Hsp70 regulates the interaction between the peroxisome targeting signal type 1 (PTS1)-receptor Pex5p and PTS1

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The peroxisome targeting signal type 1 (PTS1) receptor, Pex5p, of the tetratricopeptide repeat (TPR) motif family is located mostly in the cytosol and mediates the translocation of PTS1 proteins to peroxisomes. As a step towards understanding the mechanisms of protein import into peroxisomes, we investigated the molecular mechanisms involved in PTS1 recognition by Pex5p with regard to requirement of energy and cytosolic factors, using cell-free synthesized acyl-CoA oxidase (AOx) as a PTS1 cargo protein, together with Pex5p and heat-shock protein (Hsp)70 from rat liver. Pex5p was partly associated with peroxisomes of rat liver, was resistant to washing with a high concentration of salt and to alkaline extraction and was inaccessible to protease added externally. Pex5p bound to AOx in an ATP-dependent manner. AOx synthesized in a cell-free translating system from rabbit reticulocyte lysate was imported into peroxisomes without being supplemented with Pex5p and Hsp70, implying that peroxisome-associated Pex5p was released from the membranes and functional in this *in vitro* import assay. Antibodies against Pex5p and Hsp70 inhibited AOx import. In contrast, AOx synthesized in a wheat-germ lysate required the external addition of Pex5p for import, in which Hsp70 augmented the AOx import. The TPR domain of Pex5p was revealed to bind to the N-terminal part in an Hsp70-independent manner, whereas mutual interaction of the TPR region was noted in the presence of Hsp70. Hsp70 interacted with the TPR domain of Pex5p. Moreover, Hsp70 and ATP synergistically enhanced the binding of Pex5p to the C-terminal PTS1-containing part of AOx, implying that Pex5p recognizes its cargo PTS1 protein by chaperone-assisted as well as energy-dependent mechanisms *in vivo*.

Key words: acyl-CoA oxidase, peroxisomal protein import, tetratricopeptide repeat motif.

INTRODUCTION

To elucidate the hierarchy of highly organized biogenesis of intracellular organelles, peroxisome has been used as a model compartment in mammalian and yeast systems [1,2]. Significant progress has recently been made in our understanding of the mechanisms of peroxisome biogenesis, including the peroxisomal import of newly synthesized proteins [1]. To address such issues, we have previously used several potential approaches, such as those using protein import *in vitro* [3–5] and mammalian somatic cell mutants [1,6]. Post-translational import of peroxisomal proteins was first demonstrated *in vitro* with fatty acyl-CoA oxidase (AOx) and catalase from rat liver [7]. Since then, several important questions, including the energy and temperature dependence and the requirement for bivalent ions and a cytosolic fraction, have been addressed [8,9]. By the use of Chinese hamster ovary (CHO) cell mutants [1] and an expressed sequence tag ('EST') homology search with yeast *PEX* genes, a dozen mammalian peroxin genes, including *PEX1*, *PEX2* (formerly PAF-1), *PEX3*, *PEX5*, *PEX6*, *PEX7*, *PEX10*, *PEX12*, *PEX13*, *PEX14*, *PEX16* and *PEX19*, have been isolated [1,10]. These peroxin genes, except *PEX14* [11], were shown to be responsible for peroxisome biogenesis disorders such as Zellweger syndrome. However, underlying mechanisms by which most of the peroxins function in peroxisome biogenesis have not been well understood at the molecular level.

Peroxisomes are formed by the division of pre-existing peroxisomes after the post-translational import of newly synthesized proteins [12]. Most peroxisomal matrix proteins are mediated by well-characterized *cis*-acting peroxisome targeting signals (PTSs): the C-terminal SKL (single-letter amino acid codes) motif in PTS1 [4,13] and the N-terminal cleavable nonapeptide sequence PTS2, $(R/K)(L/V/I)X_5(H/Q)(L/A)$ [14–16]. *PEX5* and *PEX7*, encoding the receptors for PTS1 and PTS2 respectively, have been identified in both yeast and mammals, including humans (reviewed in [17]). A deficiency in Pex5p causes peroxisome biogenesis disorders of complementation group 2, manifesting as impaired protein import [18–21].

The peroxin Pex5p, the receptor for PTS1, is a relatively well characterized peroxin with regard to cell biological and biochemical functions. Pex5p translocates newly synthesized PTS1 proteins from the cytoplasm to peroxisomes. In mammals, two isoforms of Pex5p of the tetratricopeptide repeat (TPR) protein family have been identified: a shorter form, termed Pex5pS, serves as a PTS1 receptor; the longer form, Pex5pL, with an 37-amino-acid internal insertion, functions in both PTS1 and PTS2 import pathways [20–23]. Imanaka et al. [8] suggested that AOx import required ATP, not GTP, *in vitro*. A similar observation was made for peroxisomal PTS1-protein import with a semipermeabilized cell system [24]. Protein factors, if any, essential for peroxisomal protein import remained to be found.

Therefore, as a step to understanding the molecular mechanisms of Pex5p-mediated protein transport to peroxisomes, we have investigated potential factors involved in the process of PTS1-protein import. Here we report that Hsp70 interacts directly with and regulates Pex5p in an ATP-dependent manner.

Abbreviations used: AOx, acyl-CoA oxidase; CHO, Chinese hamster ovary; CI, Chinese hamster; GST, glutathione S-transferase; Hsp, heat-shock protein; PTS1 and PTS2, peroxisome targeting signal types 1 and 2; TPR, tetratricopeptide repeat.

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EXPERIMENTAL

Expression of fusion protein

A glutathione S-transferase (GST) fusion protein with a C-terminal part consisting of residues 521–661 of rat AOx [3], termed AOx-C, was constructed: a *Sal*I fragment of rat AOx cDNA in pTZ18R [3] was inserted into the *Sal*I site of pGEX4T-3 (Amersham Pharmacia Biotech, Tokyo, Japan). An expression plasmid for a GST fusion protein with Chinese hamster (Cl) Pex5pS, a shorter form of Pex5p [20], in pGEX6P-1 (Amersham Pharmacia Biotech) was as described [22,23]. A GST fusion protein with various truncated mutants of ClPex5pS was constructed as follows. For GST–ClPex5p, consisting of residues 143–595, termed GST–ClPex5p(143–595), an *Eco*RI fragment was deleted from pGEX6P-1·*ClPEX5S*. For GST–ClPex5p(290–595), an *Nco*I–*Kpn*I fragment of pGEX6P-1·*ClPEX5S* was replaced with the PCR product of *ClPex5pS* with the use of a forward primer, FW (5′-AACCGTCATAG-ACCATGGTG-3′), and a reverse primer, RV (5′-ACCATG-GACCACCACCTCAGGCTTT-3′). All plasmid constructs were assessed by nucleotide sequence analysis. GST fusion proteins with AOx-C, ClPex5p and Pex5p variants were expressed in *Escherichia coli* and purified as described [11]. AOx-C and ClPex5p derivatives were isolated from their respective GST fusion proteins by cleaving them with thrombin and PreScission protease (Amersham Pharmacia Biotech) respectively, as described [11].

