Functional studies on human Pex7p: subcellular localization and interaction with proteins containing a peroxisome-targeting signal type 2 and other peroxins

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Pex7p is a WD40-containing protein involved in peroxisomal import of proteins containing an N-terminal peroxisome-targeting signal (PTS2). The interaction of human recombinant Pex7p expressed in different hosts/systems with its PTS2 ligand and other peroxins was analysed using various experimental approaches. Specific binding of human Pex7p to PTS2 could be demonstrated only when Pex7p was formed *in vitro* by a coupled transcription/translation system or synthesized *in vivo* in Chinese hamster ovary K1 cells transfected with a construct coding for a Pex7p-green fluorescent protein (GFP) fusion protein. Apparently, no cofactors are required and only monomeric Pex7p binds to PTS2. The interaction is reduced upon cysteine alkylation and is impaired upon truncation of the N-terminus of Pex7p. Interaction of Pex7p with other peroxins could not be demonstrated in bacterial or yeast two-hybrid screens, or in pull-down binding assays. The GFP fusion proteins, tagged at either the N- or C-terminus, were able to restore PTS2 import in rhizomelic chondrodysplasia punctata fibroblasts, and Pex7p-GFP was located both in the lumen of peroxisomes and in the cytosol.

Key words: biogenesis, peroxin, protein import, thiolase, WD40.

INTRODUCTION

The functional significance of peroxisomes is highlighted by a class of inherited lethal human diseases in which no recognizable peroxisomes are present [1,2]. In recent years, much has been learned about how peroxisomes arise and how peroxisomal proteins are imported [3,4]. Most peroxisomal matrix proteins have *cis*-acting targeting signals that mediate their active import into peroxisomes, and the great majority of these proteins are targeted to the peroxisomes by a C-terminal tripeptide biochemically related to Ser-Lys-Leu, called peroxisome-targeting signal (PTS) 1 [5]. A second PTS, PTS2, is an N-terminal signal consisting of the loose consensus sequence Arg/Lys-Leu-Xaa$_5$-His/Gln-Leu, and is found in only a few matrix proteins [6]. The import of PTS1 and PTS2 proteins into peroxisomes is mediated by the hydrophilic receptors Pex5p and Pex7p, respectively [7].

Pex7p, initially discovered in *Saccharomyces cerevisiae* [8,9], has been described in various fungi, plants and mammals and is characterized by the presence of six WD40 motifs [9–14]. Phenotypically, *pex*7 mutants display impaired import of PTS2 proteins, such as 3-ketoacyl-CoA thiolase. Based on these observations and the fact that *S. cerevisiae* and *Pichia pastoris* Pex7p interact physically with the PTS2 sequences of *S. cerevisiae* and human thiolase, Pex7p is considered to be the receptor for PTS2 proteins [13,15,16]. In humans, loss of Pex7p function gives rise to a syndrome called rhizomelic chondrodysplasia punctata (RCDP) type 1. In cells derived from such patients, 3-ketoacyl-CoA thiolase, phytanoyl-CoA hydroxylase and alkyl-dihydroxyacetone phosphate synthase, all PTS2 proteins, are not imported into peroxisomes. Mislocalization and, as a consequence, eventual instability or proteolytic degradation of the two latter enzymes, explain the accumulation of phytanic acid and the deficiency of ether lipids in RCDP children. Whether the loss of thiolase has any clinical consequences is not known. A thiolase deficiency in a patient resembling a Zellweger case has been described [17], but the described clinical abnormalities, such as abnormal bile acids [18], do not fit with the current picture of β-oxidation [19] whereby bile acid intermediates and pristanic acid are processed via another peroxisomal thiolase, SCPx-thiolase [20].

The subcellular localization of Pex7p is a matter of debate. In *S. cerevisiae* and human fibroblasts, N-terminally Myc-tagged Pex7p was found predominantly in the cytosol [8,9], whereas Pex7p, C-terminally tagged with three haemagglutinin sequences, was localized entirely inside the peroxisomes in *S. cerevisiae* [10]. In *P. pastoris*, Pex7p is distributed between the cytosol and the peroxisomal matrix [13]. Depending on its subcellular localization, it has been proposed that Pex7p may function as an intraperoxisomal pulling receptor [16] or as a cytosol-to-peroxisome shuttling receptor [13,15]. In yeast, Pex18p and Pex21p are key components in the targeting of Pex7p to peroxisomes [21]. Mammalian orthologues of Pex18p and Pex21p have not been identified yet. In mammals, the PTS1 receptor, Pex5p, exists in two forms, a short form (Pex5pS), and a long form (Pex5pL) [22]. Interestingly, Pex5pL binds Pex7p directly and appears to mediate the translocation of the Pex7p–PTS2 protein complex into peroxisomes via its initial docking site, Pex14p [23,24]. Furthermore, Pex5p also functions, at least in mammals, as a cycling receptor: it delivers its cargo proteins to the peroxisomal matrix and then returns to the cytosol for another round of transport [25].

So far an interaction between human Pex7p and PTS2 proteins has not been documented by means of a yeast two-hybrid system, and to confirm the presence of a PTS2 sequence in human

Abbreviations used: PTS, peroxisome-targeting signal; GFP, green fluorescent protein; RCDP, rhizomelic chondrodysplasia punctata; GST, glutathione S-tranferase; HMG-CoA synthase, 3-hydroxy-3-methylglutaryl-CoA synthase; CHO, Chinese hamster ovary; *Hs*Pex7p, *Homo sapiens* Pex7p; Ni-NTA, Ni^{2+}-nitrilotriacetate; TBS, Tris-buffered saline; TNT, transcription/translation.

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phytanoyl-CoA hydroxylase Mihalik et al. [26] relied on the use of yeast Pex7p. This raises the possibility that species-specific bridging proteins might exist. In addition, not much is known about cofactors or other biochemical parameters influencing the binding between Pex7p and PTS2. In contrast, the critical role of some residues in the PTS2 sequence [15,16] and various mutations in Pex7p, resulting in a non-functional PTS2 receptor [10–12], are documented for the yeast and human proteins. As is the case in certain Zellweger patients, the absence of both isoforms of Pex5p in the Zellweger mouse perturbs the import of PTS2 proteins and the processing of thiolase [27]. The availability of a functional mammalian Pex7p–PTS2 interaction assay would be advantageous to document in this model the presence of other (unknown) PTS2 proteins, assuming that these proteins, like thiolase, will not be processed.

In this report, we describe an *in vitro* binding assay showing that human Pex7p, when expressed in a mammalian expression system, specifically binds to PTS2 proteins. In addition, we demonstrate that, at least under certain conditions, this functional Pex7p molecule is distributed between the cytosol and the peroxisomal matrix. Finally, we show that the peroxisomal association of Pex7p and PTS2 import is dependent on the long isoform of Pex5p.

MATERIALS AND METHODS

Generation of plasmids

The oligonucleotides and plasmids used throughout this work are compiled in Tables 1 and 2, respectively. Cloning vectors were obtained from Stratagene (pBluescript SK + vector), Amersham Bioscience (pGEX-4T-1 vector), Clontech (pAS2-1, pGAD424, pGBT9, pEGFP-C1 and pEGFP-N1 vectors), Invitrogen [pcDNA3.1(+)Myc-His, pCR2.1-TOPO, pBADHis and pYES2 vectors] and Hybrigenics (pUT18, pUT18C and pKT25 vectors).

Table 1 Oligonucleotides used in this study

Restriction sites are underlined.

