [20] are overlined in black.

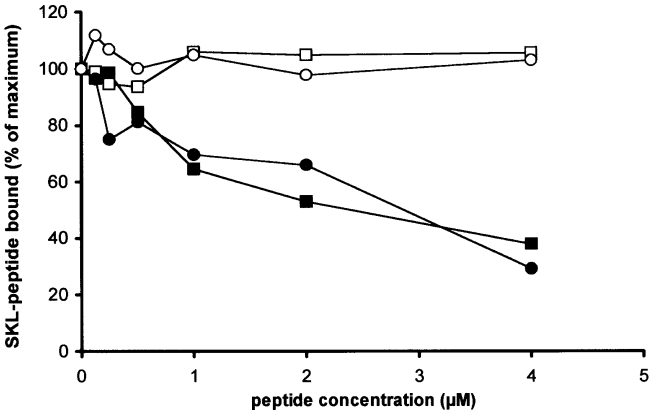


Figure 2 Peptide competition for binding to human PEX5Rp

Biotinylated human PEX5Rp (open and filled circles) and PEX5p (open and filled squares) fusion proteins were incubated in microtiter wells coated with 1 μg of HSA cross-linked to the peptide pSKL (HSA-SKL) in the presence of the indicated amounts of the peptides pSKL (filled circles and squares) or pΔSKL (open circles and squares). After extensive washings, bound PEX5Rp and PEX5p were detected photometrically as described in Table 3.

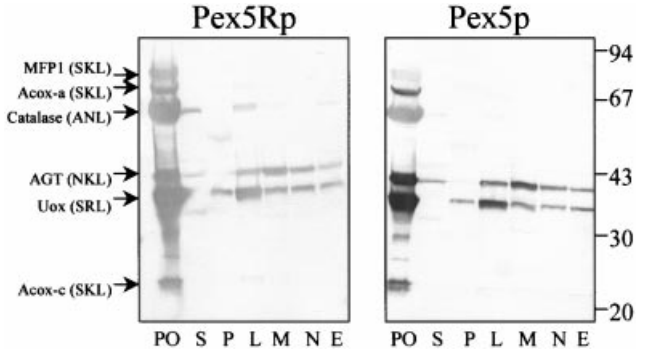


Figure 3 Pex5Rp and Pex5p recognize the same rat liver peroxisomal PTS1 proteins in a blot overlay assay

Proteins (20 μg) present in rat liver subcellular fractions or purified peroxisomes were subjected to SDS/PAGE and transferred to nitrocellulose. Individual membranes containing these proteins were incubated with a bacterial lysate expressing GST-tagged human PEX5Rp (left panel) or human PEX5p (right panel). Resultant complexes were visualized as described in the Experimental section using an anti-GST antiserum. E, postnuclear supernatant; N, nuclear fraction; M, heavy mitochondrial fraction; L, light mitochondrial fraction; P, microsomal fraction; S, cytosolic fraction; PO, purified peroxisomes. PTS1-containing proteins are indicated by arrows and the corresponding C-terminal tripeptides are in parentheses. The migration of the molecular mass markers (in kDa) is indicated on the right side. Acox-a, 70 kDa subunit of palmitoyl-CoA oxidase; Acox-c, 23 kDa subunit of palmitoyl-CoA oxidase; AGT, L-alanine:glyoxylate aminotransferase 1; MFP1, inducible multifunctional protein; Uox, urate oxidase.

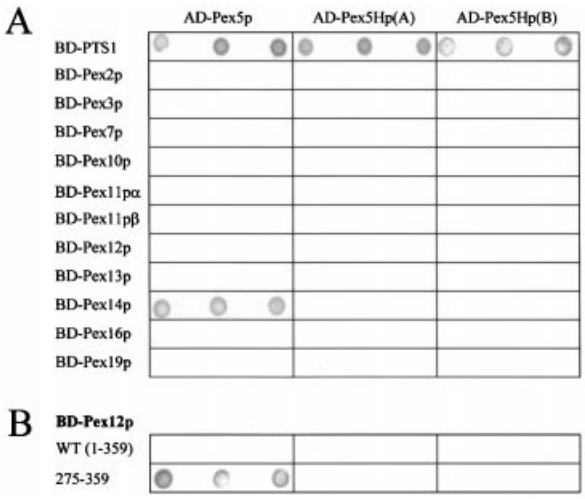


Figure 4 Pex5Rp does not interact with Pex12p and Pex14p in the yeast two-hybrid system

Pex5RpA and Pex5RpB, fused to the Gal4-activation domain (AD), were tested for interaction with PTS1 (**A**), other human peroxins (**A**) and the (mutated) RING finger domain of human PEX12p (**B**), fused to the Gal4 DNA-binding domain (BD). Double transformants (yeast strain SFY526), expressing one of the Gal4-BD-fusions and one of the Gal4-AD-fusions, were selected and β-galactosidase activity was monitored for 2 h by using a filter assay with X-gal as the substrate. Three representative independent transformants are shown. WT, wild-type human PEX12p (275–359), C-terminal 85 amino acids (including RING finger domain) of human PEX12p.

sequences to the mouse counterpart, the two clones were considered to be orthologues and were named PEX5RA (accession number AB032592) and PEX5RB (accession number AB032593) respectively. Surprisingly, the non-coding 5′-region of the liver cDNA was not present in the brain cDNAs, which coded for a larger protein of 624 amino acids (69.4 kDa) and 626 amino acids (69.7 kDa) respectively. The corresponding HsPex5RpA and HsPex5RpB molecules, which only slightly differ at their N-termini, are most likely the result of differential splicing and show 39.8% identity to the longer isoform of HsPex5p (Pex5pL) [14–16] (Figure 1). A more detailed analysis revealed that all HsPex5Rp isoforms are, like Pex5p, members of the TPR protein family. In addition, all the TPR domains are located in the C-terminal half of the Pex5Rp isoforms, and it is in this region that the similarity to Pex5p is most pronounced (57% identity; Figure 1). However, the WXXXF/Y motifs present in the N-terminal half of Pex5p [20] are not found in Pex5Rp. No other motifs, except for, presumably unphysiological, glycosylation and phosphorylation sites, appeared to be present. Note that (i) the experiments reported below were performed with all HsPex5Rp isoforms, (ii) for clarity, only the results obtained with the longer isoforms are shown and (iii) the term 'Pex5Rp' refers to all three Pex5Rp isoforms.