Antibodies

Anti-Pex5p peptide antibody was raised in rabbits by immunization with a synthetic peptide comprising the C-terminal 19-residue sequence of human Pex5p supplemented with a Cys residue at the N-terminus, which had been linked to keyhole limpet haemocyanin [25]. Rabbit antibody against full-length ClPex5pS was raised by immunization with recombinant ClPex5pS [22]. Other rabbit antibodies, including those against rat AOx [26], rat catalase [26], human Pex7p (S. Mukai and Y. Fujiki, unpublished work), Pex12p [27], Pex13p [28], Pex14p [11] and 70 kDa peroxisomal major integral membrane protein (PMP70) of rat liver [26], were as described. Rabbit anti-[bovine brain heat-shock protein (Hsp70)] antibody was purchased from Biogenesis.

Isolation of peroxisomes, Hsp70 and Pex5p from rat liver

Rat liver peroxisomes were isolated as described [4]. The cytosolic fraction was isolated from the post-light-mitochondrial fraction of rat liver by centrifugation at 150000 ***g*** for 40 min in a Beckman 55.2 Ti rotor. Hsp70 was purified from rat liver cytosol as described by Chirico et al. [29]. In brief, proteins precipitated by salting out at 30–60 %-satd $(NH_4)_2SO_4$ were dialysed against 10 mM potassium acetate/1 mM dithiothreitol/5 mM magnesium acetate/20 mM Hepes/KOH (pH 7.4). Hsp70 was isolated by two-step chromatography with DEAE-cellulose and ATP–agarose (Sigma). For isolation of Pex5p, proteins precipitated by salting out at 30 %-satd $(NH_4)_2SO_4$ were dialysed against 20 mM Tris/HCl (pH 8.0)/0.1 M NaCl/1 mM EDTA and were applied to a DEAE-cellulose column. Pex5p was purified by washing with 0.1 M NaCl/0.2 M NaCl, then by elution with a linear gradient of 0.2–0.4 M NaCl. Pex5p-containing fractions were assessed by SDS/PAGE and immunoblotting. Pex5p was further purified from these fractions by rechromatography.

Assays for intra-organellar localization of Pex5p

Freshly isolated rat liver peroxisomes (0.5 mg) in 1 ml of 0.25 M sucrose/20 mM Hepes/KOH (pH 7.4) were treated either by five cycles of freeze–thawing or with 0.5 M NaCl or 0.1 M Na_2CO_3 [30]. Peroxisomes were then separated into membrane and soluble fractions by centrifugation at 15000 rev./min (18000 ***g***) for 30 min in a Hitachi CF15D Microfuge and analysed by SDS/PAGE and immunoblotting. For the protease protection assay, rat liver peroxisomes (0.5 mg) in 1 ml of 0.25 M sucrose/20 mM Hepes/KOH (pH 7.4) were treated with various concentrations of proteinase K in the absence or presence of Triton X-100 for 30 min on ice. The reaction was terminated with 1 mM PMSF and peroxisomes were recovered by centrifugation. Peroxisomal proteins in both fractions were assessed by SDS/PAGE and immunoblotting.

Import of AOx into peroxisomes *in vitro*

The peroxisomal import assay was performed at 25 °C for 1 h, essentially as described [4], with cell-free synthesized ^{35}S-labelled AOx (10 μl) and freshly isolated rat liver peroxisomes (15 μg) in 0.25 M sucrose/0.1 % BSA/10 mM methionine (40 μl). Transcription and translation *in vitro* [3] were performed as described. As a protein-synthesizing system *in vitro*, rabbit reticulocyte lysates (Amersham Pharmacia Biotech) and wheat-germ lysates (Promega) were used. To determine whether antibodies against Hsp70 and Pex5p affect AOx import, import *in vitro* was conducted with rabbit reticulocyte lysates that had been pretreated on ice for 1 h with anti-Hsp70 antiserum or peroxisomes preincubated on ice for 1 h with anti-Pex5p antibody.

Binding assay *in vitro*

In the search for PTS1-binding protein in the cytosol, the cytosolic fraction of rat liver was dialysed overnight against 50 mM Mes/NaOH (pH 7.4)/0.1 M NaCl/5 mM $MgCl_2$/2 mM EDTA/1 mM PMSF/1 mM NaF/10 μg/ml leupeptin/10 μg/ml antipain. Binding assay mixture (1 ml), containing the protein components to be examined, including GST–AOx-C (30 μg), rat liver cytosol (4.5 mg) and GSH–Sepaharose (100 μl), was incubated for 3 h at 4 °C in the presence or absence of 2 mM $MgATP^{2-}$. Proteins bound to GSH–Sepharose were recovered by using a Mini-column (Bio-Rad), washed four times sequentially with PBS and 50 mM Tris/HCl, pH 8.0. Bound proteins were eluted differentially from the complexes by adding 0.5 M NaCl, 5 mM ATP, then 10 mM GSH to the elution buffer. Each fraction was analysed by SDS/PAGE and immunoblotting.

A binding assay of Pex5p and Hsp70 was performed with recombinant Pex5p, its variants, and rat liver Hsp70 in the presence or absence of 2 mM ATP. GST fused to Pex5p or truncated Pex5p (100 μg each) was incubated with GSH–Sepharose (100 μl). The reaction mixture (100 μl) contained the protein components to be examined, including the GST–Pex5p/GSH–Sepharose complex (5 μl) and Hsp70 (1.5 μg), in binding assay buffer A [50 mM Mes/NaOH (pH 7.5)/0.1 M NaCl/0.5 % (v/v) Nonidet P40/1 μg/ml aprotinin/1 μg/ml leupeptin/1 μg/ml pepstatin/1 μg/ml antipain/1 mM benzamidine/2 mM EDTA/1 mM NaF/2 mM dithiothreitol]. After incubation for 1 h at 4 °C, proteins bound to GSH–Sepharose were washed three times with buffer B [20 mM Hepes/KOH (pH 7.4)/0.1 M NaCl/0.01 % (v/v) Nonidet P40] and were analysed by SDS/PAGE. Hsp70 was detected by immunoblotting.