Name	Oligonucleotide
HMGCS1F-BamHI	5′-CGGGATCCACCATGCCTGGATCAC-3′
HMGCS1R-KpnI	5′-TAGGTACCGAGTATCTTAATGTTCCCC-3′
HPAHX-F2	5′-GGAATTCGCCATGGAGCAGCTTCG-3′
HPAHX-R2	5′-CCCCCGTCGACTCAAAGATTGGTTCTTTCTCC-3′
HPex7-Fwd	5′-CGGAATTCGGGATGAGTGCGGTGTGC-3′
HPex7-Rev	5′-CCACTCGAGTGTATCTCTCAAGCAGGAATAG-3′
HPTS2-F2	5′-GGATCCAGATGAAGCTGGGCTAAGGC-3′
MmPex5L-Fwd	5′-CCGCTCGAGATGGCAATGCGGGAGCTGGTGGAGG-3′
MmPex5L-Rev	5′-GCGGTACCTCACTGGGGCAGGCCAAACATAGC-3′
MmPex5L-Fwd2	5′-CCGCTCGAGCTATGGCAATGCGGGAGCTGGTGG-3′
MmPex5L-Rev2	5′-CGGCTCGAGTCACTGGGGCAGGCCAAACATAG-3′
MmPex5L-Fwd3	5′-CCGCTCGAGGATGGCAATGCGGGAGCTGGTG-3′
MVK-F-BamHI	5′-TCGGATCCGCCATGTTGTCAGAAGTCC-3′
MVK-R-KpnI	5′-TGGGTACCTCTCAGAGGCCATC-3′
Pex14.3	5′-GGGAAGATCTATGGCGTCCTCGGAGCAG-3′
Pex14.2	5′-AAACTGCAGCTACCTGGGCTCAAGCTG-3′
Pex5.1	5′-CGAGATCTGTATGGCAATGCGGGAGCTGG-3′
Pex5.2	5′-GCCCGTCGACCTGTCACTGGGGCAGGCCAAAC-3′
Pex7GFPC-Fwd	5′-GGAATTCAATGAGTGCGGTGTGCGGTG-3′
Pex7GFP-Rev	5′-CCGCTCGAGATAGCAGGAATAGTAAGACAAG-3′
Pex7WD-Fwd2	5′-CGGAATTCACCATGAGAAGCTTTGACTGGAATGATG-3′
PTS2Mut1	5′-GGGCTCGAGCGATGCATGGGCTGCAGGTAG-3′
PTS2Mut2	5′-GGGCTCGAGCGATGCATCGGCTGCAGGTAGTGCTGGGCGGCCTGG-3′
RedBglF1	5′-GCTCAGATCTGCAGGGATGGCCTCCTGGG-3′
RedEcoR1	5′-GCTCGGAATTCCTCAGAGCTTAGCTTTCTC-3′
ThioB-F-NotI	5′-TGGGACGCGCGGCCGCGATGCATCGGCTGC-3′
ThioB-F-XbaI	5′-GCTCTAGAGATGCATCGGCTGCAGG-3′
ThioB-R-BamHI	5′-TAGGATCCTCAGTTCCCAGGGTATTCA-3′
ThioB-R2-BamHI	5′-TAGGATCCAGTTCCCAGGGTATTCAAAG-3′
Thio-Fwd	5′-CGGAATTCGCAATGCAGAGGCTGCAGG-3′
Thio-Rev	5′-GCGGATCCTCACTCAGTTCCCAGGG-3

For reverse transcriptase-PCR applications, total RNA was isolated from human liver using the TRIzol protocol (Invitrogen) and first-strand cDNA synthesis was performed by means of reverse transcriptase (Boehringer Mannheim). The use of human liver (liver segment resection for hepatic tumours) was approved by the Ethics Committee of Katholieke Universiteit Leuven, Leuven, Belgium.

For PCR reactions, *Pfx* DNA polymerase (Invitrogen) or *Taq* DNA polymerase (Amersham Bioscience) was used, and amplicons were subcloned either directly after restriction or indirectly, after adding an A overhang in the case of *Pfx* DNA polymerase, into pCR2.1-TOPO. Other DNA manipulations were done according to standard laboratory methods [28].

Using yeast Pex7p [8] as a query, human expressed sequence tags containing part of the cDNA sequence of the human homologue were recovered by the BLAST program [29]. The insert of I.M.A.G.E. [30] clone 259183 was sequenced and a ^{32}P-labelled 0.9 kb *Not*I/*Eco*RI fragment of this clone was hybridized to a λgt11 human liver cDNA library (Clontech) to recover additional clones. Positive plaques were isolated, analysed by PCR and sequenced. Additional sequence information was obtained by 5′-rapid amplification of cDNA ends using human liver cDNA (Clontech) as a template. During attempts to cover the information surrounding the start codon, the cloning of the full-length human Pex7p was reported by others [10,12], allowing the design of primers to generate the complete coding sequence by PCR.

The cDNA coding for human thiolase [31] was obtained by reverse transcriptase-PCR on total RNA. The sources of the other cDNAs are indicated in Table 2. The plasmids encoding fusion proteins of peroxins other than Pex7p and Pex5pL are described elsewhere [32,33].

Purification of (recombinant) proteins

Rat thiolase (isoform A) was purified as described before [20]. $(His)_6$-tagged fusion proteins were purified by means of Ni^{2+}-nitrilotriacetate (Ni-NTA)–agarose according the manufacturer's instructions (Qiagen).

Production and affinity purification of antisera

The rabbit polyclonal antibodies directed against rat thiolase A [20], Pex14p [34] and green fluorescent protein (GFP) [35] have been described before. The anti-GFP antiserum was further affinity purified by applying it on to a Ni-NTA–agarose column (Qiagen), loaded with $(His)_6$-tagged GFP [35], followed by washing with Tris-buffered saline (TBS), containing 0.5 % (w/v) Tween-20, and elution with ImmunoPure Gentle Elution Buffer (Pierce). The 6× His monoclonal antibody was purchased from Clontech. The 1-9E10.2 hybridoma cell line producing anti-Myc antibody [36] was obtained from the A.T.C.C.

Two-hybrid analyses

For the yeast two-hybrid analyses [37] the *S. cerevisiae* strain SFY526 was transformed with vectors coding for *Homo sapiens* Pex7p (*Hs*Pex7p), other mouse or human peroxins, rat or human thiolases, or human phytanoyl-CoA hydroxylase, fused to the Gal4p DNA-binding and/or activation domains, according to the manufacturer's instructions (Clontech). Double transfor-

Table 2 Plasmids used in this study

Hs, Homo sapiens; Rn, Rattus norvegicus; Mm, Mus musculus; BD, binding domain; AD, activation domain; GST, glutathione S-tranferase; GFP, green fluorescent protein; PAHX, phytanoyl-CoA α-hydroxylase; RT-PCR, reverse transcriptase PCR.

