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Mammalian Pex14p: membrane topology and characterisation of the Pex14p–Pex14p interaction

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Abstract

Peroxisomal biogenesis is a complex process requiring the action of numerous peroxins. One central component of this machinery is Pex14p, an intrinsic peroxisomal membrane protein probably involved in the docking of Pex5p, the receptor for PTS1-containing proteins (peroxisomal targeting signal 1-containing proteins). In this work the membrane topology of mammalian Pex14p was studied. Using a combination of protease protection assays and CNBr cleavage, we show that the first 130 amino acid residues of Pex14p are highly protected from exogenously added proteases by the peroxisomal membrane itself. Data indicating that this domain is responsible for the strong interaction of Pex14p with the organelle membrane are presented. All the other Pex14p amino acid residues are exposed to the cytosol. The properties of recombinant human Pex14p were also characterised. Heterologous expressed Pex14p was found to be a homopolymer of variable stoichiometry. Finally, in vitro binding assays indicate that homopolymerisation of Pex14p involves a domain comprising amino acid residues 147–278 of this peroxin.

Keywords: Peroxin; Peroxisomal biogenesis; Coiled-coil motif; Domain mapping

1. Introduction

Peroxisomes are ubiquitous organelles of eukaryotic cells involved in a variety of metabolic processes. In mammals, examples of biochemical pathways requiring peroxisomal enzymes are: β-oxidation of very long-chain fatty acids, synthesis of cholesterol, ether phospholipids and bile acids and the catabolism of phytanic acid and pipecolic acid (reviewed in Ref. [1]). The importance of these organelles to the cellular biochemistry is illustrated by the existence of several human genetic diseases in which one or several peroxisomal functions are missing. Many of these genetic disorders (e.g., Zellweger syndrome, neonatal adrenoleukodystrophy and infantile Refsum disease) arise from mutations in genes involved in the biogenesis of the organelle (reviewed in Refs. [2,3]). This observation has fuelled intensive research on the genes and mechanisms regulating the biogenesis of peroxisomes. Presently, more than 20 peroxins (proteins involved in the biogenesis of the peroxisome) have been characterised (reviewed in Refs. [2,3]).

Peroxisomal matrix proteins are synthesised on cytosolic ribosomes and posttranslationally imported into the organelle [4]. Specific targeting of these proteins to the peroxisome is accomplished by two receptors, Pex5p and Pex7p. The vast majority of peroxisomal proteins use Pex5p as their receptor [5–9]. These proteins possess the so-called PTS1, a carboxy-terminal tripeptide with the sequence SKL (or a derivative of this) ([10] and reviewed in Ref. [11]). A minor fraction of peroxisomal matrix proteins has a different targeting signal (PTS2)—a degenerated nonapeptide present at the N terminus of the protein with the sequence (R/K)(L/V/I)X_5(H/Q)(L/A) (reviewed in Refs. [11,12]). PTS2-con-

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taining proteins are targeted to the peroxisome by Pex7p [13,14].

It is generally accepted that proteins en route to the peroxisome interact with the cognate receptor while still in the cytosol, an idea supported by the observation that both Pex5p and Pex7p are largely (soluble) cytosolic proteins (reviewed in Ref. [11]). Thus, this model also implies the existence of docking proteins at the peroxisomal membrane capable of recognising these receptor–cargo complexes. From the several membrane peroxins presently known, Pex14p seems to be the best candidate to perform this function.

In addition to Pex5p, several other peroxins interact with Pex14p. In mammals, the interactions of Pex14p with itself [15] and with Pex13p [9,16] and Pex19p [17] are well documented. In lower eukaryotes the list of Pex14p-interacting peroxins also includes Pex3p [18], Pex7p [19–22], Pex8p [22] and Pex17p [18,21–23]. Although it is difficult to imagine how a 40–60-kDa protein can interact with so many different partners (raising the possibility that some of the described interactions are not direct), these results do indicate that Pex14p is a central component of the peroxisomal import machinery. Finally, as suggested recently [24,25], Pex14p may also be involved in the turnover of the peroxisome.

In this work, we have investigated some of the properties of mammalian Pex14p. Besides refining the membrane topology model of this peroxin, we present evidence suggesting that human recombinant Pex14p is able to form homopolymers. These homopolymers are heterogeneous structures and can bind in vitro synthesised [^{35}S]Pex5p. Domain mapping of the Pex14p–Pex14p interaction revealed the existence of a binding domain comprising amino acid residues 147–278 of Pex14p. This portion of the protein, containing a predicted coiled-coil motif [9,15,26], is exposed into the cytosol.

2. Materials and methods

2.1. Protease protection assays and chemical cleavage of proteins with CNBr

Protease protection assays were done exactly as described previously [27].

Cyanogen bromide cleavage [28] of protease-treated organelles was performed as follows: after inactivating proteinase K with 500 μg/ml phenylmethylsulfonyl fluoride or PMSF (added from a 50 mg/ml stock solution in ethanol) for 5 min on ice, organelle samples were diluted with 900 μl of SEI buffer (0.25 M sucrose, 5 mM imidazole pH 7.4, 1 mM ethylenediaminetetraacetate disodium salt or EDTA/NaHO pH 7.4) supplemented with 1:100 (v/v) mammalian protease inhibitor cocktail (Sigma, USA) and centrifuged at 15 000 × *g* for 15 min at 4 °C. Peroxisomal pellets were then solubilised in 5 μl of 1% (w/v) sodium dodecyl sulfate (SDS) and subjected to CNBr cleavage by adding 40 μl of 57 mg/ml CNBr in 79% (v/v) formic acid containing 1:100 (v/v) protease inhibitor cocktail. Control samples received an equal volume of 79% (v/v) formic acid only. All the samples were incubated at room temperature for 5 h and lyophilised.