To investigate whether the N-terminal part of Pex5p interacts with the C-terminal TPR domain, GST–Pex5p(1–296) (10 μg)

and GST–Pex5p(290–595) (10 μg) were incubated with Pex5p(290–595) (5 μg) and Pex5p(1–296) (5 μg) respectively, for 30 min at 4 °C in the presence of Hsp70 (1.5 μg) and 2 mM ATP in buffer A (80 μl). The reaction continued for a further 3 h after the addition of GSH–Sepharose (20 μl) that had been pre-incubated with 0.05 % BSA/0.5 % skimmed milk/50 mM Mes/NaOH (pH 7.5). To verify the effect of Hsp70 on Pex5p-PTS1 binding, GST–AOx-C (30 μg) was also incubated with recombinant Pex5p variants (7 μg of each) in the presence and the absence of Hsp70 (1.5 μg) and 2 mM ATP. Protein complexes with GST–Pex5p variants were recovered with precoated GSH–Sepharose as described above.

AOx-C was labelled with FITC by using FITC-maleimide (Pierce), in accordance with the manufacturer's protocol. After the reaction had been terminated with 0.1 M 2-mercaptoethanol, the mixture was gel-filtered through Sephadex G-25. FITC-AOx-C was incubated for 30 min at 4 °C with Pex5p (0.5 μg) in the presence and the absence of Hsp70, in PBS/0.05 % (v/v) Nonidet P40/2 mM dithiothreitol/1 mM $MgCl_2$/1 mM EDTA/2 mM NaF (100 μl). Immunoprecipitation was performed for 1 h at 4 °C with anti-Pex5p antisera (1 μl) and formalin-fixed, 4 % (w/v) BSA-pretreated *Staphylococcus aureus* cells (Pansorbin; Calbiochem) (10 μl). Immunocomplexes were washed twice with 0.05 % (v/v) Nonidet P40/PBS. Bound FITC-AOx-C was dissociated from the immunoprecipitates by using 0.5 % SDS/0.1 M Tris/HCl (pH 8.0) (1 ml) and was quantified by measuring its fluorescence, with excitation at 490 nm and emission at 515 nm. Non-specific binding was verified as the values obtained in the presence of 0.5 mM PTS1 peptide consisting of the C-terminal 10 residues of AOx [20].

Other methods

Western blot analysis was performed on samples transferred electrophoretically to PVDF membrane (Bio-Rad), with primary antibodies and a second antibody, donkey anti-(rabbit IgG) antibody conjugated with horseradish peroxidase (Amersham Pharmacia Biotech). The antigen–antibody complex was revealed with enhanced chemiluminescence Western blotting detection reagent (ECL®; Amersham Pharmacia Biotech).

RESULTS

Anti-Pex5p antibody

We raised antiserum against Pex5p by immunizing a rabbit with the C-terminal 19-residue sequence, Pex5pC, of human Pex5pS. The antibody specifically recognized an 80 kDa protein with the apparent molecular mass of Pex5p [22], in both purified peroxisomes and the cytosolic fraction of rat liver (Figure 1A, upper panel, lanes 1 and 2). The protein band reactive to the anti-Pex5pC antibody was no longer detectable when the antiserum had been preincubated with the C-terminal peptide of Pex5p used for injection into a rabbit (Figure 1A, upper panel, lanes 3 and 4). Moreover, this anti-Pex5pC antibody was verified by the use of recombinant Pex5p-N and Pex5p-C. The antibody was reactive only to Pex5p-C, not to Pex5p-N (Figure 1A, upper panel, lanes 5 and 6). These results therefore indicate that the antibody specifically recognizes the C-terminal portion of Pex5p. Rabbit antibody against full-length ClPex5pS similarly recognized a protein, of the same size as the 80 kDa protein cross-reactive to anti-Pex5pC antibody, from both peroxisomes and cytosol (Figure 1A, lower panel, lanes 1 and 2). The anti-ClPex5pS antibody seemed to recognize the C-terminal half of Pex5pS more readily than the N-terminal half, as assessed by immunoblotting on Pex5p-N and Pex5p-C (Figure 1A, lower panel, lanes 5 and 6). Antibody against the full-length Pex5p showed a higher titre than the antibody against the C-terminal peptide (results not shown).

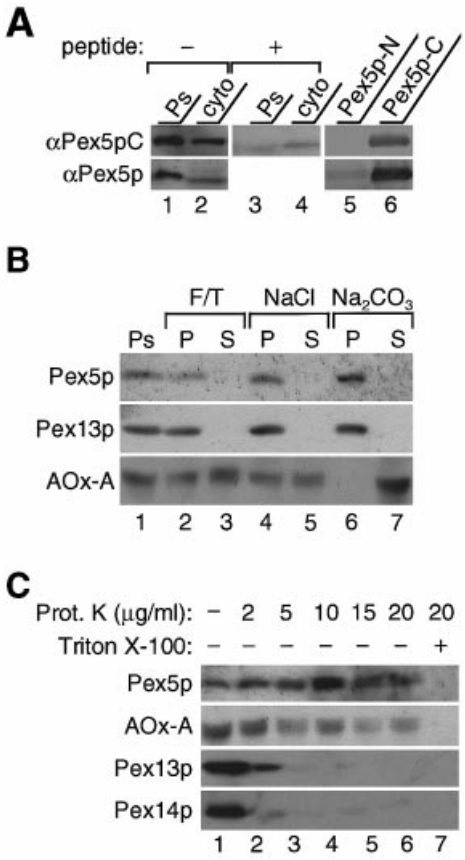


Figure 1 Subcellular localization of Pex5p

(**A**) Specificity of anti-Pex5p antibody. Western blot analysis was performed with rabbit antibodies raised against the C-terminal peptide of HsPex5p (αPex5pC) and ClPex5pS (αPex5p). Rat liver peroxisomes (lanes 1 and 3) (50 μg), cytosol (lanes 2 and 4) (70 μg) and bacterially expressed N-terminal half (residues 1–296; lane 5) (7.5 μg) as well as the C-terminal TPR domain (residues 290–595; lane 6) (0.1 μg) of ClPex5pS were probed with antisera against Pex5pC peptide (upper panel) and Pex5pS (lower panel). Anti-Pex5pC antiserum that had been preincubated with Pex5pC peptide was also used (lanes 3 and 4). (**B**) Characterization of peroxisome-associated Pex5p. Freshly isolated rat liver peroxisomes (0.5 mg) were treated by freeze–thawing (F/T), with 0.5 M NaCl or with 0.1 M Na_2CO_3 and were then separated into membrane (P) and soluble (S) fractions by centrifugation. An aliquot from peroxisomes (75 μg for Pex5p and Pex13p; 3 μg for a matrix marker, AOx) was analysed by SDS/PAGE and immunoblotting with specific antibodies, including anti-Pex5p antibody. AOx was represented by its 75 kDa AOx-A component. (**C**) Sensitivity to protease treatment. Rat liver peroxisomes (0.5 mg) were treated with various concentrations of proteinase K in the absence (lanes 1–6) or presence (lane 7) of Triton X-100. An aliquot of peroxisomes (75 μg for Pex5p, Pex13p and Pex14p; 3 μg for AOx) was analysed by SDS/PAGE and immunoblotting with specific antibodies as in (**B**).