Pex5Rp binds PTS1-containing proteins

In order to investigate whether or not Pex5Rp is functionally related to the PTS1 receptor PEX5p, we tested whether Pex5Rp was able to bind PTS1 ligands. Since the TPR domains in the C-terminal half of human PEX5p are sufficient for PTS1 binding [14], we expressed the corresponding TPR-containing domain of Pex5Rp in bacteria as a biotinylated fusion protein. *In vitro* binding studies demonstrated that this part of HsPex5Rp recognized a peptide containing the PTS1 signal (pSKL) but not other peptides such as those containing no PTS1 (pΔSKL), an amidated PTS1 (pSKL-$CONH_2$) or the C-terminal 14 amino acids of rat trihydroxycoprostanoyl-CoA oxidase (pHKM) [30] (Table 3). Furthermore, this interaction was competitively inhibited by pSKL, but not by pΔSKL (Figure 2). Similar results were obtained with dot blots on which 1 μg of HSA-pSKL (where HSA is human serum albumin) and HSA-pΔSKL were spotted (results not shown). Also, the same proteins in a subcellular fraction consisting of purified peroxisomes could be visualized in a blot overlay assay by using either bacterially expressed HsPex5p or HsPex5Rp as a ligand (Figure 3). The *in vitro* specificity of Pex5Rp for PTS1 was extended to the *in vivo*

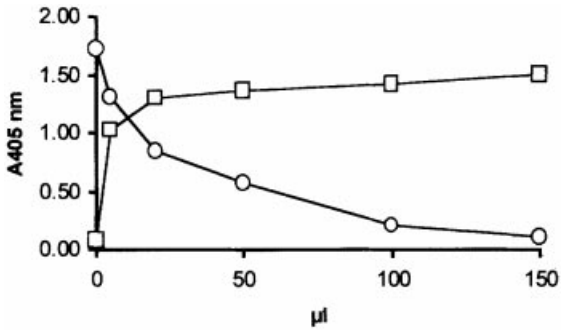


Figure 5 PEX5Rp and PEX5p compete for binding to PTS1

Bacterial lysate (10 μl) containing biotinylated human PEX5p was incubated (in duplicate) with the indicated volumes of a bacterial lysate containing GST-Pex5Rp in wells of a microtiter plate coated with 0.1 μg of HSA-SKL. After extensive washings, one of the duplicates was used to analyse the binding of biotin-Pex5p to PTS1 (open circles) by means of streptavidin-alkaline phosphatase/*p*-nitrophenyl phosphate, and the other duplicate was used to analyse the binding of GST-Pex5Rp to PTS1 (open squares) by means of a rabbit anti-GST antiserum and anti-rabbit IgG-alkaline phosphatase/*p*-nitrophenyl phosphate. After 25 min (for GST-Pex5Rp) and 5 h (for biotin-Pex5p) respectively, the amount of formed *p*-nitrophenol was measured at 405 nm. SDS/PAGE analysis of the proteins, present in 1 ml of the bacterial lysates, captured on to streptavidin beads and glutathione Sepharose beads respectively revealed that biotin-Pex5p and GST-Pex5Rp were present in approximately equal amounts (results not shown).

situation for the full-length HsPex5RpA and HsPex5RpB molecules in the yeast two-hybrid system (Figure 4A). Finally, as previously reported for Pex5p [44], binding of Pex5Rp to PTS1 was abolished by pretreatment of Pex5Rp with *N*-ethylmaleimide (results not shown).

Since it has also been reported that human PEX5p directly interacts with the human PEX5p docking protein PEX14p and the RING finger domain, but not the full-length molecule, of human PEX12p [23,45], a limited two-hybrid screen was performed to investigate whether or not full-length human PEX5Rp also interacted with other peroxins that are currently available in the laboratory (Figure 4A). As for Pex5p, an interaction between Pex5Rp and the full-length Pex2p, Pex3p, Pex7p, Pex10p, Pex11pα, Pex11pβ, Pex12p, Pex13p, Pex16p and Pex19p molecules was not observed (Figures 4A and 4B). However, in contrast with the results obtained for Pex5p, we were unable to detect an interaction between Pex5Rp and PEX14p (Figure 4A) or the RING finger motif of Pex12p (Figure 4B). These latter results strongly indicate that Pex5p and Pex5Rp are not functional homologues. Nevertheless, GST-Pex5Rp can effectively compete biotin-Pex5p out of a PTS1–Pex5p complex in an *in vitro* binding assay (Figure 5). Since Pex5p and Pex5Rp both auto-activate transcription as a DNA-binding-domain fusion protein, we used a blot overlay assay to investigate whether these two proteins interacted. A specific interaction could not be demonstrated (results not shown).