Name	Protein	Cloning vector	Insert
pKG6	Gal4pBD-*Hs* thiolase	*Eco*RI/*Bam*HI pGBT9	*Eco*RI/*Bam*HI digest of RT-PCR product generated with Thio-Fwd and Thio-Rev on total human liver RNA
pKG7	Gal4pAD-*Hs* thiolase	*Eco*RI/*Bgl*II pGAD424	*Eco*RI/*Bam*HI fragment of pKG6
pKG14	*Hs* Pex7p	*Eco*RI/*Xho*I pBluescript SK+	*Eco*RI/*Xho*I digest of RT-PCR product generated with HPex7-Fwd and Hpex7-Rev on total human liver RNA
pKG25	GST-*Hs* Pex7p	*Eco*RI/*Xho*I pGEX-4T-1	*Eco*RI/*Xho*I fragment of pKG14
pKG26	Gal4pBD-*Hs* Pex7p	*Eco*RI/*Sal*I pAS2-1	*Eco*RI/*Xho*I fragment of pKG25
pKG27	*Hs* Pex7p	*Eco*RI/*Xho*I pYES2	*Eco*RI/*Xho*I fragment of pKG25
pKG32	*Hs* Pex7p$_{(1-53)}$-GFP	*Eco*RI/*Bam*HI pEGFP-N1	*Eco*RI/*Bam*HI fragment of pKG25
pKG33	*Hs* Pex7p$_{(1-93)}$-GFP	*Eco*RI/*Pst*I pEGFP-N1	*Eco*RI/*Pst*I fragment of pKG25
pKG34	GFP-*Hs* Pex7p$_{(101-313)}$	*Pst*I/*Bam*HI pEGFP-C1	*Pst*I/*Bam*HI fragment of pKG25
pKG40	*Rn* thiolase B	*Not*I/*Bam*HI pYES2	*Not*I/*Bam*HI digest of PCR product generated with ThioB-F-NotI and ThioB-R-BamHI on pTWII [49] as a template
pKG41	(His)$_6$-*Rn* thiolase B	*Xho*I/*Hin*dIII pBADHisC	*Xho*I/*Hin*dIII fragment of pKG40
pKG43	GST-*Hs* Pex7p	*Hin*dIII/*Xho*I pKG25	*Hin*dIII/*Xho*I digest of PCR product generated with HPTS2-F2 and Pex7GFP-Rev using pKG25 as a template
pKG44	*Hs* Pex7p-GFP	*Eco*RI/*Sal*I pEGFP-N1	*Eco*RI/*Xho*I fragment of pKG43
pKG48	(His)$_6$-*Hs* PAHX	*Kpn*I/*Hin*dIII pBADHisC	*Kpn*I/*Hin*dIII fragment of pQE31/PAHX [50]
pKG51	(His)$_6$-*Hs* Pex13p	*Bgl*II/*Pst*I pBADHisA	*Bam*HI/*Pst*I fragment of pMF103 [32]
pKG52	(His)$_6$-*Hs* Pex14p	*Bgl*II/*Pst*I pBADHisB	*Bgl*II/*Pst*I fragment of pMF118
pKG58	–	pCR2.1-TOPO	PCR product generated with Pex7GFPC-Fwd and Pex7GFP-Rev on pKG44 as a template
pKG60	GFP-*Hs* Pex7p	*Eco*RI/*Sal*I pEGFP-C1	*Eco*RI/*Xho*I fragment of pKG58
pKG65	*Hs* Pex7p$_{(1-53,141-323)}$	*Bam*HI pKG44	pKG44 without 267 bp *Bam*HI fragment
pKG68	*Hs* Pex5pS	pCR2.1-TOPO	PCR product generated with Pex5.1 and Pex5.2 on pMF105 [33] as a template
pKG69	(His)$_6$-Pex5pS	*Xho*I pLA6	*Xho*I fragment of pKG68
pKG76	–	*Sac*I/*Not*I pYES2	*Sac*I/*Not*I fragment of pKG44
pKG97	(His)$_6$-*Rn* thiolase B$_{(R3G)}$	*Xho*I/*Eco*RI pKG41	*Xho*I/*Eco*RI digest of PCR product generated with PTS2Mut1 and ThioB-R-BamHI on pKG41 as a template
pKG98	(His)$_6$-*Rn* thiolase B$_{(H11G)}$	*Xho*I/*Eco*RI pKG41	*Xho*I/*Eco*RI digest of PCR product generated with PTS2Mut2 and ThioB-R-BamHI on pKG41 as a template
pKG100	(His)$_6$-*Mm* Pex5pL	*Xho*I/*Kpn*I pBADHisA	*Xho*I/*Kpn*I digest of PCR product generated with MmPex5L-Fwd and MmPex5L-Rev on pMycPEX5 [32] as a template
pKG104	*Mm* Pex5pL	pCR2.1-TOPO	PCR product generated with MmPex5L-Fwd2 and MmPex5L-Rev2
pKG107	Gal4pAD-*Mm* Pex5pL	*Sal*I pGAD424	*Xho*I fragment of pKG104
pKG108	*Hs* Pex7p-T18	*Kpn*I pUT18	*Kpn*I fragment of pKG76
pKG109	T25-*Hs* Pex7p	*Kpn*I pKT25	*Kpn*I fragment of pKG76
pKG111	T18-*Mm* Pex5pL	*Sal*I/*Kpn*I pUT18C	*Xho*I/*Kpn*I digest of PCR product generated with MmPex5L-Fwd3 and MmPex5L-Rev on pKG100 as a template
pKG112	T25-*Mm* Pex5pL	*Xho*I/*Kpn*I pMF413 [33]	*Xho*I/*Kpn*I digest of PCR product generated with MmPex5L-Fwd3 and MmPex5L-Rev on pKG100 as a template
pKG113	*Rn* thiolase B	pCR2.1-TOPO	PCR product generated with ThioB-F-XbaI and ThioB-R2-BamHI on pKG40 as a template
pKG115	T18-*Rn* thiolase B	*Xba*I/*Bam*HI pUT18C	*Xba*I/*Bam*HI fragment of pKG113
pKG116	T25-*Rn* thiolase B	*Xba*I/*Bam*HI pKT25	*Xba*I/*Bam*HI fragment of pKG113
pKG119	*Hs* Pex7p$_{(62-323)}$	*Eco*RI/*Sal*I pEGFP-C1	*Eco*RI/*Xho*I digest of PCR product generated with Pex7WD-Fwd2 and Pex7GFP-Rev on pKG25 as a template
pKG120	(His)$_6$-mevalonate kinase	*Bgl*II/*Eco*RI pBADHisB	*Bam*HI/*Eco*RI digest of pCR2.1-TOPO containing PCR product generated with MVK-F-BamHI and MVK-R-KpnI using EST AI048941 as a template
pKG122	*Rn* thiolase B	*Xho*I/*Bam*HI pEGFP-N1	*Xho*I/Ba*m*HI fragment of pKG41
pKG123	*Hs* Pex7p$_{(62-323)}$	*Eco*RI/*Xho*I pcDNA3.1mycHisC	*Eco*RI/*Xho*I digest of PCR product generated with Pex7WD-Fwd2 and Pex7GFP-Rev on pKG25 as a template
pKG126	*Hs* Pex7p$_{(62-323)}$	*Eco*RI/*Xho*I pKG27	*Eco*RI/*Xho*I fragment of pKG123
pKG128	*Hs* Pex7pC204S	pCR2.1-TOPO	PCR product generated with HPex7-Fwd and HPex7-Rev on the combined PCR products generated with HPex7-Fwd/*Hs* Pex7-C204S-F and *Hs* Pex7-C204S-R/HPex7-Rev using pKG44 as a template
pKG132	*Hs* Pex7pC204S	pYES2 (*Eco*RI/*Xho*I)	*Eco*RI/*Xho*I fragment of pKG128
pKG134	*Rn* thiolase B Mut1	pEGFP-N1 (*Xho*I/*Bam*HI)	*Xho*I/*Bam*HI fragment of pKG97
pKG135	*Rn* thiolase B Mut2	pEGFP-N1 (*Xho*I/*Bam*HI)	*Xho*I/*Bam*HI fragment of pKG98
pLA6	(His)$_6$-*Hs* Pex5p$_{(259-639)}$	*Eco*RI pBADHisB	*Eco*RI fragment of *Hs* Pex5p cDNA in Pinpoint Xa-1 vector [51]
pLA58	(His)$_6$-*trans*-2-enoyl-CoA reductase	*Bgl*II/*Eco*RI pBADHisB	*Bgl*II/*Eco*RI digest of PCR product generated with RedBglF1 and RedEcoR1 on pLA40
pMF107	Gal4AD-*Hs* Pex7p	*Eco*RI/*Sal*I pGAD424	*Eco*RI/*Xho*I fragment of pKG25
pMF118	GFP-*Hs* Pex14p	*Bgl*II/*Pst*I pEGFP-C1	*Bgl*II/*Pst*I digest of PCR product generated with Pex14.3 and Pex14.2 on I.M.A.G.E. clone 159483 (GenBank accession no. H16035) as a template

Table 2 (contd.)