Pex5p in peroxisomes

As shown in Figure 1(A), we identified Pex5p in peroxisomes, although in a smaller amount (approx. 5 %) than the cytosolically localized Pex5p (approx. 95 %), calculated based on the total amount of peroxisomes and the cytosol, representing 2–3 and 50 % respectively, of total cellular proteins in rat liver [31]. The Pex5p was recovered in membrane fraction when rat peroxisomes were subjected to several cycles of freeze–thawing or washing with 0.5 M NaCl (Figure 1B, upper panel, lanes 1–5). After

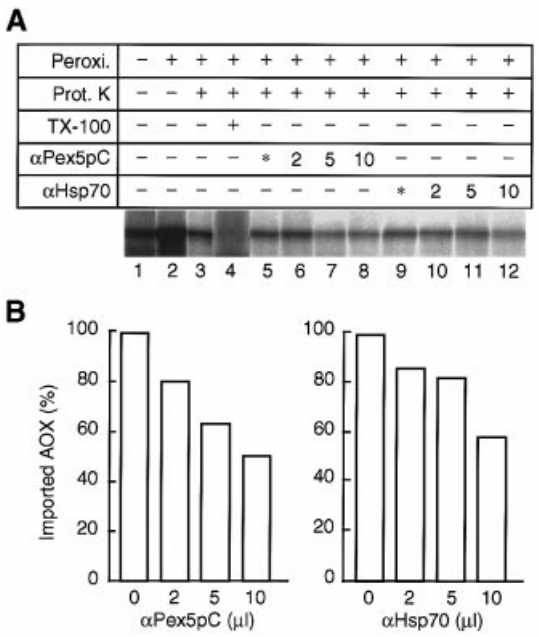


Figure 2 Inhibition of peroxisomal import of AOx by antibodies against Hsp70 and Pex5p

(**A**) Import of ^{35}S-AOx into peroxisomes *in vitro* was performed in the presence of anti-Hsp70 antibody or anti-Pex5pC antibody, as described in the Experimental section. AOx import was assessed by resistance to externally added proteinase K. Only peroxisomal fractions recovered by centrifugation are shown. Lane 1, ^{35}S-AOx input (5%); lanes 2 and 3, peroxisome-associated and protease-resistant forms of AOx respectively; lane 4, after proteinase K treatment in the presence of Triton X-100. Antisera added in the import assays are indicated: anti-Pex5pC serum (lanes 6–8); anti-Hsp70 serum (lanes 10–12); asterisks designate preimmune serum (10 μl) from each rabbit (lanes 5 and 9). (**B**) Imported ^{35}S-AOx was quantified and is shown as a percentage of untreated control (imported AOx in the presence of preimmune serum). Abbreviations are as in Figure 1(A).

treatment with 0.1 M sodium carbonate, Pex5p was in a membrane fraction (Figure 1B, upper panel, lanes 6 and 7). These results suggested that Pex5p detectable in peroxisomes is localized to and integrated into membranes. The peroxin Pex13p, an integral membrane protein [28], was in the membrane pellets after these three different treatments, whereas a matrix enzyme, AOx, was in the soluble fraction after treatment with sodium carbonate and was partly associated with the organelle fraction after the freeze–thaw and NaCl wash treatments (Figure 1B, lower panels). These results thus confirmed adequate separation of the cytosolic and membrane fractions.

To confirm the findings with regard to the peroxisomal location of Pex5p, peroxisomes were treated with proteinase K. Pex5p was resistant to the treatment with exogenously added proteinase K, as was AOx, whereas Pex13p and another membrane peroxin of peroxisomes, Pex14p [11], were readily digested under the same conditions (Figure 1C). These results were interpreted to mean that a protease-resistant form of Pex5p was inside the membrane, presumably tightly associated with other components, conceivably including peroxisomal import machinery.

Antibodies against Hsp70 and Pex5p affect AOx import

We investigated whether antibodies against Pex5p and Hsp70 had any effect on PTS1 import *in vitro*. Importing of AOx into peroxisomes was performed *in vitro* with ^{35}S-labelled AOx synthesized in a rabbit reticulocyte lysate cell-free protein-synthesizing system and was verified by resistance of ^{35}S-AOx to proteinase K (Figure 2A, lanes 1–3), as described [4]. The resistance of ^{35}S-AOx to proteinase K in the peroxisomal fraction was abolished by the addition of Triton X-100 before the digestion, confirming peroxisomal importing of AOx (Figure 2A, lane 4), which is consistent with the previous observation by several groups of investigators, including our own [3,4,7,8]. The addition of anti-Pex5p antiserum apparently decreased the level of imported AOx (Figure 2A, lanes 6–8), as verified by the resistance of ^{35}S-AOx to proteinase K, whereas preimmune serum showed no apparent effect (lane 5). The inhibition became more distinct up to approx. 50% as the amount of the antibody increased (Figure 2B, left panel). A similar inhibitory effect was also observed when anti-Hsp70 antiserum was added to the import assay mixture (Figure 2A, lanes 9–12; Figure 2B, right panel). Taken together, these results suggest that Pex5p and Hsp70 are involved in PTS1 transport.

Pex5p and Hsp70 are required and synergistically augment AOx import

Pex5p and Hsp70 were partly purified from rat liver cytosol. The Pex5p-containing fraction was revealed to be enriched approx. 20-fold over the crude cytosolic fraction, when assessed by immunoblotting (results not shown). Hsp70 was quite highly purified as judged from staining with Coomassie Blue (Figure 3A, left panel). Adequate separation of Pex5p from Hsp70 was confirmed by immunoblotting with the respective antibodies (Figure 3A, middle and right panels). Import of AOx into peroxisomes *in vitro* was performed as in Figure 2(A), except that [^{35}S]AOx was synthesized in a wheat-germ lysate cell-free protein-synthesizing system to avoid contamination with mammalian cytosolic proteins such as Hsp70 in the import assay. In the absence of Pex5p and Hsp70 in the import assay mixture, part of the ^{35}S-AOx recovered in the peroxisomal fraction was not resistant to digestion with proteinase K (Figure 3B, lanes 1–3). This result indicates that AOx was not imported into peroxisomes, implying that Hsp70 presumably present in the wheat-germ lysate is not functional in this mammalian peroxisomal import assay. AOx was imported on the addition of Pex5p or Hsp70 (Figure 3B, lanes 4 and 5), although the import efficiency was lower than that with reticulocyte lysate. This result suggests that peroxisomal membrane-associated Pex5p was functional in PTS1 import. Imported AOx was elevated severalfold by the addition of both Pex5p and Hsp70, in a Pex5p-dose-dependent manner (Figure 3B, lanes 6 and 7; Figure 3C), nearly up to the level noted in the imported AOx in the import assay *in vitro* with a rabbit reticulocyte lysate system (see Figure 2).