2.2. Expression of fusion proteins

The rat Pex2p cDNA encoding amino acids 1–305 was amplified from rat liver total RNA by reverse transcription polymerase chain reaction (RT-PCR) using the primers 5′-CGCGAATTCATGGCTGCCAGAGAAGAGAGTACA-3′ and 5′-CGGCTGCAGTGGTTTCTAAAGAGCATTCACTTCTG-3′, designed according to the published sequence [29]. The amplified cDNA fragment was digested with *Eco*RI and *Pst*I and cloned into the pBluescript II KS vector (Stratagene, USA). This recombinant plasmid was digested with *Eco*RI and *Not*I, and the insert obtained was cloned into the pGEX-5X-1 expression vector (Amersham Biosciences, USA).

The cDNA encoding amino acids 1–373 of human Pex3p was amplified from human skin fibroblasts total RNA by RT-PCR, using the primers 5′-CGCGTCGACATGCTGAGGTCTGTATG-3′ and 5′-CGCGTCGACTCATTTCTCCAGTTGCT-3′, designed according to the published sequence [30]. The Pex3p cDNA was then cloned into the pGEM®-T Easy vector, following the manufacturer's instructions (Promega, USA). This recombinant plasmid was digested with *Sal*I, and the insert was cloned in pGEX-4T-3 expression vector (Amersham Biosciences).

The amplification and cloning into the pGEM®-T Easy vector of a cDNA encoding amino acids 1–639 of human Pex5p was described previously [27]. This plasmid, hereafter referred to as pGEMT–Pex5p, was used as a template in a PCR reaction using the primers 5′-GGCGTCGACTGGAGATCTATGGCAATGCGGGAGCTGGTGGA-3′ and 5′-GGGTCTAGAGCGGCCGCGTCGACCTGTCACTGGGGCAGGCC-3′. This product was digested with *Bgl*II and *Not*I and inserted into the *Bam*HI and *Not*I sites of the pGEX-4T-3 expression vector.

The cDNA encoding amino acids 1–247 of human Pex11p was amplified from human skin fibroblasts total RNA by RT-PCR, using the primers 5′-CGCGTCGACGCCATGGACGCCTTCACC-3′ and 5′-CGCAAGCTTCCTAAAAACACCCTAACG-3′, designed according to the published sequence [31]. This cDNA fragment was then cloned into the pGEM®-T Easy vector. The recombinant plasmid was digested with the restriction enzymes *Sal*I and *Not*I and the insert was cloned into the pGEX-4T-3 expression vector.

The human Pex19p cDNA encoding amino acids 1–299 was amplified from human kin fibroblasts total RNA by RT-PCR, using the primers 5′-CGCGGATCCAAGATGGCCGCCGCTGAGGAA-3′ and 5′-CGCAAGCTTGTTTCACATGATCAGACACTG-3′, according to the published sequence [32]. After cloning this cDNA fragment into the

pGEM®-T Easy vector, the recombinant plasmid was digested with the restriction enzymes *Bam*HI and *Not*I and the insert was cloned into the pGEX-4T-3 expression vector.

The glutathione-S-transferase–Pex14p (GST–Pex14p) encoding plasmid was obtained as described previously [33].

All the GST fusion proteins were expressed in the XL_1-Blue strain of *Escherichia coli*. GST–Pex2p, GST–Pex3p and GST–Pex11p fusion proteins were obtained as inclusion bodies and further purified by preparative SDS-polyacrylamide gel electrophoresis (SDS-PAGE). This purification method was also applied to the insoluble pool of GST–Pex14p. GST–Pex5p and GST–Pex19p were obtained as soluble proteins and purified by affinity chromatography using glutathione Sepharose 4B (Amersham Biosciences).

The soluble pool of the GST–Pex14p fusion protein was prepared using the following procedure: pelleted *E. coli* cells from 100-ml induced culture were resuspended in 2 ml of phosphate-buffered saline (PBS), 1 mM EDTA, 5 mM dithiothreitol (DTT), 1:500 (v/v) mammalian protease inhibitor cocktail and 50 μg/ml PMSF, and sonicated two times for 25 s (with 1-min interval on ice) using a Vibra Cell/Sonics and Materials sonicator (model VC130). The sonicated cells were then subjected to a clarifying spin (15 000 × *g*, 15 min, 4 °C) to remove cell debris and insoluble material. About 70% of the total GST–Pex14p fusion protein was recovered in the supernatant.

2.3. Sedimentation analysis of recombinant human Pex14p

The soluble pool of GST–Pex14p (1 ml of the supernatant obtained as described above) was applied on the top of a sucrose gradient (2.2 ml of 10%, 2 ml of 15.5%, 1.8 ml of 21%, 1.6 ml of 25%, 1.3 ml of 30% and 1 ml of 35% (w/v) sucrose in 50 mM Tris(hydroxymethyl)aminomethane or Tris/acetic acid pH 8.0, 0.1% (w/v) Triton X-100, 1 mM EDTA, 1 mM DTT and 1:500 (v/v) mammalian protease inhibitor cocktail), and centrifuged at 185 000 × *g* for 14 h at 4 °C in the TST41.14 rotor (Sorvall® Instruments, USA). Thirteen aliquots of 850 μl were collected from the bottom of the tube. Equal portions of each fraction were then precipitated with trichloroacetic acid and analysed by SDS-PAGE.

In order to analyse the sedimentation properties of Pex14p alone, GST–Pex14p present in fractions 5 and 6 of the previous gradient was first concentrated by adding a saturated ammonium sulfate solution (pH 7.0 with NH_4OH) to 35% saturation. After 15 min on ice, the suspension was centrifuged (15 000 × *g* for 15 min at 4 °C) and the pellet was resuspended in 300 μl of 50 mM Tris/acetic acid pH 8.0, 0.1% (w/v) Triton X-100, 2.5 mM $CaCl_2$, 1 mM EDTA and 5 mM DTT. GST–Pex14p was then cleaved with 5 Units of thrombin (Sigma T4648; stock solution 5 mg of solid/ml of 50 mM Tris/HCl pH 7.5) by incubating for 1 h at room temperature. The protease was inactivated by adding 1:500 (v/v) mammalian protease inhibitor cocktail and PMSF to 50 μg/ml. Thrombin-cleaved GST–Pex14p was then applied on the top of a sucrose density gradient as described above.