PEX5R is expressed preferentially in brain

The expression level of PEX5R mRNA in human tissues was examined by Northern (dot) blot analysis with a DNA fragment corresponding to a common 5′-region of PEX5RA and PEX5RB cDNA as a probe. Three hybridizing bands (8.3 kb, 4.3 kb and 3.3 kb respectively) were detected (Figure 6). Signals were strongest in brain, but weaker signals were also detected in pancreas (Figure 6A). Hybridizing a dot blot containing RNA obtained from 50 tissues with the same probe confirmed that the PEX5R mRNA is expressed preferentially in brain, and also in pancreas (Figures 6B and 6C). In addition, these dot blots revealed that the PEX5R mRNA was present in all parts of the brain, and also, albeit to a lower level, in testis and the pituitary gland. When a probe corresponding to the 3′-end of PEX5R cDNA was used, a similar mRNA expression pattern was seen in the multiple human tissue Northern blot (results not shown). In addition, in heart and skeletal muscle a weak signal (6.9 kb) was detected (results not shown). Thus, it seems that the 8.3 kb signal represents an intact Pex5Rp mRNA and that the expression is highest in brain. It should be noted that, although the expression level of the PEX5R cDNA in most tissues, including liver, was below the detection limit with both probes (Figures 6B and 6C), the PEX5RS cDNA encoding the 344 amino acid isoform (but not the cDNAs encoding the longer isoforms) was isolated from a liver cDNA library by PCR, indicating the presence of mRNA.

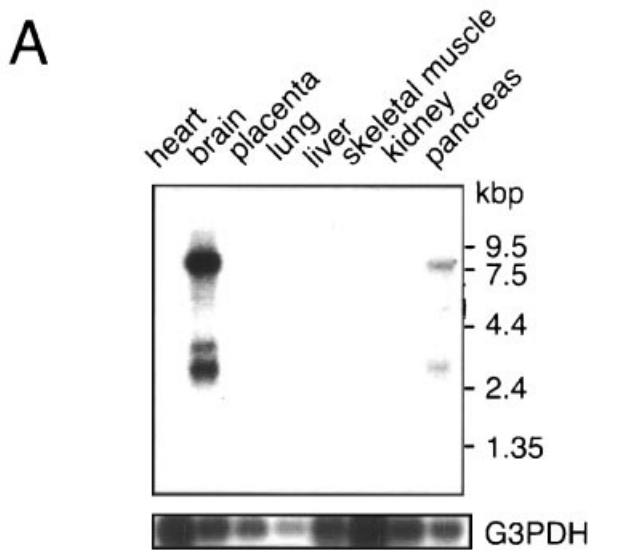
To examine *PEX5R* expression at the protein level, homogenates of multiple mouse tissues (brain, liver, kidney, pancreas and testis) were immunoblotted using a polyclonal antiserum raised against Pex5Rp. Although the sensitivity of this antiserum was high enough to detect 5 ng of recombinantly expressed Pex5Rp, no endogenous Pex5Rp could be detected in any of the examined tissues (results not shown).

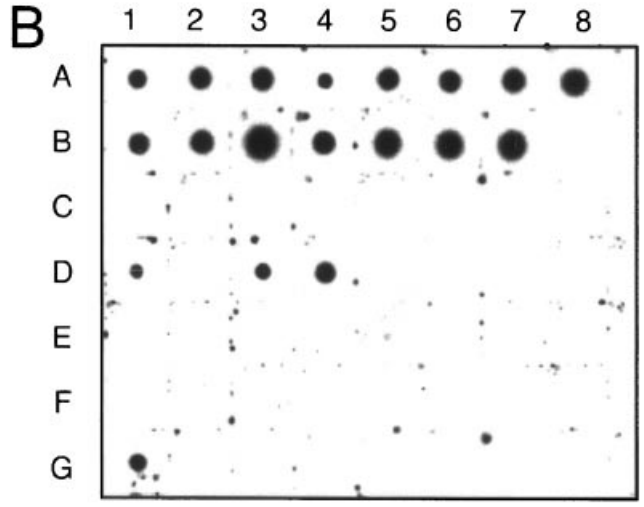
Expression of PEX5R cannot restore PTS1 protein import in *Pex5*$^{-/-}$ mouse fibroblasts

To test whether or not PEX5R cDNA could rescue PTS1 protein import in immortalized *Pex5*$^{-/-}$ mouse fibroblasts [17], we co-transfected these cells with plasmids coding for the reporter protein EGFP-KSKL and HsPex5RpA (Figure 7F), HsPex5RpB (Figure 7G), His_6-HsPex5RpA, FLAG-HsPex5RpA or HsPex5RpA-FLAG (results not shown). As judged by the mislocalization of the PTS1 reporter protein to the cytosol, the expression of the HsPex5Rp (fusion) proteins did not restore the peroxisomal protein import defect, in contrast to Myc-MmPex5p (Figure 7E), His_6-HsPex5p, FLAG-HsPex5p and HsPex5p-FLAG (results not shown). Yet, in order to be sure that the peroxisomal marker protein as well as HsPex5Rp are co-expressed in at least some cells, *Pex5*$^{-/-}$ mouse fibroblasts were co-transfected with plasmids encoding DsRed-KSKL, a red fluorescent peroxisomal marker protein, and EGFP-HsPex5RpA (Figures 7I and 7J) or HsPex5RpA-EGFP (results not shown). As expected, no complementation was observed in cells expressing both HsPex5RpA-EGFP and DsRed-KSKL (Figures 7I and 7J). Consequently, although Pex5Rp binds to PTS1 proteins, it cannot functionally replace Pex5p. Interestingly, although DsRed-KSKL is localized to the peroxisomes in *Pex5*$^{+/-}$ mouse fibroblasts (Figure 7C), it displays a mitochondrial-like distribution pattern (Figure 7D) in the majority of the transfected *Pex5*$^{-/-}$ mouse fibroblasts. Also no complementation was observed when *Pex5*$^{-/-}$ fibroblasts were transfected with a construct coding for a chimaeric protein composed of the N-terminal 298 amino acids of HsPex5p and the 300 C-terminal amino acids of HsPex5Rp (Figure 7H). In this context, it is interesting to note that (i) the N-terminal 298 amino acids of HsPex5p still contain the binding region for its docking protein Pex14p [20], but not for the RING finger domain of Pex12p (results not shown) and (ii) the 300 C-terminal amino acids of HsPex5Rp are sufficient to bind PTS1 ligands (Figure 2), but they do not bind the RING finger domain of Pex12p (Figure 4B).