Name	Protein	Cloning vector	Insert
pCR2.1/PAHX	PAHX	pCR2.1-TOPO	[52]
pVF104	Gal4pBD-*Hs* PAHX	*Eco*RI/*Sal*I pGBT9	*Eco*RI/*Sal*I digest of PCR product generated with HPAHXF2 and HPAHXR2 on pCR2.1/PAHX as a template
pVF105	Gal4pAD-*Hs* PAHX	*Eco*RI/*Sal*I pGAD424	*Eco*RI/*Sal*I digest of a PCR product generated with HPAHXF2 and HPAHXR2 on pCR2.1/PAHX as a template

mants were selected on synthetic drop-out medium lacking tryptophan and leucine. β-Galactosidase activity was measured by a filter assay as described by the manufacturer (Clontech).

For the bacterial two-hybrid analyses, plasmids coding for proteins fused to the T18 and/or T25 fragments of adenylate cyclase [38] were transformed into competent *Escherichia coli* BTH101 cells. Transformants were plated on indicator plates to study complementation as described [38].

Transfection studies

Chinese hamster ovary (CHO) K1 cells, control human and mouse fibroblasts, *Pex5*$^{-/-}$ mouse fibroblasts [27] or human fibroblasts derived from RCDP type 1 patients, obtained from the NIGMS Human Genetic Mutant Cell Repository (Coriell Institute for Medical Research, Camden, NY, U.S.A.) or kindly provided by Dr G. Dodt (University of Bochum, Bochum, Germany), were cultured in modified Eagle's alpha medium or Dulbecco's modified Eagle's medium, each supplemented with 10% (v/v) fetal bovine serum (Invitrogen) and an antibiotics/anti-mycotics solution [100 units/ml penicillin (base)/100 μg/ml streptomycin (base)/0.25 μg/ml amphotericin B; Invitrogen]. Transfections of CHO-K1 cells were done with the cationic polymer polyethyleneimine as described [39]. Mouse and human fibroblasts were transfected with Lipofectamine Plus (Invitrogen) according to the manufacturer's protocol. After transfection (2 days), the cells were analysed by fluorescence microscopy, either directly or after immunostaining. For immunostaining, monolayers were fixed with 4% (w/v) paraformaldehyde for 10 min and incubated sequentially with 1% (w/v) Triton X-100 for 5 min, the primary polyclonal rabbit antibody (anti-GFP; diluted 1:600), anti-Pex14p (1:200) or anti-thiolase (1:100), or concentrated hybridoma medium (anti-Myc) for 1 h, and the secondary antibody for 1 h [FITC-conjugated anti-rabbit (1:400), Cy3-conjugated anti-rabbit (1:20000) or Cy3-conjugated anti-mouse (1:5000) antibody; all from Sigma]. Extensive washing with PBS was performed between the incubations. Fluorescence was observed under a Leica microscope equipped with FITC/RSGFP/BODIPY®/Fluo3/DiO and Texas Red filters (Leica).

Binding assays

Blot-overlay assays

For the first approach, CHO-K1 cells, grown in 100 mm plastic dishes, were transfected with pKG44 as described above. Then, 2 days later, monolayers were washed twice with PBS and detached from the plastic by gentle scraping with a disposable cell scraper (Sarstedt) in lysis buffer [50 mM Tris/HCl, pH 8.0, 300 mM NaCl, 5 mM EDTA and 1% (w/v) Triton X-100; 1 ml/dish]. Following lysis for 20 min on ice, the solution was centrifuged at 20000 ***g*** for 15 min at 4 °C. The supernatant (1–5 ml) was mixed with binding-assay buffer [PBS containing 1% (w/v) Protifar (Nutricia); 5–10 ml final volume, depending on the size of the blot] and added to nitrocellulose blots, containing immobilized purified recombinant thiolase B, and incubated for 1 h. On some occasions, cell lysates were dialysed for 1 h against 500 vol. of lysis buffer without Triton X-100 using Spectra/Por® 1 membranes (6–8 kDa molecular-mass cut off; Spectrum). For fractionation studies, CHO-K1 cells, collected via trypsinization, were homogenized in homogenization medium [0.25 M sucrose, 0.1% (v/v) ethanol, 5 mM Mops, pH 7.2, and 1 mM EDTA] by two up-and-down strokes in a steel homogenizer (Kontes). Nuclear fractions were obtained by centrifugation (500 ***g***, 5 min at 4 °C) of these cell homogenates and washed twice. The combined post-nuclear supernatants were separated into soluble and particulate fractions by centrifugation (100000 ***g***, 40 min at 4 °C).

For the second approach, ^{35}S-labelled *Hs*Pex7p was generated by an *in vitro* transcription/translation reaction (TNT® T7 Coupled Reticulocyte Lysate System; Promega) according to the manufacturer's protocol in the presence of TRAN^{35}S-label (ICN) and using suitable vectors (pKG27, pKG126 or pKG132) as templates. From the labelled reaction mixture, 10–50 μl aliquots were diluted with binding-assay buffer to probe blots containing immobilized thiolase for 1 h.

Blots were washed three times with PBS, and binding of *Hs*Pex7p was analysed with affinity-purified anti-GFP antiserum or by autoradiography (Kodak Scientific Imaging Film) using an intensifying screen (Kodak BioMax TranScreen-LE).

Pull-down assay

For this *in vitro* binding assay, $(His)_6$-tagged fusion proteins present in crude lysates were bound to Ni-NTA resin. After washing the resin three times with 1 ml of washing buffer (TBS with 10 mM imidazole, pH 7.5), 25 μl of the loaded resin was incubated with ^{35}S-labelled *Hs*Pex7p [10–15 μl of the *in vitro* transcription/translation (TNT) reaction] for 1 h at 4 °C. The loaded resin was washed three times with 1 ml of washing buffer and the bound proteins were released by boiling the agarose beads in 25 μl of sample buffer containing 3% (w/v) SDS. Proteins, present in 20 μl of the SDS solution, were separated by SDS/PAGE and electroblotted on to a nitrocellulose membrane. Blots were analysed by autoradiography, followed usually by immunostaining of the $(His)_6$-tagged fusion proteins as described above. Alternatively, a second aliquot of the Ni-NTA beads was analysed for the presence of the $(His)_6$-tagged fusion proteins without being incubated with the TNT mixture.

In some experiments, the TNT mixture (50 μl) was subjected to gel filtration (Superdex 75 column, 3.2 mm × 30 cm; Amersham Bioscience) on a Smart platform using TBS as a solvent (flow rate, 50 μl/min; fraction size, 100 μl). For alkylation tests, the *in vitro* TNT mixture (containing approx. 4.5 mM free thiol groups; K. Ghys and P. P. Van Veldhoven, unpublished work) was fortified with *N*-ethylmaleimide (final concentration, 10 mM) and allowed to react at 4 °C or room temperature for 30 min.

RESULTS AND DISCUSSION

Interaction of human Pex7p with its PTS2 ligand

The interaction of yeast Pex7p with yeast thiolase has been documented in various ways [8,13,16], but the main technique employed has been the yeast two-hybrid system. Initially, we also used this system to test the interaction between human Pex7p, fused to the Gal4p DNA-binding domain, and a PTS2 ligand, either human thiolase or human phytanoyl-CoA hydroxylase, fused to the Gal4p activation domain, or vice versa in the case of phytanoyl-CoA hydroxylase. No induction of the *lacZ* reporter gene was obtained after co-expression of the corresponding fusion proteins in the *S. cerevisiae* strain SFY526 (results not shown). In addition, we tested the Pex7p–thiolase interaction in a bacterial two-hybrid system, based on functional complementation between two adenylate cyclase fragments [38]. Also in this system, although the interaction occurs in the cytosol, Pex7p did not bind thiolase (results not shown). The fusion moiety of the hybrid proteins may occlude the normal site of interaction or prevent proper folding of the hybrid protein. Also, instability of the hybrid protein or, in case of the yeast two-hybrid system, failure to target the hybrid proteins to the nucleus, can cause negative results. Our attempts to show expression of the hybrid proteins failed, but we can currently not distinguish the reason; this may be due to either the instability of the fusion proteins or expression levels below the detection limit.