2.4. In vitro synthesis of [^{35}S]-labelled proteins by coupled transcription/translation

The human Pex5p cDNA was obtained by cleaving pGEMT–Pex5p with *Sal*I and inserted on the *Sal*I site of pGEM-4 vector (Promega). This recombinant plasmid was linearised with *Nhe*I and transcribed in vitro using T7 RNA polymerase, according to the manufacturer's instructions (T7 Cap-Scribe, Roche, Germany). Radiolabelled Pex5p was synthesised in a rabbit reticulocyte lysate in the presence of [^{35}S]methionine, according to the manufacturer's protocol (Translation Kit Reticulocyte Type II, Roche).

The cDNA encoding full-length Pex14p inserted into the pGEM®-T Easy vector [33] was extracted from this plasmid by digestion with *Sal*I and *Sph*I and cloned into the pGEM-4 vector, yielding pGEM4–Pex14p. This plasmid was linearised with the restriction enzyme *Hin*dIII and transcribed in vitro using SP6 RNA polymerase, according to the manufacturer's protocol (SP6 Cap-Scribe, Roche). Radiolabelled Pex14p was synthesised as described above.

Truncated forms of [^{35}S]Pex14p were obtained using an expression-PCR (E-PCR) strategy, as described [34]. Two sequential PCRs were done to amplify the relevant portion of the cDNA and to introduce a T7 RNA polymerase promoter sequence upstream of Pex14p sequences. In the first PCR, the pGEM4–Pex14p plasmid was subjected to amplification using the pairs of primers listed on Table 1. All the upper primers contained a linker sequence 'GGGAGAGCCACC' before the initiation codon; the lower primers contained three 'ATG' codons before the stop codon to improve the signal in the autoradiography. The cDNA fragments obtained in this way were then subjected to a second PCR amplification using the respective lower primers and an upper primer (5′-GAATTCCTAATACGACTCACTATAGGGAGAGCCACCATG-3′) containing the T7 RNA polymerase promoter sequence followed by the linker sequence. These DNA fragments were then subjected to in vitro transcription/translation.

Table 1
Primers used in expression-PCR of truncated Pex14p proteins

Truncated Pex14p proteins	Primer sequence (5′ → 3′)
Pex14p (1–155)	GGGAGAGCCACCATGGCGTCCTCGGAGCAG
	CTACATCATCATCAGCTCAGAGAGACCGGC
Pex14p (1–79)	GGGAGAGCCACCATGGCGTCCTCGGAGCAG
	CTACATCATCATGGACGAAGGCTCATCGGC
Pex14p (147–377)	GGGAGAGCCACCATGGAGGCCGGTCTCTCT
	CTACATCATCATGTCCCGCTCACTCTCGTT
Pex14p (140–278)	GGGAGAGCCACCATGGACAGAAAGCAGCTGGAG
	CTACATCATCATCTTCCCAGGCGAGGACGA
Pex14p (262–377)	GGGAGAGCCACCATGGACATCTCACCTGTCAG
	CTACATCATCATGTCCCGCTCACTCTCGTT

2.5. Blot-overlay assays

GST–Pex2p, GST–Pex3p, GST–Pex5p, GST–Pex11p, GST–Pex14p and GST–Pex19p were resolved by SDS-PAGE (1–2 μg/lane) and blotted onto nitrocellulose membranes (Schleicher & Schuell, USA). These membranes were incubated in renaturing buffer containing 50 mM Tris/HCl pH 7.5, 100 mM potassium acetate/acetic acid pH 7.2, 150 mM NaCl, 1 mM DTT, 5 mM $MgCl_2$, 1 mM EDTA, 100 μM $ZnCl_2$, 100 mM methionine, 0.3% (w/v) Tween-20 and 5% (w/v) nonfat dry milk for 2 h at 4 °C, as described [9]. Individual membranes were probed with equal amounts (as estimated by fluorography of dried SDS polyacrylamide gels) of the different [^{35}S]Pex14p proteins—1 to 4 μl of reticulocyte lysates in 2 ml of renaturing buffer. Following an incubation overnight at 4 °C, the membranes were washed two times with renaturing buffer and dried for autoradiography.

2.6. Miscellaneous

Isolation of highly pure peroxisomes from mouse and rat liver by differential centrifugation and Nycodenz gradient purification was performed as described [35], with minor modifications [33].

Alkaline extraction of organelles was done according to Fujiki et al. [36].

Proteins were measured by the Lowry method using bovine serum albumin as standard [37].

SDS-PAGE was performed in 1.0-mm-thick, 12% polyacrylamide gels using the Laemmli discontinuous buffer system [38]. Urea–SDS polyacrylamide gels [39] were used in some experiments as indicated in the figure legends.

Western blotting onto nitrocellulose membranes was performed according to the manufacturer's instructions (Schleicher & Schuell).

The preparation and characterisation of the antibodies against Pex5p [27], Pex14p(1–134) [26] and Pex14p [33] were described previously. A monoclonal antibody (clone 7H10-BD4) directed to the α subunit of the mitochondrial ATPase, a peripheral membrane protein [40], was purchased from Molecular Probes (USA).

The molecular mass markers used to calibrate the sucrose density gradients were bovine serum albumin (66 kDa), beef heart lactate dehydrogenase (140 kDa) and beef liver catalase (232 kDa).

3. Results

3.1. Characterisation of the membrane topology of mammalian Pex14p

In a first attempt to define the membrane topology of mammalian Pex14p, we have performed a protease protection assay. For this purpose, purified rat liver peroxisomes were treated with increasing amounts of proteinase K. After inactivating the protease, protein samples were analysed by Western blotting using antibodies directed to Pex14p(1–134) [26]. The behaviour of Pex5p was also monitored in these experiments. As shown previously [27], peroxisomal Pex5p is completely degraded at low proteinase K concentrations when the peroxisomal compartment is not intact. On the other hand, when intact organelles are used in these experiments, only a short fragment of approximately 2 kDa can be clipped off from Pex5p by the action of proteinase K. Thus, Pex5p provides the means to control both the proteolytic activity of proteinase K and the intactness of the organelles used in these experiments.