Pex5Rp is localized to the cytosol

Since the sensitivity of our anti-Pex5Rp antiserum was not high enough to detect endogenous Pex5Rp, the subcellular





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	1	2	3	4	5	6	7	8
A	whole brain	amygdala	caudate nucleus	cere-bellum	cerebral cortex	frontal lobe	hippo-campus	medulla oblongata
B	occipital lobe	putamen	substantia nigra	temporal lobe	thalamus	nucleus accumbens	spinal cord	
C	heart	aorta	skeletal muscle	colon	bladder	uterus	prostate	stomach
D	testis	ovary	pancreas	pituitary gland	adrenal gland	thyroid gland	salivary gland	mammary gland
E	kidney	liver	small intestine	spleen	thymus	peripheral leukocyte	lymph node	bone marrow
F	appendix	lung	trachea	placenta				
G	fetal brain	fetal heart	fetal kidney	fetal liver	fetal spleen	fetal thymus	fetal lung	

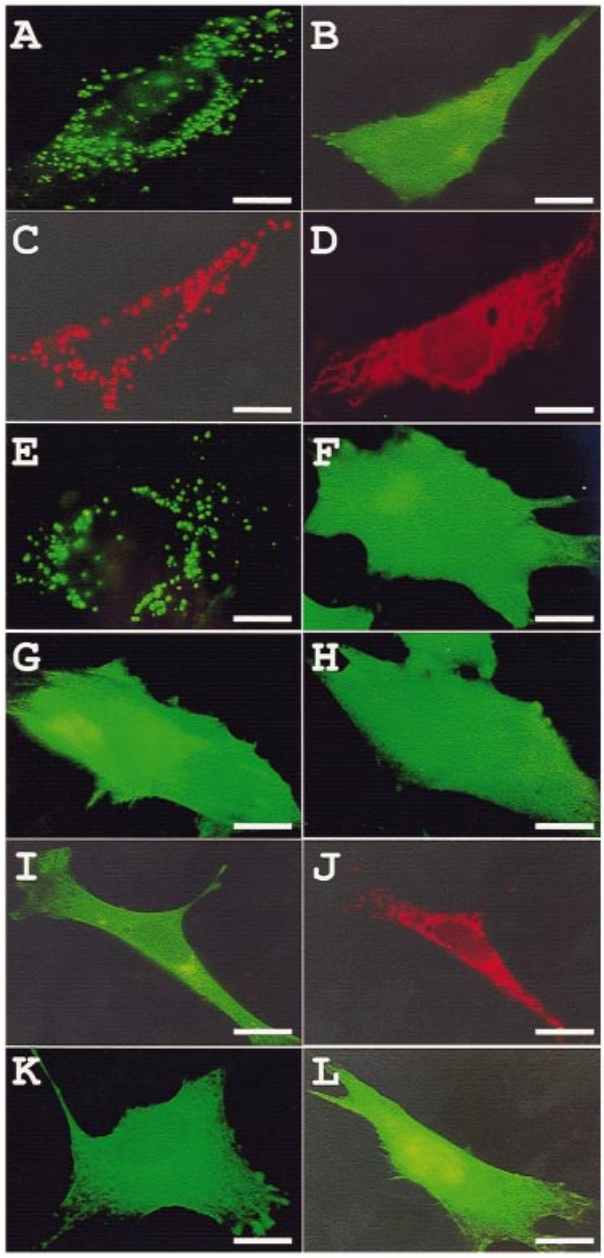

Figure 7 PEX5Rp is a cytosolic protein that can't restore PTS1 import in *Pex5*$^{-/-}$ mouse fibroblasts

Immortalized *Pex5*$^{+/-}$ (**A**,**C**,**K**,**L**) or *Pex5*$^{-/-}$ mouse fibroblasts (**B**,**D**,**E**,**F**,**G**,**H**,**I**,**J**) were transiently transfected with plasmids encoding EGFP-KSKL (**A**,**B**), DsRed-KSKL (**C**,**D**), EGFP-HsPex5RpA (**K**) or HsPex5RpA-EGFP (**L**), or co-transfected with plasmids encoding MmPex5p (**E**), HsPex5RpA (**F**), HsPex5RpB (**G**), a chimaeric protein composed of the N-terminal 298 amino acids of HsPex5p and the 300 C-terminal amino acids of HsPex5Rp (**H**) or EGFP-HsPex5RpA (**I**,**J**) and EGFP-KSKL (**E**,**F**,**G**,**H**) or DsRed-KSKL (**I**,**J**). Two days after transfection the cells were processed for direct fluorescence using the appropriate filters. Scale bar = 10 μm.

Figure 6 Tissue distribution of human PEX5R mRNA

^{32}P-labelled Pex5R cDNA was hybridized to a human multiple tissue Northern blot (**A**, top panel) and a human RNA dot blot (**B**). The origin of the various RNAs on the dot blot are indicated in (**C**). The same Northern blot was re-hybridized with human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA as a probe to document the amount of RNA (**A**, lower panel).