Hsp70 binds to Pex5p TPR domains in an ATP-dependent manner

We next investigated underlying mechanisms by which Pex5p and Hsp70 interact with PTS1 protein, with a GST fusion protein with the 141-residue C-terminal part of AOx (AOx-C) carrying the PTS1 SKL motif, and rat liver cytosol. Proteins bound to GST–AOx-C were probed with antibodies against Pex5p and Hsp70. Pex5p was detected and apparently decreased in amount in the presence of 2 mM ATP, whereas no Pex5p was detectable in the fraction recovered with GST (Figure 4A, upper panel, lanes 1–3), indicative of specific binding of Pex5p. Hsp70 was also present in the GST–AOx-C fraction, where ATP similarly decreased the level of bound Hsp70 (Figure 4A, lower panel). The complexes comprising Pex5p, Hsp70 and GST–AOx-C were characterized further. Neither Pex5p nor Hsp70 was released from these complexes by a salt wash (Figure 4B, lanes 1 and 2). Hsp70 was mostly eluted from the complexes by the

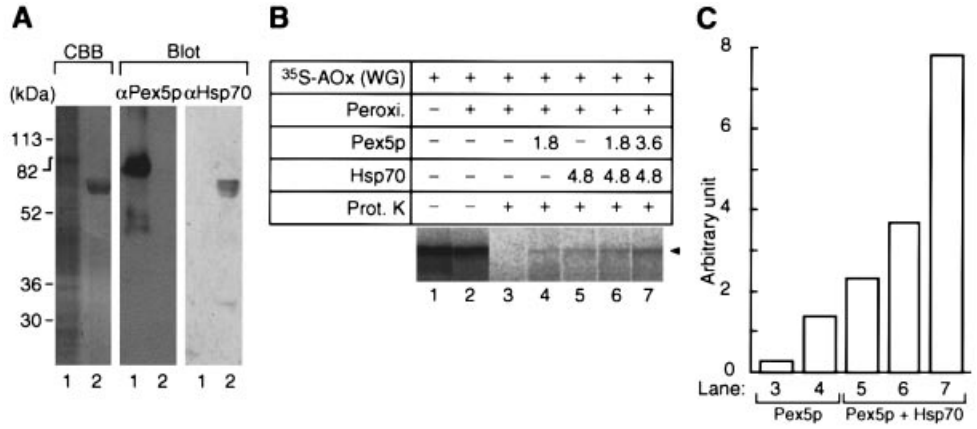


Figure 3 Peroxisomal import of PTS1 protein requires Pex5p and is enhanced by Hsp70

(**A**) Purification of Pex5p and Hsp70 from rat liver cytosol. Partly purified Pex5p (lane 1) and Hsp70 (lane 2) were assessed by SDS/PAGE followed by staining with Coomassie Blue (left panel) and by immunoblotting with antibodies against Pex5p (middle panel) and Hsp70 (right panel). Note that Pex5p and Hsp70 were separated by this purification. The positions of molecular mass markers are indicated at the left. (**B**) Import of AOx *in vitro* was performed with ^{35}S-AOx (10 μl) synthesized in a cell-free protein-synthesizing system from wheat-germ lysate, freshly isolated rat liver peroxisomes (15 μg), rat liver Pex5pS and Hsp70 (in μg), as indicated at the top. The arrowhead indicates ^{35}S-AOx. ^{35}S-AOx input (5%) was loaded in lane 1. (**C**) Imported AOx was quantified and is shown as described in the legend to Figure 2(B); abbreviations are as in Figure 1(A).

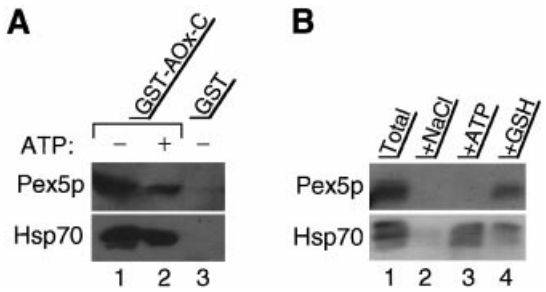


Figure 4 Identification of PTS1-interacting proteins in rat liver cytosol

(**A**) A binding assay *in vitro* was performed with GST–AOx-C (the C-terminal 141 residues of AOx) and rat liver cytosol in the presence (+) or absence (−) of 1 mM ATP. Proteins bound to GST–AOx-C were pulled down with GSH–Sepharose and were analysed by SDS/PAGE and immunoblotting with antibodies against Pex5p (upper panel) and Hsp70 (lower panel). Lane 1, GST–AOx-C; lane 2, GST–AOx-C plus ATP; lane 3, GST. (**B**) ATP is involved in the interaction of PTS1 protein with Hsp70. GST–AOx-C and rat liver cytosol were incubated in the absence of ATP as in (**A**). Bound proteins recovered with GSH–Sepharose beads (lane 1) were eluted sequentially from the complexes by the addition of 0.5 M NaCl (lane 2), 5 mM ATP (lane 3), then 10 mM GSH (lane 4) to the elution buffer. Each fraction was analysed by SDS/PAGE and immunoblotting.

addition of 2 mM ATP (Figure 4B, lane 3), suggesting that Hsp70 in the AOx–Pex5p–Hsp70 complexes might be in an ADP-bound form. In contrast, Pex5p was eluted only by GSH (Figure 4B, lane 4), together with the remaining Hsp70 and presumably with GST–AOx-C concomitantly released from GSH–Sepharose beads.

Next, to determine the interaction of Hsp70 with Pex5p, we constructed various GST fusion proteins with truncated Pex5p (Figure 5A) and incubated them with Hsp70 in the presence and the absence of 2 mM ATP. Hsp70 was detectable with full-length Pex5p but not bound to GST (Figure 5B, lanes 1 and 10). Hsp70 also bound to GST–AOx-C (Figure 5B, lane 9). The addition of ATP decreased the level of Hsp70 bound to Pex5p (Figure 5B, lane 2). Deletion of the N-terminal 142 residues of Pex5p (GST–Δ1–142) resulted in an interaction with Hsp70 (Figure 5B, lanes 3 and 4). Pex5p deleted in TPR domains 4–7 interacted with Hsp70, apparently in an ATP-independent manner, whereas TPR4–7 bound weakly to Hsp70 (Figure 5B, lanes 5–8). Moreover, other types of deletion mutant of Pex5p were verified for interaction with Hsp70, in the absence of ATP (Figure 5C). The C-terminal half of Pex5p containing the TPR domain (Pex5p-C) bound strongly to Hsp70, whereas the N-terminal half comprising residues 1–295, excluding the TPR region (Pex5p-N), no longer interacted with Hsp70 (Figure 5C, lanes 2 and 1). Hsp70 binding was also observed with GST–Pex5p truncated in TPR1–3 (GST–ΔTPR1–3; Figure 5C, lane 5); this binding was similarly retained for Pex5p deleted in TPR4–7 (see Figure 5B, lane 5). These results suggest strongly that Hsp70 interacts with TPR1–3 and TPR4–7 of Pex5p. It is noteworthy that mutant forms of Pex5p derived from CHO *pex5* mutants ZP105 and ZP139, containing $Gly^{298} \rightarrow Glu$ (G298E) and $Gly^{485} \rightarrow Glu$ (G485E) mutations respectively [22], interacted with Hsp70, whereas Pex5p-G298E bound PTS1 weakly but Pex5p-G485E showed no binding to PTS1 protein [22].