All attempts to express recombinant Pex7p in bacteria, as a biotinylated, $(His)_6$-tagged or glutathione S-tranferase (GST) fusion protein, resulted in the formation of insoluble proteins (results not shown), which also precluded the use of bacterial lysates for blot-overlay assays.

Human Pex7p, expressed in CHO-K1 cells and tagged with GFP at its C-terminus (*Hs*Pex7p-GFP), however, was able to bind to unprocessed rat thiolase B immobilized on a nitrocellulose membrane. Binding was specific and PTS2-dependent, as no binding to mature thiolase, purified from rat liver, occurred (Figure 1). The interaction between *Hs*Pex7p-GFP and precursor thiolase was not abolished after dialysis of the CHO-K1 cell lysates (results not shown), ruling out the need for small soluble co-factors (unless these would be supplemented by the dried milk powder used in the assay medium). *Hs*Pex7p-GFP recovered from both the cytosolic or particulate fractions of the transfected cells was active (results not shown). Of the different GFP-tagged proteins tested (Figure 2A), only full-length *Hs*Pex7p-GFP appeared functional. With extracts of cells expressing the full-length *Hs*Pex7p with GFP fused to its N-terminus (GFP-*Hs*Pex7p), no interaction with full-length rat thiolase B could be revealed (results not shown), but immunoblot analysis showed that the majority of the fusion protein was degraded in the CHO-K1 cells (Figure 2B).

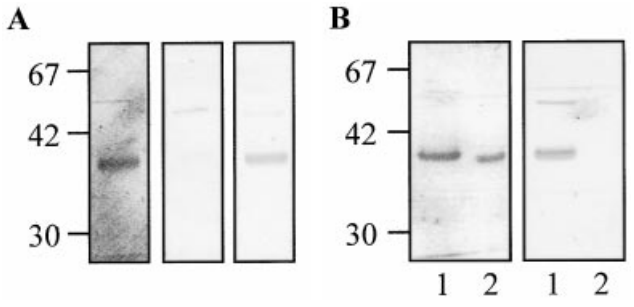

Figure 1 Interaction of Pex7p-GFP with PTS2 proteins

(**A**) The bacterially expressed and purified precursor form of rat thiolase B was subjected to SDS/PAGE and electroblotted on to nitrocellulose, followed by Ponceau S staining (left-hand panel) or incubated with a lysate of CHO-K1 cells (middle panel, non-transfected; right-hand panel, transfected with a construct coding for *Hs*Pex7p-GFP). After washing, binding of *Hs*Pex7p-GFP was detected using an antibody raised against GFP (middle and right-hand panels). With lysates of cells expressing only GFP, no binding was noticed (results not shown). (**B**) The experimental set-up was analogous to that described for (**A**) with the purified heterologously expressed precursor form of rat thiolase B (lanes 1) or thiolase (isoform A) purified from rat liver (lanes 2). The left-hand panel reveals the Ponceau S staining; the right-hand panel the detection of bound *Hs*Pex7p-GFP, after incubation of the blots with lysates of CHO-K1 cells expressing *Hs*Pex7p-GFP. The migration of the molecular-mass markers (in kDa) is indicated on the left.

Binding of Pex7p to its PTS2 ligand was also demonstrated in an independent manner by following the retention of ^{35}S-Pex7p, produced *in vitro* by a coupled TNT system, on Ni-NTA resin loaded with $(His)_6$-tagged fusion proteins. Labelled Pex7p was retained when the resin was charged with $(His)_6$-PTS2-thiolase, but not when $(His)_6$-*trans*-2-enoyl-CoA reductase [40,41], a peroxisomal protein ending in a PTS1, was used as a ligand (Figure 3A). In an analogous experiment whereby $(His)_6$-tagged fusion proteins of thiolase with or without mutations at position 1 or 8 of the PTS2 consensus sequence, changing Arg and His into Gly, respectively, were loaded on to a Ni-NTA resin, ^{35}S-Pex7p was only retained by the wild-type form (Figure 3B).

Gel-filtration experiments showed that ^{35}S-Pex7p is present as both a monomer and a (likely) dimer in the *in vitro* TNT mixture, but that only monomeric ^{35}S-Pex7p recognized PTS2 (Figure 3C). These data also prove that the binding was not dependent on small cofactors present in the reticulocyte lysate. The fact that *N*-ethylmaleimide pretreatment of ^{35}S-Pex7p reduced its PTS2 binding (Figure 3D) and the inability of dimeric Pex7p to bind PTS2 suggest a critical role for a free cysteine residue. Alignment of the different Pex7p sequences reveals only one conserved Cys residue, located at position 204 of the human protein and present within the fourth WD40 repeat. Upon mutation of Cys-204 to Ser, binding to PTS2 was reduced (Figure 3E). Dimerization of Pex7p in the TNT reaction was rather efficient (approx. half of the formed protein was dimeric) and might be an intrinsic property of Pex7p and of physiological importance, especially as it seems to occur also in CHO-K1 cells expressing the GFP full-length fusion proteins (see Figure 2B). Not many data are available on oligomerization of proteins in TNT reactions. Dimerization of the transcription factor cAMP-response-element-binding (CREB) protein, which occurs via a leucine-zipper domain, is hardly detectable, even after glutaraldehyde treatment of the TNT mixture [42].

For a functional PTS2 interaction, almost the entire Pex7p sequence seems to be required. A ^{35}S-labelled Pex7p deletion mutant, lacking the first 61 N-terminal amino acids, lost the ability to bind to PTS2 (results not shown), which is in agreement with the result obtained with the corresponding GFP-tagged Pex7p deletion mutant in the blot-overlay assay. In these constructs the first five amino acids of the first WD40 motif have been removed, pointing to the importance of this repeat.

We examined also the binding of ^{35}S-Pex7p to $(His)_6$-tagged fusions of human 3-hydroxy-3-methylglutaryl-CoA synthase (HMG-CoA synthase) and mevalonate kinase. According to Olivier et al. [43], HMG-CoA synthase contains a new PTS2 variant sequence, Ser-Val-Xaa_5-Gln-Leu, a sequence that is also found in mevalonate diphosphate decarboxylase, whereas mevalonate kinase would be targeted via a putative PTS2, Lys-Val-Xaa_5-His-Ala. However, ^{35}S-Pex7p was not retained by Ni-NTA resin loaded with either $(His)_6$-HMG-CoA synthase or $(His)_6$-mevalonate kinase (results not shown). These findings place some doubt on Ser-Val-Xaa_5-Gln-Leu and Lys-Val-Xaa_5-His-