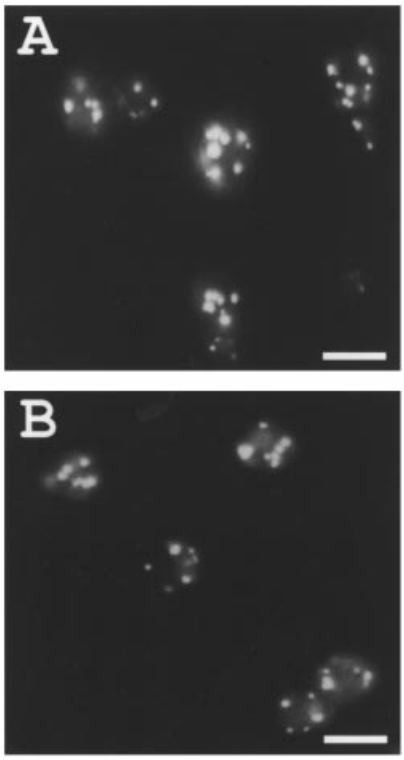

Figure 8 Yeast cells devoid of the putative Pex5Rp homologue, YMR018wp, still import PTS1 and assemble peroxisomes

The wild-type *S. cerevisiae CB80* strain (**A**) as well as the *YMR018w* deletion strain (**B**) were transformed with a construct coding for the EGFP-SKL reporter protein. Transformed cells were grown and processed for direct fluorescence as described [32]. Scale bar = 10 μm.

localization of Pex5Rp was indirectly determined by immunofluorescence microscopy. For this, *Pex5*$^{-/-}$ mouse fibroblasts were transiently transfected with constructs coding for HsPex5RpA fused either N- or C-terminally to EGFP. Direct fluorescence microscopy indicated that both fusion proteins were distributed throughout the cytoplasm (Figures 7K and 7L). Similar results were obtained when CHO-cells were transfected with constructs coding for EGFP- or FLAG-tagged HsPex5Rp-fusion proteins (results not shown). The possibility that the distribution of the over-expressed EGFP- and FLAG-tagged Pex5Rp-fusion proteins differs from the subcellular distribution of the untagged proteins expressed at normal levels can currently not be excluded.

Identification and characterization of a putative yeast homologue of Pex5Rp

By using yeast Pex5p as query to search the *S. cerevisiae* database, a hypothetical protein (open reading frame *YMR018w*; accession number Q04364) of roughly similar size and 27.1 % identity was found (results not shown). This 59.1 kDa protein of 514 amino acids was 25 % identical to human PEX5p (results not shown). To determine if *YMR018w* is required for PTS1 protein import in *S. cerevisiae*, the entire open reading frame of *YMR018w* was replaced by a PCR-generated *kanMX4* cassette [36]. Since peroxisome assembly mutants (and fatty acid oxidation mutants) do not grow on media that contain fatty acids as sole carbon source [46], the wild-type and the *ΔYMR018w* deletion strain were streaked on to oleic acid plates. However, both the *YMR018w* deletion strain and the wild-type strain were capable of growing on oleic acid plates (results not shown). As a result, the *YMR018w* gene is dispensable for growth on oleic acid and, as a consequence, the corresponding mutant will not display the phenotype of a peroxisome assembly (or fatty acid oxidation) mutant [46]. Additional evidence that *YMR018w* does not function in PTS1 protein import was obtained from the observation that (i) the EGFP-PTS1 reporter protein in the wild-type as well as in the *YMR018w* deletion strain displayed a peroxisomal fluorescence pattern (Figure 8) and (ii) YMR018wp did not bind PTS1 either *in vitro* or *in vivo* (results not shown).

DISCUSSION

By independent approaches, three related human cDNAs were identified coding for a TPR-containing Pex5-like protein. The proteins encoded by the brain-derived cDNAs were of a similar size as Pex5p, the one from liver cDNA contained only the C-terminal TPR-domain. Binding studies revealed that the cloned proteins not only recognized peptides containing the C-terminal tripeptide serine-lysine-leucine, but, in addition, displayed the same binding specificity as Pex5p for peptides and proteins containing other PTS1 variants. Therefore, we called these novel PTS1-binding proteins Pex5p-related proteins (abbreviated Pex5Rp). Analysis of genomic bacterial artificial chromosome clones revealed that the mRNAs encoding the three isoforms are most probably the result of alternative splicing and that the corresponding gene, *PEX5R*, is localized on chromosome 3q26.2–3q27. Northern-blot analysis demonstrated that *PEX5R* is not ubiquitously expressed. Rather, PEX5R mRNA is preferentially expressed in brain, and to a far lower extent in testis and pituitary gland. *PEX5*, on the other hand, is expressed in all tissues [15,16]. Unfortunately, we were unable to demonstrate the presence of Pex5Rp, either the long or the short form, in these tissues, most likely because of their low abundance. In this context, it is interesting to note that EST database searches revealed that the *PEX5R* gene is poorly transcribed compared with the *PEX5* (*PXR1*) gene. These observations may also explain why screening a human cDNA yeast two-hybrid library for PTS1-binding proteins [14] and probing the EST database with yeast Pex5p [15,16] only resulted in the identification of Pex5p, and not Pex5Rp. Human PEX5p and Pex5RpA display 26.4 % (31.7 %) and 25 % (27.8 %) sequence identity to *S. cerevisiae* (*P. pastoris*) Pex5p respectively. Given the presence of two genes encoding PTS1-binding proteins, at least in mammals, cloning of new Pex5ps in different species or organisms, based on homology or functional identification, should be controlled more carefully.

In order to define a function for Pex5Rp, we determined its subcellular localization and possible interactions with other peroxins. Since we were unable to detect endogenous Pex5Rp either in various mouse tissues or in subcellular fractions of rat liver, mouse fibroblasts and CHO cells, the intracellular localization of Pex5Rp was determined by expressing this molecule, tagged with EGFP or FLAG at its N- or C-terminus, in mouse fibroblasts and CHO cells. These experiments demonstrated that the tagged Pex5Rp molecules were largely distributed throughout the cytosol, even in cells expressing intermediate or low levels of the protein. With the caveat that overexpression of a protein can affect its subcellular localization, we conclude that Pex5Rp is a cytosolic molecule. Pex5p, however, is present not only in the cytosol. A small, but significant part of this molecule is also associated with peroxisomes [19,25], and this peroxisome-associated pool most likely represents Pex5p bound to the peroxisomal integral membrane protein

Pex14p [22,47]. In this context, it is interesting to note that Pex5Rp does not interact with Pex14p. Moreover, no interaction could be observed with the RING finger domain of Pex12p, a protein that is reported to act downstream of the Pex5p-receptor docking event [48].