As shown in Fig. 1A, when intact peroxisomes are treated with low concentrations of proteinase K (1–5 μg/ml), Pex14p is degraded to a 31-kDa fragment. A very similar result was described recently for human Pex14p [26]. However, this fragment is still partially protruding into the cytosolic side of the peroxisomal membrane. Indeed, at higher proteinase K concentration this fragment is completely degraded giving rise to a smaller fragment of 16 kDa. Increasing the protease concentration up to 0.5 mg/ml has no further effect on the cleavage profile of Pex14p (data not shown).

Mammalian Pex14p behaves as an intrinsic membrane protein [9,15,26]. Considering the results presented above it is plausible to assume that the 16 kDa Pex14p fragment obtained after proteinase K digestion of intact peroxisomes contains a major (if not the only) membrane association domain. Indeed, when protease-treated intact peroxisomes are subjected to alkaline extraction in order to separate intrinsic membrane proteins from peripheral membrane and soluble proteins, this 16-kDa fragment remains in the intrinsic membrane protein fraction (Fig. 1B). Thus, at least a small domain of the 16 kDa fragment is embedded in the peroxisomal membrane. But does this fragment cross completely the membrane of the organelle? In an attempt to answer this question, we performed proteinase K digestions of peroxisomal proteins using disrupted peroxisomes. Two different methods were used to provide access of the protease to the lumenal domains of peroxisomal proteins, as described before [27]: the organelles were either solubilised using mild detergents before protease treatment or they were disrupted by sonication in the presence of proteinase K. As shown in Fig. 1A (lanes 7–8), when Triton X-100-solubilised peroxisomes are incubated in the presence of low concentrations of proteinase K, no immunodetectable fragment of Pex14p can be obtained. Essentially the same results were observed when 1% (w/v) digitonin instead of Triton X-100 was used in these experiments (data not shown). This result could suggest that at least a small portion of the 16-kDa Pex14p fragment is exposed to the lumenal side of the peroxisomal membrane. However, this interpretation is not corroborated by the second experiment in which peroxisomes were sonicated in the presence of proteinase K. Under these conditions, no further cleavage of

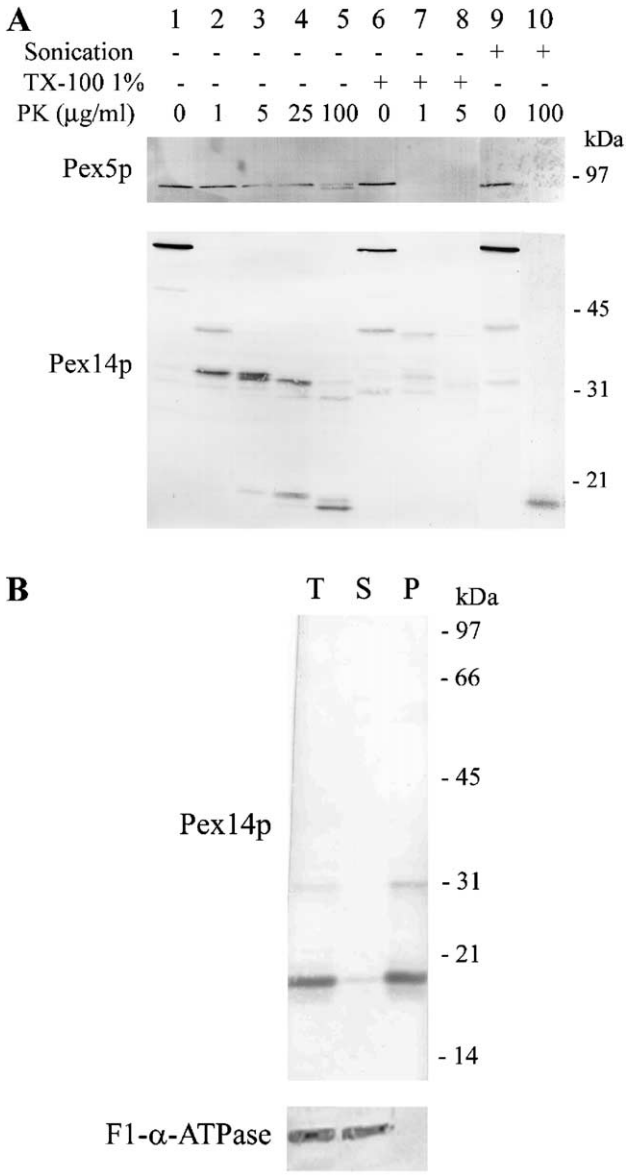

Fig. 1. Membrane topology of Pex14p. (A) Forty micrograms of purified rat liver peroxisomes was incubated in the presence of different concentrations of proteinase K (PK). After inactivation of the protease, protein samples were precipitated with trichloroacetic acid, resolved by urea–SDS-PAGE and analysed by immunoblotting using anti-Pex5p and anti-Pex14p(1–134) antibodies. Organelles solubilised with Triton X-100 (TX-100) were also subjected to protease treatment as a control. Some of the samples were sonicated immediately after the addition of proteinase K. (B) Eighty micrograms of purified peroxisomes was treated with proteinase K (100 μg/ml final concentration). After inactivating the protease, the organelles were subjected to alkaline extraction. Half of the sample was separated into membrane (P) and soluble (S) fractions by centrifugation. The other half was used for determination of recoveries (T). After trichloroacetic acid precipitation, proteins were analysed by immunoblotting using antibodies against Pex14p(1–134) and the α subunit of mitochondrial ATPase. The positions of the molecular mass markers are shown.

the 16-kDa fragment was observed. Again, as described above, we tested higher proteinase K concentrations (0.5 mg/ml), but virtually the same result was obtained (data not shown). Many different hypotheses can be envisaged to explain this discrepancy (see Discussion). However, the bottom line here is that the membrane topology of this Pex14p fragment cannot be inferred from these experiments.