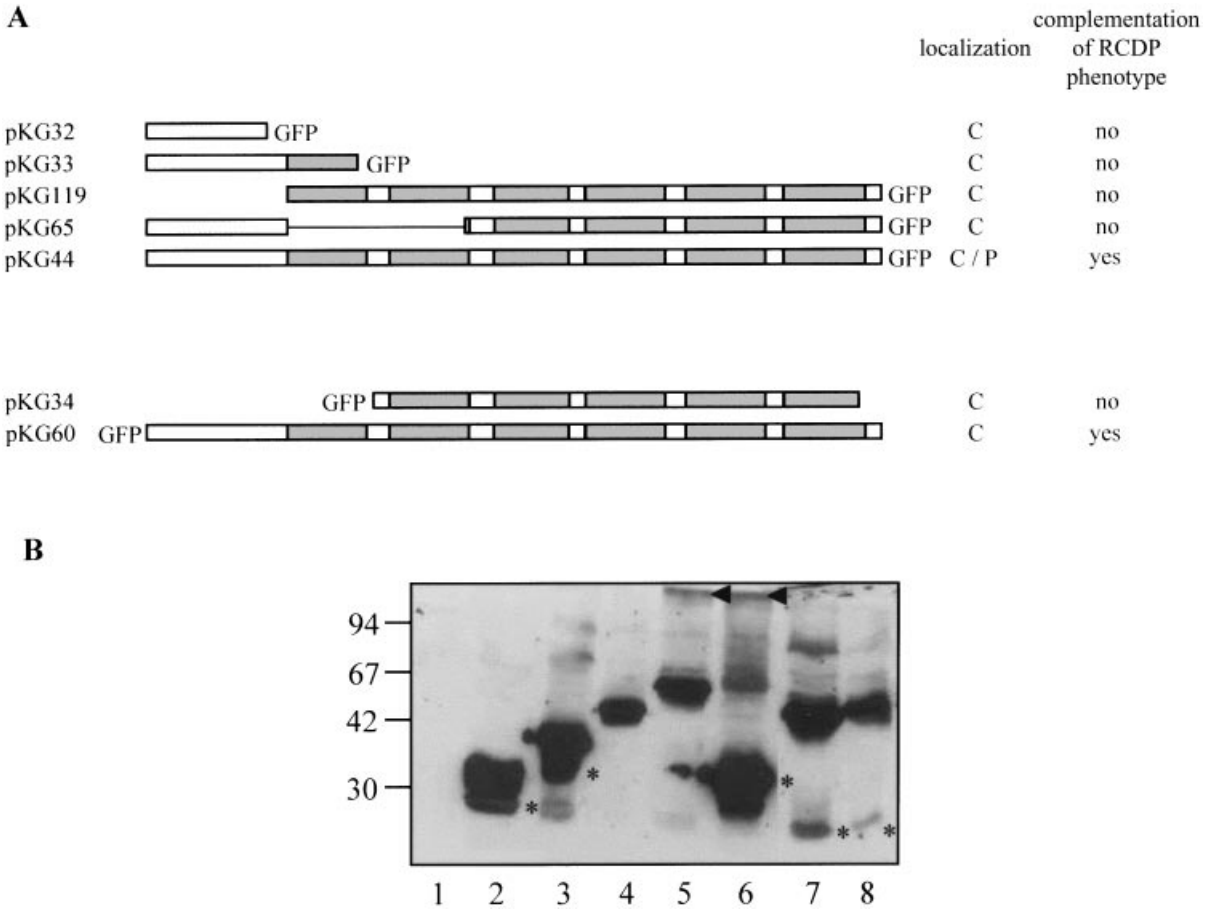

Figure 2 Localization and expression analysis of different GFP-tagged Pex7p deletion mutants

(**A**) Schematic presentation of different Pex7p-GFP fusion proteins used in this study, together with their subcellular localization, as determined via fluorescence microscopy on transfected CHO-K1 cells and human fibroblasts, and their ability to complement the RCDP type 1 phenotype. WD40 motifs of Pex7p are indicated by grey boxes; C, cytosol; P, peroxisome. (**B**) Cell lysates of untransfected CHO-K1 cells (lane 1) and CHO-K1 cells transfected with pKG32 (lane 2), pKG33 (lane 3), pKG34 (lane 4), pKG44 (lane 5), pKG60 (lane 6), pKG65 (lane 7) or pKG119 (lane 8), were separated by SDS/PAGE and analysed by Western blotting using an affinity-purified anti-GFP antibody and the Supersignal® West Pico chemiluminescent substrate (Pierce). The calculated molecular masses of the GFP fusion proteins encoded by pKG32, pKG33, pKG34, pKG44, pKG60, pKG65 and pKG119 were 32.6, 38.3, 53.3, 64.3, 64.0, 54.1 and 58.1 kDa, respectively. Degradation products (containing the GFP moiety or part of it) are marked with asterisks. The weak signals visible in lanes 5 and 6 at around 120 kDa (indicated by arrowheads) probably represent dimeric forms of the fusion proteins. Similar analysis were performed with human fibroblasts, but due to their lower transfection efficiencies the presence/intactness of the fusion proteins could not be shown.

Ala truly being PTS2 sequences. A possible explanation for the deficiency of HMG-CoA synthase and mevalonate diphosphate decarboxylase in human fibroblasts deficient in PTS2 protein import [43] and for the reduced mevalonate kinase, at the protein and activity levels in cells and tissues obtained from patients with peroxisome deficiency diseases [44], may be that they are imported into peroxisomes via 'piggy-backing' with a PTS2 protein.

Interaction of human Pex7p with other peroxins

To investigate whether or not human Pex7p interacts with other mammalian peroxins (Pex2p, Pex3p, Pex5pS, Pex5pL, Pex10p, Pex11pα, Pex11pβ, Pex12p, Pex13p, Pex14p, Pex16p and Pex19p) and with itself, we used the transcription-based yeast two-hybrid system [37] and a non-transcription-based bacterial two-hybrid system [38]. Although all these peroxins, with the exception of Pex2p and Pex11pα, are functionally active in one or both of these systems [33,35], no interaction of human Pex7p with these peroxins could be demonstrated using these systems. During the course of our investigations, the long isoform of Pex5p was reported to interact with Pex7p [23]. Therefore, the interaction of Pex7p with peroxins of interest (Pex5pL and Pex14p) was tested in another manner by analysing the binding of ^{35}S-Pex7p to $(His)_6$-tagged fusion proteins bound to Ni-NTA beads. Under our assay conditions, ^{35}S-Pex7p was not retained in a convincing or consistent manner by the long or short isoform of Pex5p or by Pex14p (results not shown). Although a direct interaction between Pex7p and Pex14p in mammals was shown by others [45], its relevance has since been questioned, since in CHO-K1 cells with a mutated form of Pex5pL, impaired in Pex7p binding, PTS2 protein import is blocked completely [24].

Localization of *Hs*Pex7p

As mentioned above, expression of soluble recombinant bacterial full-length or truncated Pex7 proteins was rather difficult. Against some of the fusion proteins, purified under denaturing conditions,