A detailed comparison of the primary structures of Pex5Rp and Pex5p revealed that the C-terminal TPR-containing regions of Pex5p, which are responsible for PTS1 binding, are highly conserved. In addition, the asparagine at position 526 in Pex5pL, which is reported to be essential for PTS1 protein import [15], is conserved in Pex5Rp. Alkylation of Pex5Rp also destroys the binding to PTS1. Interestingly, Pex5Rp contains three cysteine residues, located in three different TPRs (assigned TPR2, TPRII and TPR5; see Figure 1), and the surrounding amino acids are well conserved between Pex5Rp and other (mammalian) PEX5ps. However, the WXXXF/Y pentapeptide motifs and the TPR IV motif (Figure 1) required for binding of Pex5p to Pex14p [20] and Pex12p (M. Fransen and P. P. Van Veldhoven, unpublished work) respectively, are absent in the Pex5Rp isoforms. No interactions could be demonstrated between the Pex5Rp isoforms and Pex2p, Pex3p, Pex5p, Pex7p, Pex10p, Pex11pα, Pex11pβ, Pex12p, Pex13p, Pex14p, Pex16p or Pex19p.

In another series of experiments, the yeast protein database was screened for a putative orthologue. These searches yielded a yeast open-reading frame, *YMR018w*, encoding a hypothetical protein with unknown function of roughly similar size and 25% identity to Pex5Rp. However, *in vitro* and *in vivo* binding experiments clearly demonstrated that the protein encoded by *YMR018w* did not interact with PTS1. In addition, a *ΔYMR018w* deletion strain was capable of growing on oleic acid plates. These results indicate that the corresponding mutant does not display the phenotype of a peroxisome assembly (or fatty acid oxidation) mutant [46], a result that could be confirmed by the observation that in this deletion strain the EGFP-PTS1 reporter protein displays a peroxisomal fluorescence pattern. In conclusion, these data prove that YMR018wp, the closest-related yeast protein based on sequence homology, is not an orthologue of Pex5Rp. Also, based on sequence similarities, no *PEX5R* homologues were found in plants and in insects.

A comparison of the partially available genomic structures of *PEX5* (*PXR1*) and *PEX5R* showed that both genes most probably consist of 15 exons (results not shown). In addition, the positions of the introns, although their lengths are considerably larger in the *PEX5R* gene, are largely identical (results not shown). This similar organization favours the idea that *PXR1* and *PEX5R* are evolutionarily related and originate from a common ancestral gene. Whether such a gene evolution can explain why we did not find an orthologue of Pex5Rp in *S. cerevisiae*, remains to be established.

Although we have identified three isoforms of a Pex5p-related novel protein that all specifically and directly bind PTS1 proteins, the absence of a well-defined gene-related phenotype (e.g. a knock-out strain of a yeast homologue, a patient cell line or a mouse model) or other known interaction partners makes it difficult to define a functional role for these proteins. Smith and Johnson [49] recently reported that a portion of a loop in the homeodomain of the yeast DNA-binding protein α2 is not only recognized by the co-repressor Ssn6, a TPR-protein, but also by the TPR domains of yeast Pex5p. Apparently, this interaction is mediated by SRI, a PTS1-like sequence although not possessing a free carboxy group. Presumably, this is an example of a nonphysiological interaction for Pex5p. With respect to Pex5Rp, there is currently no reason to argue that the displayed binding specificity of Pex5Rp and Pex5p for exactly the same set of peroxisomal proteins is purely coincidental. Thus, based on our findings that the different isoforms of Pex5Rp (i) display the same binding specificity for PTS1 proteins as they do for Pex5p, (ii) can compete with Pex5p for PTS1 binding *in vitro*, (iii) are localized exclusively to the cytosol, the site where PTS1 proteins are synthesized and (iv) are not ubiquitously expressed, we speculate that in certain tissues, e.g. fetal brain, the *PEX5R* gene products may play a regulating role in an early step of the PTS1 protein import process.

Note added in proof (received 14 June 2001)

Since this paper was originally submitted and revised, Chen et al. [51] reported on the identification of the rat orthologue of Pex5Rp, which they termed TRIP8b and which was identified as a Rab8b-interacting protein using the yeast two-hybrid system. The authors claimed that TRIP8b, in contrast with our data, does not bind PTS1-ligands and they proposed a role for this protein in regulated secretion.

We thank Mrs. C. Brees and Mrs. V. Brys for their excellent technical support, Dr A. Hartig (Institut für Biochemie und Molekulare Zellbiologie, Universität Wein, Wein, Austria) for the yeast strain CB80 and the plasmid pJR233, and Karen Ghys (Katholieke Universiteit Leuven, Campus Gasthuisberg, Afdeling Farmakologie, Leuven, Belgium) and Dr Myriam Baes for the pKG26 and pMycPex5p/pEGFP-KSKL plasmids respectively. L. Amery is supported by a fellowship from the Vlaams Instituut voor de Bevordering van het Wetenschappelijk-Technologisch Onderzoek in de Industrie (IWT) and M. Fransen is a postdoctoral fellow of the Fonds voor Wetenschappelijk Onderzoek – Vlaanderen (FWO). This work is supported by a Geconcerteerde Onderzoeksacties grant (GOA/99/09) from the Flemish government.