Taking into account that the anti-Pex14p antibody used in these experiments was produced against amino acid residues 1–134 of this peroxin, we still considered the possibility that the 16-kDa fragment detected in the protease-protection experiments could represent a domain of Pex14p lacking some N-terminal residues. Thus, we have mapped the region of Pex14p corresponding to this 16-kDa fragment. This was accomplished using the specific methionine-cleavage chemical, CNBr. As shown in Fig. 2, when CNBr-treated peroxisomal proteins from rat liver are analysed by Western-blotting using the anti-Pex14p antibody, an 18-kDa fragment is observed (lane 3). This fragment corresponds to amino acid residues 2–148 of rat Pex14p (the second methionine in the rat Pex14p primary structure appears at position 148 [15]). When intact rat liver peroxisomes are first subjected to proteinase K in order to degrade Pex14p to its 16-kDa fragment and afterwards treated with CNBr, no further cleavage is observed (Fig. 2, lane 4). There are two possibilities to explain this result: (1) proteinase K cleaves rat Pex14p in the neighbourhood of Met-148 and clips approximately 2 kDa from the N terminus of the protein; or (2) proteinase K hydrolyses rat Pex14p in a region located approximately 2 kDa before Met-148. To clarify this point, we explored the fact that mouse Pex14p (GenBank accession number AAF04616) has an additional methionine at position 116. When mouse liver peroxisomal proteins are treated with CNBr, two fragments arising from Pex14p can be detected upon Western blot analysis using the anti-Pex14p antibody (Fig. 2, lane 7). Clearly, CNBr

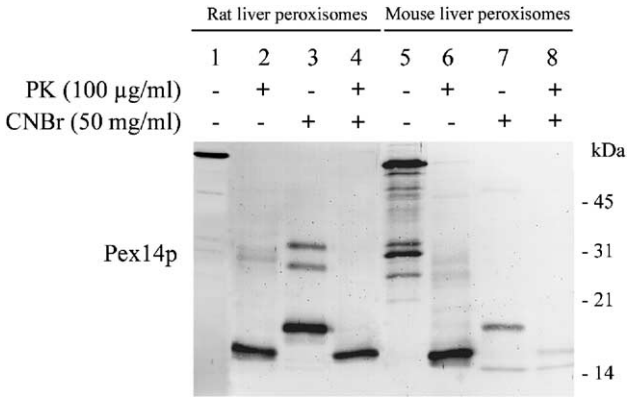

Fig. 2. The proteinase K-resistant 16-kDa fragment of Pex14p corresponds to the N-terminal 130 amino acid residues. Forty micrograms of purified liver peroxisomes from rat or mouse was treated with proteinase K (PK), at the indicated concentrations. After inactivating the protease, samples were diluted with SEI buffer and centrifuged to pellet the organelles. Peroxisomal proteins were then subjected to CNBr cleavage in 70% (v/v) formic acid. Control samples received the same amount of the formic acid solution. After 5 h at room temperature, protein samples were lyophilised and subjected to urea-SDS-PAGE. The antibody anti-Pex14p(1–134) was used in the immunoblotting analysis. The positions of the molecular mass markers are shown.

cleavage was not complete at Met-116 of mouse Pex14p although exactly the same conditions were used to cleave both the rat and mouse proteins. Different cleavage efficiencies of methionines in different protein contexts are a well-documented characteristic of the CNBr chemistry [28]. Thus, the 18-kDa fragment corresponds to amino acid residues 2–148 of mouse Pex14p; the 15-kDa fragment observed corresponds to amino acid residues 2–116 of mouse Pex14p. When this procedure is repeated with mouse peroxisomes that were previously subjected to a protease protection assay in order to degrade Pex14p to its 16-kDa fragment (see Fig. 2, lane 6), a 15-kDa fragment is now observed (lane 8). Thus, the 16-kDa fragment obtained after proteinase K digestion of Pex14p still contains Met-116. Furthermore, since a 15-kDa fragment is obtained after CNBr cleavage in both the proteinase K digested (Fig. 2, lane 8) and undigested (Fig. 2, lane 6) samples, this experiment also implies that no significant number of N-terminal residues are removed from Pex14p by the action of proteinase K (proteolytic removal of five residues or more from the N terminus of Pex14p would have been easily detected in the gel presented in Fig. 2). Thus, the 16-kDa Pex14p fragment obtained after proteinase K digestion corresponds approximately to amino acid residues 1–130.

Taken together, the data presented here indicates that the first 130 amino acid residues of mammalian Pex14p are highly resistant to proteinase K when the peroxisomal membrane is intact (i.e., in the absence of detergents). Our results also show that this domain alone displays the characteristics of an intrinsic membrane protein. Finally, our data also suggest that the C-terminal two thirds of Pex14p are largely exposed into the cytosol. Evidence in support of this conclusion comes from the experiments presented in Figs. 1 and 3. When using intact rat liver peroxisomes and low concentrations of proteinase K, four Pex14p fragments can be detected (see lanes 3 and 4 of Fig. 1A). All these fragments (still containing the epitopes recognised by the anti-Pex14p antibody) can be degraded into the smaller 16-kDa fragment when the proteinase K concentration is increased. Thus, they correspond to C-terminal truncated forms of Pex14p. The same observation can be made in a more impressive way when intact purified rat liver peroxisomes are incubated with increasing concentrations of trypsin (see Fig. 3). In this case, multiple C-terminal truncated forms of Pex14p can be observed.

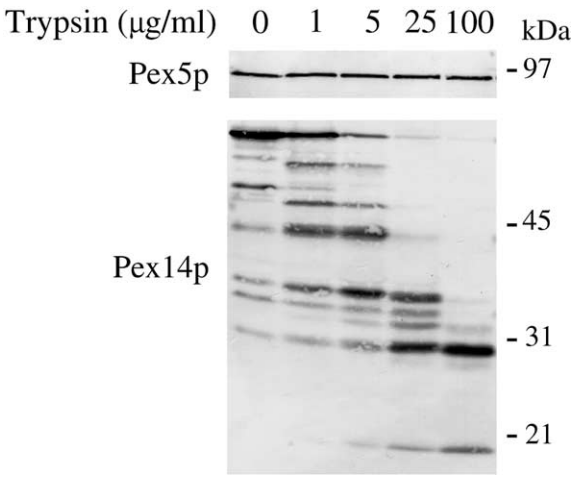

Fig. 3. The C-terminal domain of Pex14p is exposed to the cytosol. Forty micrograms of purified rat liver peroxisomes was digested with different amounts of trypsin, as indicated. After inactivation of the protease, protein samples were precipitated with trichloroacetic acid, resolved by urea-SDS-PAGE and analysed by immunoblotting using anti-Pex5p and anti-Pex14p(1–134) antibodies. The positions of the molecular mass markers are shown.