Hsp70 modulates interaction between Pex5p and PTS1 protein

Full-length and various truncated forms of Pex5p were incubated with GST–AOx-C in the presence or absence of Hsp70 and ATP. Pex5p was detectable in the absence of Hsp70, whereas binding of Pex5p to GST–AOx-C was made more distinct by the addition of Hsp70 (Figure 6, top left panel, lanes 1 and 2); on the addition of ATP, the level of bound Pex5p was significantly decreased (lane 3), suggesting that Hsp70 modulated the binding of Pex5p to PTS1 in an ATP-dependent manner. A similar requirement for Hsp70 and an ATP-dependent, milder decrease in Pex5p–PTS1 interaction were discernible for TPR4–7 of Pex5p (Figure 6, lower left panel). An Hsp70-dependent increase in Pex5p binding to AOx-C was observed with Pex5pΔ1–142, in which ATP enhanced the binding to AOx-C (Figure 6, middle left panel). Moreover, a more distinct requirement for Hsp70 was observed for the interaction of Pex5pΔTPR1–3 with AOx-C, whereas no difference was noted for Pex5p-C containing the entire TPR1–7 (Figure 6, right panels, lanes 4 and 5).

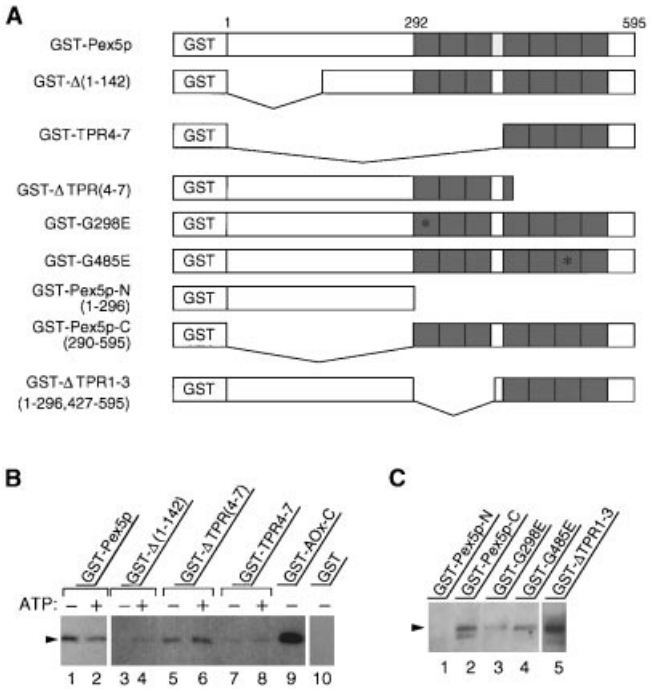


Figure 5 Domain mapping of Pex5p

(**A**) Schematic representation of Pex5pS variants fused to GST. GST–G335E and GST–G522E represent GST fused to Pex5pS with G298E and G485E mutations (asterisks) derived from CHO *pex5* cell mutants ZP105 and ZP139 respectively [20]. GST fusion proteins with the N-terminal half (residues 1–296) and the TPR domain (residues 290–595) of Pex5pS were termed GST–Pex5p-N and GST–Pex5p-C respectively. (**B**) GST fusion proteins with full-length and various truncated mutants of Pex5pS were incubated with Hsp70 in the absence (−) or presence (+) of 1 mM ATP. Binding of Hsp70 to GST–AOx and GST was also verified (lanes 9 and 10). Bound Hsp70 (arrowhead) was detected as in Figure 4(A). (**C**) GST fusion proteins with other Pex5pS mutants were incubated with Hsp70 in the absence of ATP. Arrowhead, Hsp70.

N-terminal part of Pex5p interacts with C-terminal TPR region

The N- and C-terminal halves of Pex5p, each fused to GST, were verified for binding to Pex5p-N and Pex5p-C in the presence and the absence of Hsp70 and ATP. The C-terminal half of Pex5p (residues 290–595) bound to GST–Pex5p-C in an Hsp70-dependent manner (Figure 7, lanes 3 and 4). ATP significantly elevated the level of bound Pex5p-C (Figure 7, lane 5). In contrast, Pex5p-C bound to GST–Pex5p-N (residues 1–296) in the absence of Hsp70 (Figure 7, lane 6). The addition of Hsp70 did not change the amount of Pex5p-C bound to GST–Pex5p-N (Figure 7, lane 7). More strikingly, Hsp70 plus ATP markedly decreased the level of Pex5p-C (Figure 7, lane 8). No apparent effects of Hsp70 and ATP were observed in the interaction between Pex5p-N with GST–Pex5p-N (Figure 7, lanes 9–11). We interpret these results to mean that the C-terminal domain of Pex5p interacts with the C-terminal half TPR region in an ATP- and Hsp70-dependent manner. The interaction between the N-terminal region and the TPR domains does not require Hsp70. It is more likely that Hsp70 dissociates the C-terminal TPR domain from the N-terminal region, rendering the TPR part available to bind to the PTS1-containing part of cargo proteins.

Hsp70 augments the binding of Pex5p to PTS1

We determined whether Hsp70 augmented the binding of Pex5p to PTS1 by using FITC-labelled AOx. The binding profile of

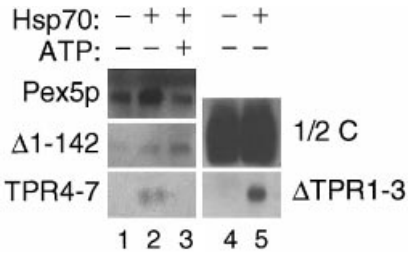


Figure 6 PTS1-binding region of Pex5p

Full-length and various truncated variants of Pex5pS were incubated with GST–AOx-C in the absence (−) or presence (+) of Hsp70 and ATP (lanes 1–3) or in the absence or presence of Hsp70 alone, without ATP (lanes 4 and 5). Pex5p was detected with anti-Pex5p antibody.