REFERENCES

1 Blatch, G. L. and Lassle, M. (1999) The tetratricopeptide repeat: a structural motif mediating protein-protein interactions. Bioessays **21**, 932–939
2 Corpet, F., Gouzy, J. and Kahn, D. (1999) Browsing protein families via the 'Rich Family Description' format. Bioinformatics **15**, 1020–1027
3 Haucke, V., Horst, M., Schatz, G. and Lithgow, T. (1996) The Mas20p and Mas70p subunits of the protein import receptor of yeast mitochondria interact via the tetratricopeptide repeat motif in Mas20p: evidence for a single hetero-oligomeric receptor. EMBO J. **15**, 1231–1237
4 Young, J. C., Obermann, W. M. and Hartl, F. U. (1998) Specific binding of tetratricopeptide repeat proteins to the C-terminal 12-kDa domain of hsp90. J. Biol. Chem. **273**, 18007–18010
5 McCollum, D., Monosov, E. and Subramani, S. (1993) The *pas8* mutant of *Pichia pastoris* exhibits the peroxisomal protein import deficiencies of Zellweger syndrome cells – the PAS8 protein binds to the COOH-terminal tripeptide peroxisomal targeting signal, and is member of the TPR protein family. J. Cell Biol. **121**, 761–774
6 Van der Leij, I., Franse, M. M., Elgersma, Y., Distel, B. and Tabak, H. F. (1993) PAS10 is a tetratricopeptide-repeat protein that is essential for the import of most matrix proteins into peroxisomes of *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. U.S.A. **90**, 11782–11786
7 Van der Klei, I. J., Hilbrands, R. R., Swaving, G. J., Waterham, H. R., Vrieling, E. G., Titorenko, V. I., Cregg, J. M., Harder, W. and Veenhuis, M. (1995) The *Hansenula polymorpha PER3* gene is essential for the import of PTS1 proteins into the peroxisomal matrix. J. Biol. Chem. **270**, 17229–17236
8 Szilard, R. K., Titorenko, V. I., Veenhuis, M. and Rachubinski, R. A. (1995) Pay32p of the yeast *Yarrowia lipolytica* is an intraperoxisomal component of the matrix protein translocation machinery. J. Cell Biol. **131**, 1453–1469
9 De Walque, S., Kiel, J. A., Veenhuis, M., Opperdoes, F. R. and Michels, P. A. (1999) Cloning and analysis of the PTS-1 receptor in *Trypanosoma brucei*. Mol. Biochem. Parasitol. **104**, 106–119
10 Jardim, A., Liu, W., Zheleznova, E. and Ullman, B. (2000) Peroxisomal targeting signal-1 receptor protein PEX5 from *Leishmania donovani*. Molecular, biochemical, and immunocytochemical characterization. J. Biol. Chem. **275**, 13637–13644
11 Kragler, F., Lametschwandtner, G., Christmann, J., Hartig, A. and Harada, J. J. (1998) Identification and analysis of the plant peroxisomal targeting signal 1 receptor *NtPEX5*. Proc. Natl. Acad. Sci. U.S.A. **95**, 13336–13341
12 Wimmer, C., Schmid, M., Veenhuis, M. and Gietl, C. (1998) The plant PTS1 receptor: similarities and differences to its human and yeast counterparts. Plant J. **16**, 453–464
13 Brickner, D. G., Brickner, J. H. and Olsen, L. J. (1998) Sequence analysis of the cDNA encoding Pex5p, a peroxisomal targeting signal type 1 receptor from *Arabidopsis*. Plant Physiol. **118**, 330