3.2. Recombinant human Pex14p forms homopolymers

In a previous study we described the production of a recombinant GST–Pex14p fusion protein with the purpose of raising antibodies [33]. During the course of that work, it was noticed that only a fraction of the recombinant GST–Pex14p fusion protein was found in inclusion bodies after overexpression in *E. coli* cells. Indeed, more than 70% of the recombinant protein could be easily recovered as a soluble protein when these cells were disrupted by mild sonication in the presence of 0.1% (w/v) Triton X-100 (data not shown). Similar observations regarding the solubility of human recombinant Pex14p were made recently [17,26]. Since it has been shown that Pex14p interacts with itself using in vitro binding assays [15] and the two-hybrid system [19–23], we tried to explore the solubility of this recombinant protein to study in more detail the Pex14p–Pex14p interaction. The aim was to obtain data concerning the degree of homopolymerisation of Pex14p.

We first tried to determine the molecular mass of the GST–Pex14p protein. For this purpose, *E. coli* cells expressing the fusion protein were gently sonicated in order to release soluble proteins. After a clarifying spin to remove cell debris and insoluble proteins, the supernatant was subjected to sucrose gradient centrifugation. As shown in Fig. 4, GST–Pex14p can be found almost over the entire gradient—from fraction 9 (representing dimeric protein) to fraction 1 (corresponding to a very high molecular mass). It is important to note that no other *E. coli* protein presents this kind of sedimentation profile. Thus, the observed behaviour represents a specific property of the fusion protein alone, implying that recombinant GST–Pex14p is a homopolymer displaying variable stoichiometries.

Since GST itself is a dimeric protein [41], most of the intermolecular interactions inferred from the previous experiment could be the result of GST–GST interactions. In order to remove this uncertainty, we cleaved the GST–Pex14p fusion protein with thrombin (this protease cleaves the GST moiety from the fusion protein) and reanalysed the sedimentation behaviour of the recombinant human peroxin. We selected for this experiment GST–Pex14p present in fractions 5 and 6 (apparent molecular mass 250–500 kDa) of a sucrose gradient similar to the one described above. (For

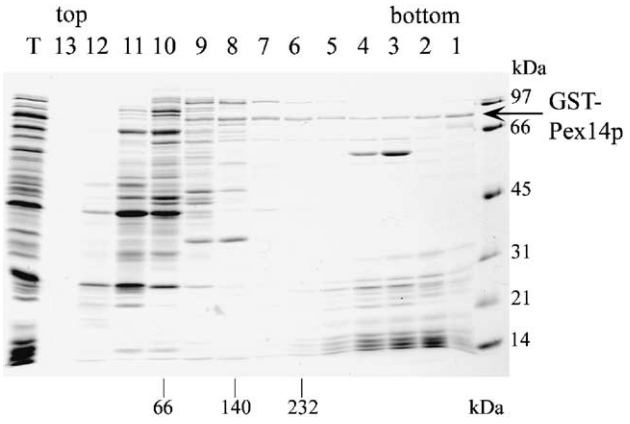

Fig. 4. GST–Pex14p is a homopolymer of heterogeneous size. Total soluble proteins obtained from *E. coli* cells expressing GST–Pex14p were subjected to sucrose gradient centrifugation as described in Materials and methods. The gradient was fractionated into 13 aliquots, starting from the bottom of the tube. Equal portions from each aliquot (corresponding to 1 ml of the initial culture) were analysed by SDS-PAGE. A Coomassie blue-stained gel is shown. The identity of the GST–Pex14p fusion protein (indicated on the right-hand side of the gel) was confirmed by immunoblotting analysis (data not shown). Lane T—total soluble proteins corresponding to 0.5 ml of the initial culture. The positions of the molecular mass markers are shown. The numbers at the bottom indicate the positions of the molecular mass markers used to calibrate the gradients.

reasons that will be explained below, 5 μg of thrombin-cleaved GST–Pex14p was pre-incubated with a radiochemical amount of in vitro synthesised human [^{35}S]Pex5p before loading onto the sucrose gradient.) As shown in Fig. 5A, the majority of human Pex14p derived from fractions 5–6 of the first gradient are now detected in fractions 5–8 of the second sucrose gradient. This observation has two implications: first, GST–GST interactions are not the main driving force for the homopolymerisation of the GST–Pex14p fusion protein; second, Pex14p homopolymers are stable entities under the experimental conditions used. Of relevance is also the fact that the GST moiety of the thrombin-cleaved fusion protein is found in fractions 10–11 of the gradient (representing monomeric/dimeric protein), suggesting that at least the N-terminal 1/3 of the GST–Pex14p fusion protein was in a correctly folded conformation.

As stated above, we included in the sucrose gradient sedimentation analysis of Pex14p a radiochemical amount of human [^{35}S]Pex5p. The aim was to assess the conformational state of the recombinant Pex14p protein. As shown in Fig. 5B, in the absence of Pex14p, in vitro synthesised human [^{35}S]Pex5p behaves as a monomeric protein (Fig. 5B, fraction 10 of the gradient). A similar result was described recently for rat liver cytosolic Pex5p [27]. However, when [^{35}S]Pex5p is pre-incubated with recombinant Pex14p before the sucrose gradient centrifugation step, a quite different result is obtained—Pex5p is now detected as a high molecular mass complex. Thus, at least a small fraction of the Pex14p homopolymers are able to bind [^{35}S]Pex5p, suggesting that the recombinant peroxin is in a correct folded state.