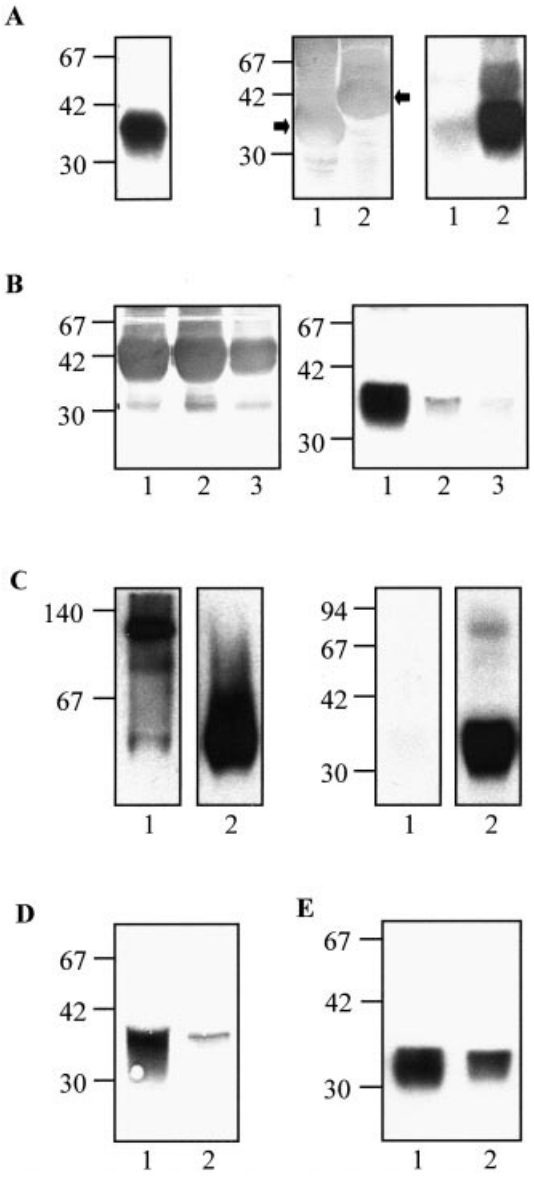

Figure 3 Interaction of ^{35}S-Pex7p with PTS2 proteins

(**A**) Lysates of bacteria expressing $(His)_6$-tagged *trans*-2-enoyl-CoA reductase (lanes 1) or rat thiolase B (lanes 2) were loaded on Ni-NTA–agarose followed by incubation with ^{35}S-labelled *Hs*Pex7p. After washing, bound proteins were separated by SDS/PAGE and transferred on to nitrocellulose in duplicate. One blot was immunostained with a purified mouse 6× His monoclonal antibody [Clontech; middle panel; the positions of $(His)_6$-tagged fusion proteins are indicated by arrows], the other was subjected to autoradiography overnight (right-hand panel). The left-hand panel represents an autoradiogram of a blot containing 10% of the amount of ^{35}S-*Hs*Pex7p used in this experiment. (**B**) The interaction of ^{35}S-*Hs*Pex7p with $(His)_6$-tagged wild-type thiolase (lanes 1), mutated $(His)_6$-thiolase$_{R3G}$ (lanes 2) or $(His)_6$-thiolase$_{H11G}$ (lanes 3) was analysed in a similar manner as described for (**A**). Left-hand panel, immunoanalysis using anti-rat thiolase; right-hand panel, autoradiography. (**C**) The *in vitro* TNT reaction mixture was subjected to gel filtration, and one-sixth of the fractions corresponding to the dimeric (lanes 1) and monomeric (lanes 2) forms of human ^{35}S-Pex7p was subjected to native gel electrophoresis, transferred on to nitrocellulose and subjected to autoradiography (left-hand panel). The remaining parts of the fractions were allowed to interact with $(His)_6$-tagged thiolase B in a similar manner as described for (**A**) and detected by autoradiography (right-hand panel). (**D**) The *in vitro* TNT reaction mixture was treated with water (lane 1) or *N*-ethylmaleimide (final concentration, 10 mM; lane 2) and allowed to interact with $(His)_6$-tagged thiolase as described in (**A**), followed by detection of the amount of bound ^{35}S-Pex7p by autoradiography. (**E**) The interaction of the TNT-made wild-type ^{35}S-*Hs*Pex7p (lane 1) or ^{35}S-*Hs*Pex7p$_{C204S}$ (lane 2) with $(His)_6$-tagged wild-type thiolase was analysed in a similar manner as in (**A**) and detected by autoradiography. Migration of the molecular-mass markers (in kDa) is indicated.

antisera were raised, but none appeared to offer the sensitivity or selectivity necessary for immunocytochemistry. Therefore we relied on transfection studies in cultured mammalian cells using different GFP-tagged *Hs*Pex7 proteins. CHO-K1 cells, control human and mouse fibroblasts (Figure 4) transfected with a plasmid coding for *Hs*Pex7p-GFP showed non-uniform patterns: both cytosolic and peroxisomal fluorescence was seen, whereby the ratio of cytosolic to peroxisomal staining differed from cell to cell and the range of staining patterns went from exclusively cytosolic to purely peroxisomal. In *Pex5*$^{-/-}$ mouse fibroblasts, however, Pex7p-GFP was exclusively cytosolic. In agreement with data obtained in Pex5p-deficient human [46] and CHO [47] cells, PTS2 protein import in the *Pex5*$^{-/-}$ mouse fibroblasts was restored after re-introduction of the long isoform of Pex5p, but not upon expression of the short isoform (Figure 4, middle panel). These data emphasize the importance of the long isoform of Pex5p in PTS2 protein import and provide indirect proof for a Pex5pL–Pex7p interaction. During the reviewing of this paper, Dodt et al. [48] documented this interaction by means of a mammalian two-hybrid system in human skin fibroblasts. All deletion mutant and full-length fusion proteins, except *Hs*Pex7p-GFP, showed an exclusive cytosolic staining pattern in both CHO-K1 cells and human fibroblasts (Figure 2A). Immunoblot analysis of the transfected CHO-K1 cells revealed that the GFP fusion proteins were formed at the expected size, and, except for GFP-Pex7p, minimally degraded (Figure 2B). In contrast with the situation in yeast, where the first 56 amino acids appear to be sufficient to target *S. cerevisiae* Pex7p to peroxisomes [16], in humans, even the first 93 amino acids of *Hs*Pex7p fused to GFP are located in the cytosol. Probably, the PTS2 receptor, like the PTS1 receptor [22], functions as a cycling receptor and is normally mainly present in the cytosol. Subramani and co-workers [13], who successfully raised a useful antibody, found most of Pex7p to be present in the cytosol in *P. pastoris*. All other literature reports on the localization of Pex7p in different organisms were based on studies using (overexpressed) fusion proteins. Mainly cytosolic localization for Pex7p was always found in the case of N-terminal tagging [8,10,13], whereas peroxisomal localization was found in the case of C-terminal tagging [10]. Our observations, although perhaps not reflecting the correct fate or localization of the endogenous Pex7p, were similar. *Hs*Pex7p tagged at its C-terminus with either GFP (Figure 4) or the Myc epitope (results not shown) was partially peroxisomal. With the N-terminal GFP-tagged *Hs*Pex7p cytosolic fluorescence was noticed (Figure 4, upper panel). However, due to the partial degradation of the latter fusion protein (Figure 2B) we cannot exclude the possibility that the cytosolic fluorescence is associated with the cleaved protein and/or obscuring the fluorescence of the intact GFP fusion protein. The differences in proteolytic stability of the full-length GFP fusion proteins are remarkable, and seem to suggest a more rigid folding of the C-terminally tagged Pex7p. As a consequence, C-terminal tagging might cause impaired recycling, which would be consistent with the peroxisomal localization of Pex7p-GFP or Pex7p-Myc.

Semi-permeabilization experiments in CHO-K1 cells demonstrate that Pex7p-GFP enters the peroxisomal lumen (Figure 4, upper panel). This finding supports the idea that Pex7p, like Pex5p [25], functions as a cycling receptor that enters the peroxisomal matrix. Since human Pex7p interacts with Pex5pL, the Pex7p–PTS2 protein complex is most likely shuttled to the matrix by Pex5pL. This might explain the absence of interactions of human Pex7p with peroxins of the docking and translocation complexes, since in mammals the point of convergence of the two PTS pathways is Pex5pL and the import cascade happens via interactions with this protein.