14 Fransen, M., Brees, C., Baumgart, E., Vanhooren, J. C., Baes, M., Mannaerts, G. P. and Van Veldhoven, P. P. (1995) Identification and characterization of the putative human peroxisomal C-terminal targeting signal import receptor. J. Biol. Chem. **270**, 7731–7736
15 Dodt, G., Braverman, N., Wong, C., Moser, A., Moser, H. W., Watkins, P., Valle, D. and Gould, S. J. (1995) Mutations in the PTS1 receptor gene, *PXR1*, define complementation group 2 of the peroxisome biogenesis disorders. Nat. Genet. **9**, 115–125
16 Wiemer, E. A., Nuttley, W. M., Bertolaet, B. L., Li, X., Francke, U., Wheelock, M. J., Anne, U. K., Johnson, K. R. and Subramani, S. (1995) Human peroxisomal targeting signal-1 receptor restores peroxisomal protein import in cells from patients with fatal peroxisomal disorders. J. Cell Biol. **130**, 51–65
17 Baes, M., Gressens, P., Baumgart, E., Carmeliet, P., Casteels, M., Fransen, M., Evrard, P., Fahimi, D., Declercq, P. E., Collen, D. et al. (1997) A mouse model for Zellweger syndrome. Nat. Genet. **17**, 49–57
18 Otera, H., Okumoto, K., Tateishi, K., Ikoma, Y., Matsuda, E., Nishimura, M., Tsukamoto, T., Osumi, T., Ohashi, K., Higuchi, O. and Fujiki, Y. (1998) Peroxisome targeting signal type 1 (PTS1) receptor is involved in import of both PTS1 and PTS2: studies with *PEX5*-defective CHO cell mutants. Mol. Cell. Biol. **18**, 388–399
19 Subramani, S. (1998) Components involved in peroxisome import, biogenesis, proliferation, turnover, and movement. Physiol. Rev. **78**, 171–188
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 Albertini, M., Rehling, P., Erdmann, R., Girzalsky, W., Kiel, J. A., Veenhuis, M. and Kunau, W. H. (1997) Pex14p, a peroxisomal membrane protein binding both receptors of the two PTS-dependent import pathways. Cell **89**, 83–92
22 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
23 Terlecky, S. R. and Fransen, M. (2000) How peroxisomes arise. Traffic **1**, 465–473
24 Powers, J. M. and Moser, H. W. (1998) Peroxisomal disorders: genotype, phenotype, major neuropathologic lesions, and pathogenesis. Brain Pathol. **8**, 101–120
25 Dodt, G. and Gould, S. J. (1996) Multiple *PEX* genes are required for proper subcellular distribution and stability of Pex5p, the PTS1 receptor: evidence that PTS1 protein import is mediated by a cycling receptor. J. Cell Biol **135**, 1763–1774
26 Warren, D. S., Morrell, J. C., Moser, H. W., Valle, D. and Gould, S. J. (1998) Identification of *PEX10*, the gene defective in complementation group 7 of the peroxisome-biogenesis disorders. Am. J. Hum. Genet. **63**, 347–359
27 Urquhart, A. J., Kennedy, D., Gould, S. J. and Crane, D. I. (2000) Interaction of Pex5p, the type 1 peroxisome targeting signal receptor, with the peroxisomal membrane proteins Pex14p and Pex13p. J. Biol. Chem. **275**, 4127–4136
28 Lametschwandtner, G., Brocard, C., Fransen, M., Van Veldhoven, P. P., Berger, J. and Hartig, A. (1998) The difference in recognition of terminal tripeptides as peroxisomal targeting signal 1 between yeast and human is due to different affinities of their receptor Pex5p to the cognate signal and to residues adjacent to it. J. Biol. Chem. **273**, 33635–33643
29 Amery, L., Brees, C., Baes, M., Setoyama, C., Miura, R., Mannaerts, G. P. and Van Veldhoven, P. P. (1998) C-terminal tripeptide Ser-Asn-Leu (SNL) of human D-aspartate oxidase is a functional peroxisome-targeting signal. Biochem. J. **336**, 367–371
30 Baumgart, E., Vanhooren, J. C., Fransen, M., Marynen, P., Puype, M., Vandekerckhove, J., Leunissen, J. A., Fahimi, H. D., Mannaerts, G. P. and Van Veldhoven, P. P. (1996) Molecular characterization of the human peroxisomal branched-chain acyl-CoA oxidase: cDNA cloning, chromosomal assignment, tissue distribution, and evidence for the absence of the protein in Zellweger syndrome. Proc. Natl. Acad. Sci. U.S.A. **93**, 13748–13753
31 Nagase, T., Ishikawa, K., Nakajima, D., Ohiri, M., Seki, N., Miyajima, N., Tanaka, A., Kotani, H., Nomura, N. and Ohara, O. (1997) Prediction of the coding sequences of unidentified human genes. VII. The complete sequences of 100 new cDNA clones from brain which can code for large proteins *in vitro*. DNA Res. **4**, 141–150
32 Brocard, C., Lametschwandtner, G., Koudelka, R. and Hartig, A. (1997) Pex14p is a member of the protein linkage map of Pex5p. EMBO J. **16**, 5491–5500
33 Gietz, D., St-Jean, A., Woods, R. A. and Schiestl, R. H. (1992) Improved method for high efficiency transformation of intact yeast cells. Nucleic Acids Res. **20**, 1425
34 Lennon, G., Auffray, C., Polymeropoulos, M. and Soares, M. B. (1996) The I.M.A.G.E. Consortium: an integrated molecular analysis of genomes and their expression. Genomics **33**, 151–152
35 Carninci, P. and Hayashizaki, (1998) High-efficiency full-length cDNA cloning. Methods Enzymol. **303**, 19–44
36 Wach, A., Brachat, A., Pohlmann, R. and Philippsen, P. (1994) New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. Yeast **10**, 1793–1808
37 Elgersma, Y., van den Berg, M., Distel, P. and Tabak, H. F. (1993) An efficient positive selection procedure for the isolation of peroxisomal import and peroxisome assembly mutants of *Saccharomyces cerevisiae*. Genetics **135**, 731–740
38 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 using the biotinylated PTS1-receptor. Anal. Biochem. **242**, 26–36
39 Boussif, O., Lezoualc'h, F., Zanta, M. A., Mergny, M. D., Scherman, D., Demeneix, B. and Behr, J. P. (1995) A versatile vector for gene and oligonucleotide transfer into cells in culture and *in vivo*: polyethylenimine. Proc. Natl. Acad. Sci. U.S.A. **92**, 7297–7301
40 Amery, L., Fransen, M., De Nys, K., Mannaerts, G. P. and Van Veldhoven, P. P. (2000) Mitochondrial and peroxisomal targeting of 2-methylacyl-CoA racemase in humans. J. Lipid Res. **41**, 1752–1759
41 Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25**, 3389–3402
42 Corpet, F. (1988) Multiple sequence alignment with hierarchical clustering. Nucleic Acids Res. **16**, 10881–10890
43 Huang, X. (1994) On global sequence alignment. Comput. Appl. Biosci **10**, 227–235
44 Fransen, M., Marynen, P., Baumgart, E., Vanhooren, J. C., Mannaerts, G. P. and Van Veldhoven, P. P. (1996) Further characterization of the human peroxisomal C-terminal targeting signal protein import receptor. Ann. N.Y. Acad. Sci. **804**, 672–673
45 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
46 Erdmann, R., Veenhuis, M., Mertens, D. and Kunau, W. H. (1989) Isolation of peroxisome-deficient mutants of *Saccharomyces cerevisiae*. Proc. Natl Acad. Sci. U.S.A. **86**, 5419–5423
47 Otera, H., Harano, T., Honsho, M., Ghaedi, K., Mukai, S., Tanaka, A., Kawai, A., Shimizu, N. and Fujiki, Y. (2000) The mammalian peroxin Pex5pL, 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
48 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
49 Smith, R. L. and Johnson, A. D. (2000) A sequence resembling a peroxisomal targeting sequence directs the interaction between the tetratricopeptide repeats of Ssn6 and the homeodomain of alpha 2. Proc. Natl. Acad. Sci. U.S.A. **97**, 3901–3906
50 Fransen, M., Brees, C., 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
51 Chen, S., Liang, M. C., Chia, J. N., Ngsee, J. K. and Ting, A. E. (2001) Rab8b and its interacting partner TRIP8b are involved in regulated secretion in AtT20 cells. J. Biol. Chem. **276**, 13209–13216

Received 11 December 2000/12 April 2001; accepted 16 May 2001