3.3. Domain mapping of the Pex14p–Pex14p interaction

The primary structure of Pex14p from several organisms reveals the existence of a coiled-coil motif in the middle region of the peroxin [9,15,19,20,22,26]. Coiled-coil motifs are involved in protein–protein interactions and can give rise to dimeric, trimeric, tetrameric and, possibly, pentameric structures [42]. Thus, it seems likely that this type of interaction is involved in the homopolymerisation process observed for human Pex14p. However, no experimental data supporting this hypothesis were yet provided.

In an attempt to define the domain(s) involved in the Pex14p–Pex14p interaction, we have synthesised in vitro several truncated forms of the human Pex14p. The capacity of these shorter versions of [^{35}S]Pex14p in binding full-length recombinant Pex14p was then assessed by a blot-overlay assay. Several other recombinant peroxins were also used as controls, namely, Pex2p, Pex3p, Pex5p, Pex11p and Pex19p. The results of this experiment are shown in Fig. 6. No binding to Pex2p, Pex3p or Pex11p was detected with any of the in vitro synthesised [^{35}S]Pex14p forms used in this assay. In contrast, a very strong binding to Pex5p was observed with all the [^{35}S]Pex14p forms containing at least amino acid residues 1–79. This result is in agreement with the Pex5p-binding domain mapping data reported previously for human Pex14p [43]. Human Pex14p also binds Pex19p [17]. As shown recently [17], Pex14p amino acid residues 108–147 are necessary for the interaction with Pex19p. Our results are compatible with these data: amino acid residues 79–155 of Pex14p are necessary for the interaction with Pex19p.

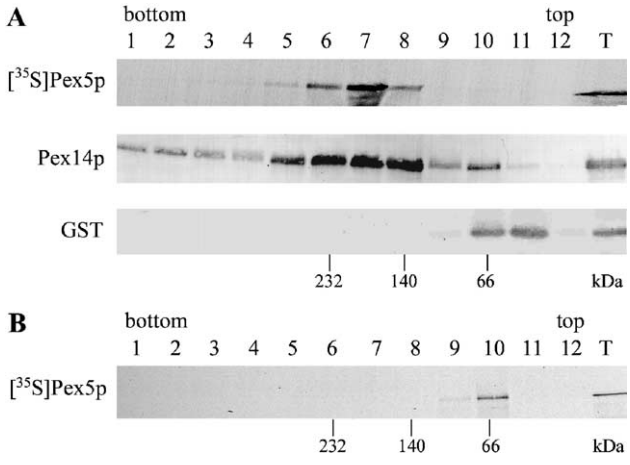

Fig. 5. Homopolymeric recombinant Pex14p binds Pex5p. In vitro synthesised [^{35}S]Pex5p was pre-incubated with (panel A) or without (panel B) human recombinant Pex14p (obtained as described in Materials and methods) and subjected to sedimentation analysis. The gradients were fractionated into 12 aliquots, starting from the bottom of the tube. Equal portions from each fraction were analysed by immunoblotting using the anti-Pex14p and anti-GST antibodies. [^{35}S]Pex5p was detected by autoradiography of the dried blot. Lane T—one fifth of the samples was loaded on the sucrose gradients. The numbers at the bottom indicate the positions of the molecular mass markers used to calibrate the gradients.

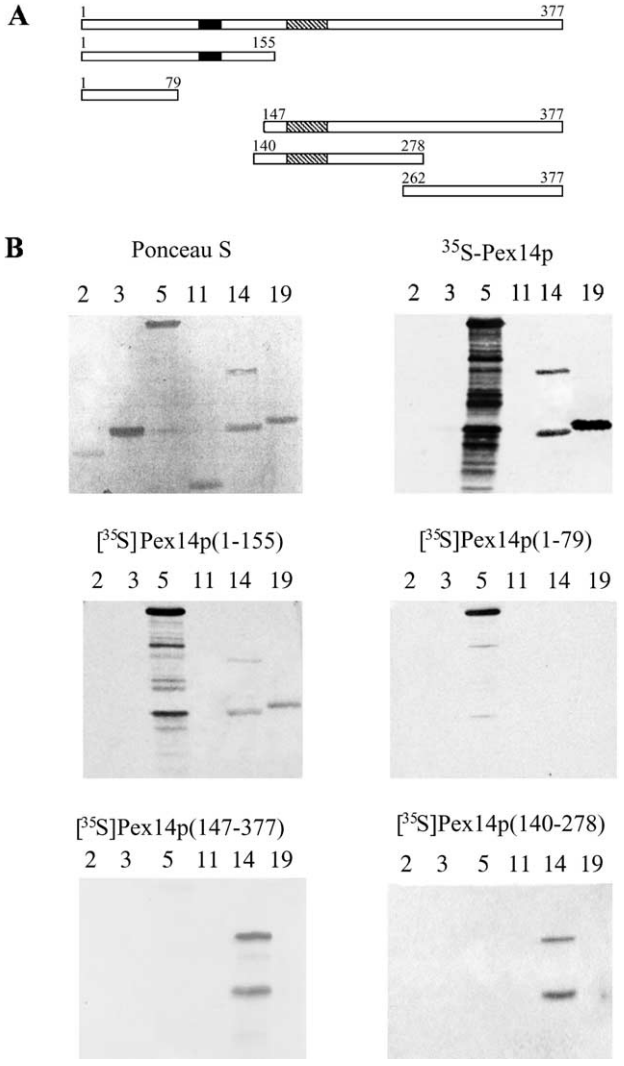

Fig. 6. Domain mapping of Pex14p–Pex14p interaction. (A) Schematic representation of Pex14p and the truncated versions of it used in blot-overlay assays. The black and hatched boxes represent the putative transmembrane and coiled-coil domains, respectively. (B) One to two micrograms of GST–Pex2p, GST–Pex3p, GST–Pex5p, GST–Pex11p, GST–Pex14p (see below) and GST–Pex19p (indicated in the blots by 2, 3, 5, 11, 14 and 19, respectively) was subjected to SDS-PAGE and transferred to nitrocellulose membranes (a Ponceau S-stained membrane is shown). Individual membranes containing the recombinant proteins were incubated with the following radiolabelled proteins: [^{35}S]Pex14p, [^{35}S]Pex14p(1–155), [^{35}S]Pex14p(1–79), [^{35}S]Pex14p(147–377) and [^{35}S]Pex14p(140–278). The interactions were detected by autoradiography. A GST–Pex14p preparation partially cleaved with thrombin was used in these experiments. The protein band displaying the higher apparent molecular mass detected in lanes "14" represents GST–Pex14p; the faster migrating protein band corresponds to full-length Pex14p.