AOx to Pex5p is sigmoidal (Figure 8). Hsp70 augmented the binding of AOx to Pex5p in a concentration-dependent manner, apparently with first-order kinetics, particularly at lower concentrations of AOx. This effect was no longer distinct at higher concentrations of AOx; instead, equimolar binding was noted. It is likely that Hsp70 modulates the interaction of Pex5p and PTS1 without changing the level of maximal binding of Pex5p. These findings suggest that Hsp70 is required for the recognition of PTS1 protein by Pex5p, probably when PTS1 cargoes are at a constitutively expressed level. An induced level of PTS1 proteins such as those by peroxisome proliferators might be readily transported to peroxisomes by Pex5p. Alternatively, if the physiological concentration of these induced cargo PTS1 proteins were lower than that set here (maximum at approx. 10 μg/ml), Hsp70 might be involved in their transport to peroxisomes.

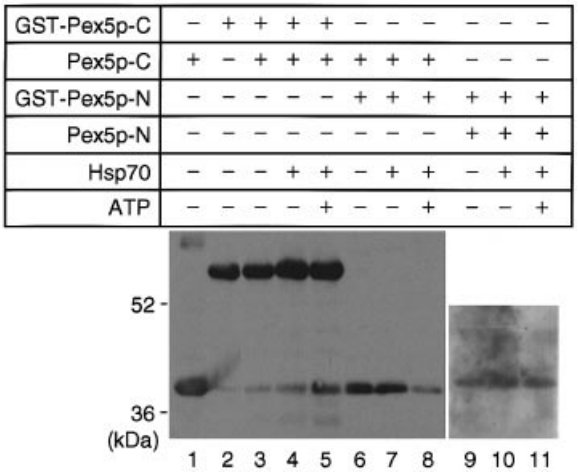


Figure 7 Hsp70-dependent interaction of N-terminal and C-terminal half-domains of Pex5p

The GST fusion proteins GST–Pex5p-N and GST–Pex5p-C (5 μg of each) were incubated with Pex5p-N and Pex5p-C (2.5 μg of each) in the presence (+) or absence (−) of Hsp70 (1.5 μg) and 2 mM ATP, as indicated. One half of each of the bound proteins was analysed by SDS/PAGE and immunoblotting with antibodies against Pex5pC peptide (lanes 1–8) and Pex5pS (lanes 9–11). The positions of molecular mass markers are indicated at the left. Lanes 1 and 2, Pex5p-C (1 μg) and GST–Pex5p-C (2 μg) probed with anti-Pex5pC antibody; lanes 3–5, GST–Pex5p-C incubated with Pex5p-C; lanes 6–8, GST–Pex5p-N incubated with Pex5p-C; lanes 9–11, GST–Pex5p-N incubated with Pex5p-N.

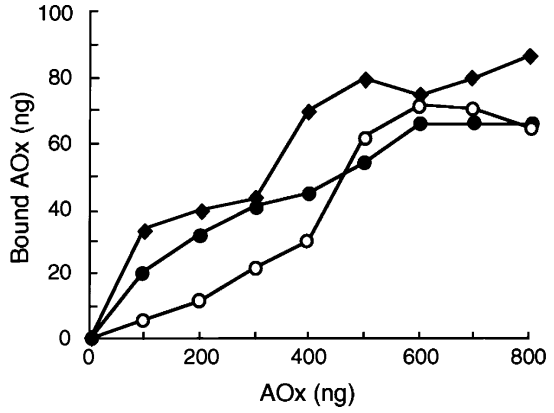


Figure 8 Hsp70 regulates binding of Pex5p to PTS1 protein

FITC-labelled AOx-C was incubated with Pex5p in the absence (○) or presence of Hsp70 (●, 1.5 μg; ◆, 4.8 μg). Pex5p–AOx–C complexes were recovered by immunoprecipitation with anti-Pex5p antibody bound to formalin-fixed *S. aureus* cells. Bound FITC-AOx-C was determined spectrophotometrically and is plotted as a function of the amount of AOx-C added.

DISCUSSION

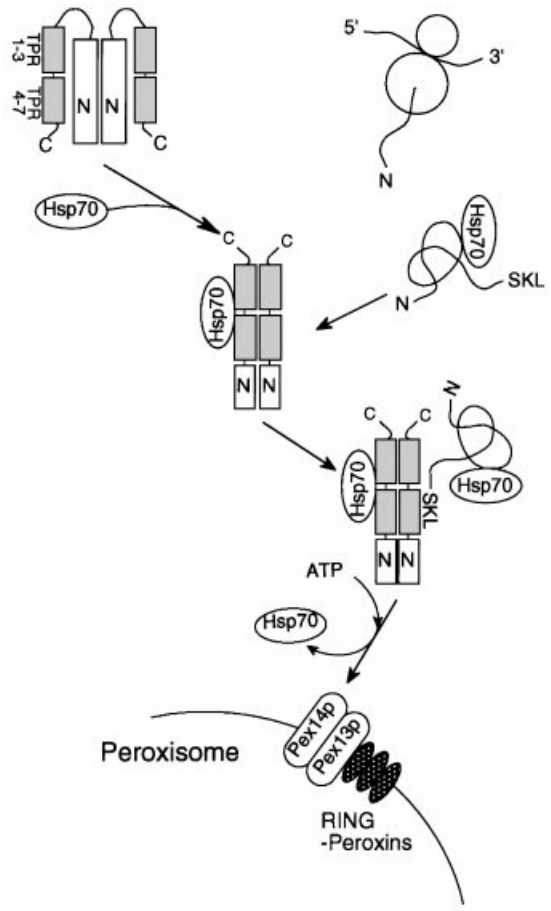
A current view of peroxisomal protein import is as follows [22,32]. The receptor and cargo complexes, including Pex5p dimer–PTS1 proteins, Pex5p–Pex7p–PTS2 proteins and Pex5p–Pex7p–cargo proteins, each with PTS1 or PTS2, are formed in the cytosol, travel to peroxisomes, bind to Pex14p in the potential translocation machinery, and translocate to Pex13p and subsequently to the RING peroxins. After emerging at the matrix side of the machinery, PTS1 and PTS2 proteins are released from the receptors. In the present study, to study the molecular mechanism of peroxisomal protein import, we investigated underlying mechanisms of the way in which Pex5p recognizes PTS1 protein and also how such steps are regulated. In protein transport to intracellular organelles, chaperones such as Hsp70 have been shown to function in unfolding cargo proteins to yield an import-competent form [29,33–35]. The peroxisomal import of matrix proteins also requires Hsp70 [36,37]. It is noteworthy that there have been several reports of import of folded and oligomeric proteins into peroxisomes [38–40]. This implies a functional significance of Hsp70 in protein transport to peroxisomes, beyond the unfolding of cargo proteins.