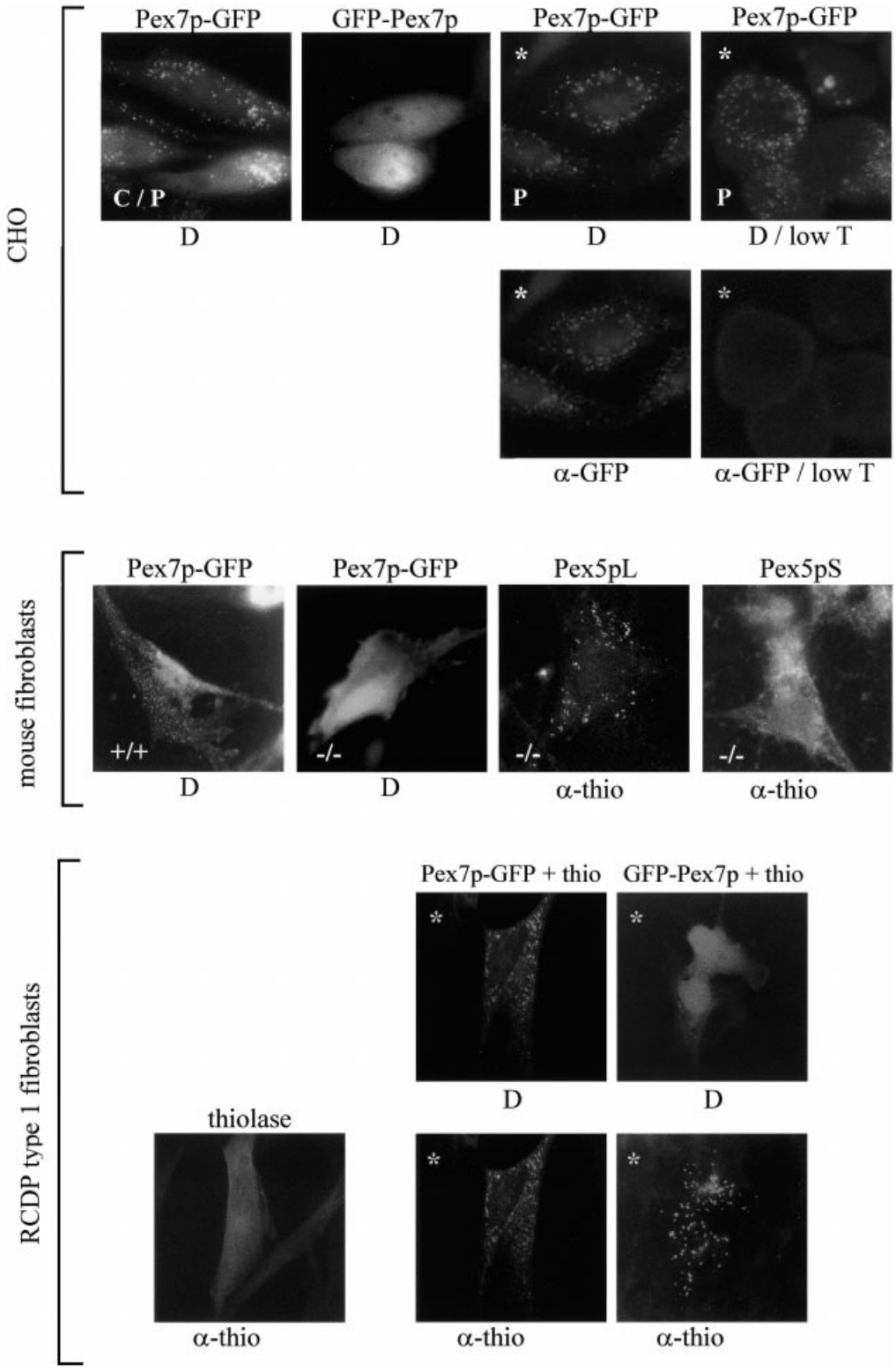

Figure 4 Localization and functional analysis of Pex7p in different cell types

CHO-K1 cells, mouse fibroblasts (*Pex5*$^{+/+}$ or *Pex5*$^{-/-}$ genotype) or RCDP type 1 patient fibroblasts were transfected with plasmids encoding the protein(s) indicated above the panels. After fixation the cells were analysed by either direct fluorescence microscopy (D) and/or indirect immunofluorescence microscopy using a primary antiserum, directed against the antigen indicated below the panels, and a Cy3- or FITC-labelled secondary anti-rabbit antibody. Panels marked with an asterisk document co-localization studies. For indirect immunofluorescence microscopy the cells were incubated routinely with 1% Triton X-100. In some cases, the detergent was reduced to 0.01% (indicated below the panels as low T). For CHO cells, representative pictures are selected showing cytosolic/peroxisomal (C/P) or peroxisomal (P) GFP staining. thio, thiolase; α-thio, anti-thiolase antibody.

Complementation of RCDP type 1 phenotype

To investigate whether or not the different tagged Pex7p fusion proteins could functionally complement the import defect in fibroblasts derived from RCDP type 1 patients, their influence on PTS2 protein import was studied. As seen in other cell types, *Hs*Pex7p-GFP was both cytosolic and peroxisomal in the human skin fibroblasts. Since endogenous thiolase could not be detected immunocytochemically in fibroblasts, probably due to its rather low expression level (results not shown), the subcellular localization of PTS2 protein was delineated in cells, transfected with a plasmid encoding rat thiolase B. Thiolase B was located in the cytosol of the RCDP type 1 fibroblasts (Figure 4, lower panel), confirming the PTS2 protein import defect. However, upon co-transfection with a plasmid encoding *Hs*Pex7p-GFP (Figure 4, lower panel) or *Hs*Pex7p-Myc (results not shown), thiolase B showed up in the peroxisomes. These results demonstrate that *Hs*Pex7p-GFP/*Hs*Pex7p-Myc is able to restore the PTS2 import defect in RCDP type 1 fibroblasts. Thiolase B with a mutation at position 1 or 8 of the PTS2 consensus sequence, however, remained in the cytosol after co-transfection with *Hs*Pex7p-GFP (results not shown). This is not surprising, since these mutations in the PTS2 consensus sequence result in loss of Pex7p binding, as was demonstrated in the pull-down assay. Co-transfection with a plasmid encoding GFP-*Hs*Pex7p also resulted in peroxisomal localization of thiolase B in some of the cells (Figure 4, lower panel). Different from *Hs*Pex7p-GFP, RCDP cells transfected with a construct encoding GFP-*Hs*Pex7p showed a cytosolic fluorescence. As reported for the other cell types, an exclusively cytosolic localization was seen for all the *Hs*Pex7p deletion mutants that were tested and none of these mutants were able to complement the RCDP type 1 phenotype. Due to the low transfection efficiency, the fate of these fusion proteins in the transfected RCDP cells was difficult to document by immunoblotting experiments. If we assume similar proteolytic stability of the fusion proteins as seen in CHO-K1 cells, the cytosolic fluorescent staining observed in the GFP-Pex7p-expressing fibroblasts could be due to degraded fusion protein. On the other hand, the amount of the intact GFP-*Hs*Pex7p appeared sufficient to complement the defect in RCDP type 1 fibroblasts, or alternatively, the proteolytically released Pex7p portion is functional. Most probably, in the *in vitro* overlay assays discussed above, the quantity of full-length GFP-Pex7p might become a limiting factor, explaining the negative data.

In conclusion, it seems that only mammalian and not bacterial or yeast expression systems are useful to obtain a functionally active form of human Pex7p. Two functional binding assays for *Hs*Pex7p were developed that each displayed selectivity for PTS2, based on ^{35}S-Pex7p, prepared *in vitro*, or on Pex7p-GFP synthesized *in vivo* in transfected CHO-K1 cells. Pex7p binds as a monomeric protein to the PTS2 ligand, apparently without the need for small co-factors. Mutations at position 1 or 8 of the PTS2 nonapeptide destroy the interaction. Although PTS2-binding competent, the GFP-fused or labelled Pex7p does not interact with bacterially expressed Pex14p or with the isoforms of Pex5p, human Pex7p tagged at either its N- or C-terminus was able to restore PTS2 import in RCDP type 1 cells. In different cell types, the Pex7p-GFP was localized partially to the peroxisomes and partially to the cytosol. We demonstrate further that Pex7p-GFP is present inside the peroxisomes, suggesting that Pex7p functions as a cycling receptor, which enters the peroxisomal matrix together with its PTS2 cargo.

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