When the Pex14p–Pex14p interaction is analysed, a specific binding to recombinant Pex14p is observed with either the full-length version of the protein, [^{35}S]Pex14p (147–377), or [^{35}S]Pex14p(140–278). Thus, amino acid residues 147–278 are involved in the Pex14p–Pex14p interaction. This is the region containing the coiled-coil domain of Pex14p (amino acid residues 163–198, as predicted by the SMART program [44,45]). Interestingly, a weak but specific interaction with recombinant Pex14p is also detected with [^{35}S]Pex14p(1–155), but not with the shorter [^{35}S]Pex14p(1–79) form. It is not clear at this moment whether this result derives from the existence of a second Pex14p–Pex14p binding domain or if Pex14p(1–155) contains at its C terminus additional (unpredicted) portions of the coiled-coil domain.

No specific interactions with any of the analysed peroxins were detected when [^{35}S]Pex14p(262–377) was used in this assay (data not shown).

4. Discussion

It is a generally accepted fact that mammalian Pex14p is an intrinsic component of the peroxisomal membrane [9,15,26]. However, data regarding the membrane topology of this peroxin are scarce. In fact, there are only two studies dealing with this problem [15,26]. In one of these studies [15], it was concluded that both termini of rat Pex14p are exposed into the cytosol. These results were obtained using N- and C-terminal epitope-tagged versions of the rat peroxin and, therefore, no conclusions regarding the membrane topology of the remaining domains of Pex14p could be withdrawn from those experiments. Experimental data suggesting that the C terminus of human Pex14p is at least partially exposed to the cytosol were provided in the second study [26]—upon proteinase K treatment of intact organelles, human Pex14p could be degraded to a 31-kDa fragment still containing epitope(s) recognised by an anti-Pex14p antibody directed to amino acid residues 1–134 (the antibody used in this work). Here, we present data suggesting, first, that the domain comprising amino acids residues 130–377 of rat Pex14p is completely exposed to the cytosol, and second, that the first 130 amino acid residues of rat Pex14p are highly protected from proteolytic attack by the peroxisomal membrane itself. In spite of several attempts, we were unable to degrade this 16-kDa domain of Pex14p even when access to the protease was provided through the matrix side of the peroxisomal membrane. Our data suggest that this characteristic does not derive from an intrinsic resistance of the 16-kDa peptide to proteolysis by proteinase K. Indeed, proteolysis of this domain could be easily accomplished when the peroxisomal membrane was first solubilised using mild detergents. On the other hand, we show that this N-terminal domain per se behaves as an intrinsic membrane component. These results do not necessarily mean that all the residues present in this domain are embedded in the membrane. In fact, with the exception of residues 109–127, the primary structure of Pex14p reveals that this region is quite hydrophilic. It is possible, however, that these hydrophilic residues are lying so close to the membrane or other proteins that by sterical hindrance no proteolytic cleavage can occur. Two peroxins, Pex5p and Pex19p, have been shown to bind to this region

of Pex14p (this paper and Refs. [17,43]). A hypothetical model for such a Pex14p/Pex5p complex was previously proposed [27]. In agreement with this interpretation, it is noteworthy that antibodies directed to the N terminus of Pex14p [26] or to an N-terminal tagged version of it [15] fail to detect this peroxin upon immunofluorescence analysis of mammalian cells when the peroxisomal membrane is left intact.

How is the folding of this N-terminal domain? If we assume that the data obtained with epitope-tagged versions of Pex14p [15] reflect the topology of the endogenous peroxin (i.e., the N terminus of Pex14p is facing the cytosol), and that, as shown here, all the C-terminal domains starting at approximately amino acid residue 130 are exposed into the cytosol, then there are only two possibilities: (1) the N-terminal domain comprising amino acid residues 1–130 of mammalian Pex14p never crosses completely the peroxisomal membrane—it just associates laterally with the membrane—or (2) this domain crosses the peroxisomal membrane an even number of times. Discriminating between these two possibilities will surely be a demanding task.

It is known for long that Pex14p interacts with itself [15]. However, the precise domain(s) involved in this interaction and the degree of polymerisation (i.e., if this interaction results in dimers, trimers, etc.) were never determined. In this work, data concerning the first problem are provided. Using a blot-overlay assay, we show that amino acid residues 147–278, a domain containing a coiled-coil motif [9,15,26], are involved in the Pex14p–Pex14p interaction. Unfortunately, and in spite of several attempts using different experimental conditions and analytical systems, we were unable to assess the polymerisation degree of this peroxin—Pex14p was always found as an heterogeneous entity, a property that was also observed by electron microscopy analysis of negatively stained preparations of recombinant Pex14p (data not shown). There are many different possibilities to explain this behaviour, going from nonspecific association of coiled-coil complexes (as described recently for human heat shock binding factor 1 [46]) to the existence of additional Pex14p–Pex14p interaction domain(s) not characterised in our experiments. Obviously, it could also be argued that the recombinant Pex14p protein used in this work is not correctly folded. Although we cannot completely exclude this possibility, several observations suggest that this is not the case. First, misfolded proteins are mostly insoluble. Second, we would not expect a misfolded protein aggregate to be so efficiently cleaved by thrombin giving rise to monomeric/dimeric GST. Finally, the Pex14p polymers detected in our sedimentation analysis can form stable complexes with in vitro synthesised Pex5p, suggesting that the conformation of recombinant Pex14p mimics, at least partially, the folding state of the endogenous Pex14p. Thus, if credit is given to the properties of recombinant Pex14p, our results raise the interesting possibility that Pex14p may be a tethering protein bringing together, at the same site of the peroxisomal membrane, many of the components involved in the biogenesis/turnover of the organelle.

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