In the present study we found that antibody against Hsp70 inhibited the import of PTS1 protein into peroxisomes *in vitro*. Import of PTS1 AOx was similarly inhibited by anti-Pex5p antibody (see Figure 2). A similar inhibitory effect was observed on the addition of PTS1 peptide to an import assay *in vitro* [4]. We also found that a small part of Pex5p is tightly associated with peroxisomes, behaving as though it were an integral membrane protein as assessed by resistance to the sodium carbonate treatment. A similar membrane integrity of Pex5p was observed recently [41]. These observations imply that Pex5p associated with peroxisomes might function as the PTS1 receptor. Moreover, *in vitro* import of AOx was not seen when cell-free protein synthesis was performed with a wheatgerm lysate system, despite the presence of Hsp70 in the lysate [37]. This finding suggested that homologous mammalian chaperones are required for such import *in vitro*. The import was indeed re-established when rat liver Hsp70 or Hsp70 plus Pex5p were added, whereas the stimulatory effect was slight with Pex5p alone. This suggested that Hsp70 might interact directly with Pex5p.

The C-terminal part of Pex5p contains a domain of seven TPRs involved in the recognition of PTS1 [18,42]. Of the several modules comprising multiple TPRs in one polypeptide, each frequently interacts with different proteins, as noted in Cdc16p, Cdc23p and Cdc27p [43]. TPR1–3 and TPR4–7 are separated by a 14-residue sequence in the human and Chinese hamster forms [18–20,44]. Both TPR1–3 and TPR4–7 recognize PTS1 and bind directly to Hsp70 (the present study). It is known that Hsp70 and Hsp90 modulate the steroid hormone receptor via the TPR domain [45]. The TPR-mediated interaction of Pex5p and Hsp70 resembles a TPR co-chaperone Hip that regulates the ATPase activity of Hsp70 [46]. We suspected that the binding of Pex5p to PTS1 might be regulated by direct interaction with Hsp70. Indeed, the binding of Pex5p to AOx was enhanced by Hsp70. This effect was observed in Pex5p lacking N-terminal residues 1–142 but not in a Pex5p variant lacking the entire N-terminal portion, despite binding to Hsp70. Moreover, Pex5p(1–295) was pulled down with TPR1–7 in the absence of Hsp70. We interpreted these observations to mean that the N-terminal part has a role in suppressing the PTS1-binding activity of the TPR1–7 (Scheme 1). Hsp70 releases this suppressive N-terminal region from TPR domains with the aid of ATP. The homomeric interaction of TPR1–7 requires Hsp70 and ATP, whereby Pex5p(1–295) cannot interact with TPR1–7.

Schliebs et al. [47] reported that the N-terminal part of Pex5p forms an oligomer and that TPR1–7 exists as a monomer. Pex5p occasionally aggregates through the N-terminal part. It is therefore possible that Hsp70 dissociates such aggregates. Instead, Hsp70-dependent interaction of the N-terminal domain of Pex5p with the TPR region implies that Hsp70 might regulate the recognition of PTS1 by Pex5p by binding to TPR, giving rise to changes in the configuration of Pex5p. By interacting with Hsp70, the TPR domain induces a configuration of Pex5p that is competent in binding PTS1. In this way, TPR 1–7 might readily bind to the N-terminal part of Pex5p when the cargo PTS1 protein is not available, implying that the N-terminal part and PTS1 are competitive in binding to the TPR domain. This interaction is supported by the Hsp70-mediated augmentation of Pex5p binding to FITC-labelled AOx, in which the molar ratio of Pex5p to AOx was approx. 1 at binding saturation (see Figure 8). Pex5p–cargo complexes, including Pex5pL–Pex7p–PTS2 [20], are formed in the cytoplasm, are translocated and bind to Pex14p, the initial docking site of Pex5p. The higher affinity of Pex5p–cargo for Pex14p than for Pex13p [48] is consistent with this model. Furthermore, the N-terminal part carries multiple Pex14p-binding sites [47]. Under these conditions, the interaction of the N-terminal part of Pex5p with Pex14p is enhanced on release from Pex5p-C mediated by Hsp70 and/or PTS protein.

In the present study we found two PTS1-binding sites, TPR1–3 and TPR4–7, in the TPR region of Pex5p. TPR1–3 or TPR1–7 of yeast Pex5p, and human TPR1–7, were shown to interact with synthetic SKL-containing dodecapeptide, revealing a first-order kinetic saturation curve [42], whereas we observed a sigmoidal response with the AOx C-terminal 141-residue portion. A smaller peptide might readily reach the binding site(s), which are normally regulated by Hsp70 (see Scheme 1). The observation that the amount of AOx bound to Pex5p increased in an Hsp70-dependent manner at a lower concentration of AOx might reconcile a notion that the binding kinetics was no longer sigmoidal at a higher concentration of PTS1 protein. It is more likely that Pex5p regulates binding capacity by using TPR1–7. The formation of Hsp70–Pex5p–PTS1 complexes is suppressed by ATP. However, once these complexes are formed, the interaction seems to be strong and does not dissociate even by salt-washing. ATP releases Hsp70 from the complexes but Pex5p remains



Scheme 1 Schematic view of peroxisomal protein import mediated by PTS1 receptor Pex5p and Hsp70

The Hsp70-assisted PTS1 recognition of Pex5p occurs in the cytosol. The C-terminal TPR region of Pex5p interacts with the N-terminal part of Pex5p in an intramolecular or intermolecular manner. Pex5p forms a dimer [22] by mutual interaction of the N-terminal part. Hsp70 binds the C-terminal TPR domain of Pex5p, whereby Pex5p is regulated in PTS1 binding. Hsp70 also binds to PTS1 proteins. The Hsp70–Pex5p–PTS1 complexes traverse to peroxisomes; Hsp70 dissociates from the complexes by hydrolysing ATP. The Pex5p-PTS1 complexes bind to the Pex5p-docking peroxin Pex14p of the potential 'import machinery', comprising Pex14p, Pex13p and the RING peroxins Pex12p, Pex10p and Pex2p [22]. The signal receptor and cargo complexes might temporarily translocate through a potential binding partner Pex13p and associate with the RING peroxins Pex10p–Pex12p complex [22,49]. PTS1 proteins are released at the inner surface and/or the inside of peroxisomes. Pex5p then shuttles back to the cytosol.

bound to PTS1. The physiological consequences of both intermolecular and intramolecular binding include: (1) the regulation of binding to PTS1, (2) the protection of Pex14p, the initial docking site of Pex5p and cargo complexes such as Pex5pL–Pex7p–PTS2, from binding of cargo-unloaded Pex5p, and (3) the blocking of rebinding of cargo proteins such as PTS1. The receptor–cargo complexes enter peroxisomes; the cargo protein then needs to be unloaded. Chaperone-like factor(s) might have a role in this step as well. The mechanisms involved in these import processes remain to be defined.

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