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The *Pichia pastoris* peroxisomal protein PAS8p is the receptor for the C-terminal tripeptide peroxisomal targeting signal

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The peroxisomal targeting signal 1 (PTS1), consisting of a C-terminal tripeptide (SKL and variants), directs polypeptides to the peroxisome matrix in evolutionarily diverse organisms. Previous studies in the methylotrophic yeast *Pichia pastoris* identified a 68 kDa protein, PAS8p, as a potential component of the PTS1 import machinery. We now report several new properties of this molecule which, taken together, show that it is the peroxisomal PTS1 receptor. (i) PAS8p is localized to and tightly associated with the cytoplasmic side of the peroxisomal membrane, (ii) peroxisomes of wild-type, but not of *pas8*Δ (null) mutant, *P.pastoris* cells bind a PTS1-containing peptide (CRYHLKPLQSKL), (iii) CRYHLKPLQSKL can be cross-linked to PAS8p after binding at the peroxisome membrane and (iv) purified PAS8p binds CRYHLKPLQSKL with high affinity (nanomolar dissociation constant). In addition, the tetratricopeptide repeat (TPR) domain of PAS8p is identified as the PTS1 binding region.

Keywords: cross-linking/peroxisomes/*Pichia pastoris*/protein targeting

Introduction

Peroxisomes are ubiquitous intracellular organelles which house nearly 50 enzymes and are involved in a variety of metabolic processes (Subramani, 1993). Polypeptides destined for the peroxisome matrix contain specific molecular determinants (Subramani, 1993) which are recognized post-translationally (Lazarow and Fujiki, 1985). Two evolutionarily conserved peroxisomal targeting signals (PTSs) have been described. PTS1, used by the vast majority of peroxisomal polypeptides, consists of the C-terminal tripeptide SKL or closely related variants (Gould *et al.*, 1989). PTS2, initially identified in rat 3-ketoacyl-CoA thiolase (Osumi *et al.*, 1991; Swinkels *et al.*, 1991), is an N-terminal undecapeptide related to MHRLQVVLGHL. The topogenic signals utilized by peroxisomal membrane polypeptides have not been defined precisely (McCammon *et al.*, 1994).

In order to understand the molecular mechanism and to identify the requisite biochemical components of peroxisomal polypeptide import, several groups have reconstituted the process *in vitro* (Subramani, 1993). These efforts have led to significant advances in our understanding of the import pathway. For example, import requires cytosolic factors (Wendland and Subramani, 1993a), including members of the 70 kDa heat shock protein family (hsp70s) (Walton *et al.*, 1994). The pathway is energy-dependent, requiring ATP hydrolysis, although there is no requirement for GTP hydrolyzing proteins or a membrane potential (Imanaka *et al.*, 1987; Rapp *et al.*, 1993; Wendland and Subramani, 1993a). Peroxisomal import is inhibited by the sulfhydryl alkylating agent *N*-ethylmaleimide (NEM) and the sensitive polypeptides have been localized to the peroxisome membrane (Wendland and Subramani, 1993a). Also, prior to import substrate polypeptides bind to peroxisomal membranes in an ATP-independent step (Imanaka *et al.*, 1987; Wendland and Subramani, 1993a). The import process is saturable (Wendland and Subramani, 1993a), although the rate limiting step has not been determined. In addition, the role of protein unfolding has been examined and appears not to be a prerequisite for peroxisomal import (Glover *et al.*, 1994; McNew *et al.*, 1994; Walton *et al.*, 1995). Despite this considerable progress, however, no components of a receptor or translocation machinery have been purified or characterized biochemically.

The failure to import polypeptides into peroxisomes has lethal consequences. Indeed, several human genetic diseases, including cerebro-hepato-renal (Zellweger) syndrome, hyperpipecolic acidemia, the neonatal form of adrenoleukodystrophy and infantile Refsum's disease, are characterized by an inability to import matrix enzymes and to assemble functional organelles (reviewed in Wiemer and Subramani, 1994). In certain complementation groups of these diseases the defect appears to be in the import of PTS1-containing polypeptides (Walton *et al.*, 1992; Wendland and Subramani, 1993b; Motley *et al.*, 1994). In the methylotrophic yeast *Pichia pastoris* a peroxisome assembly (*pas*) mutant has been isolated which has the same import deficiency phenotype as that exhibited by cells from certain Zellweger syndrome patients; specifically, no import of PTS1-containing polypeptides, but apparently normal import of the PTS2-containing polypeptide thiolase (McCollum *et al.*, 1993). This mutant has been characterized and the complementing gene, *PAS8*, cloned (McCollum *et al.*, 1993). *PAS8* codes for a 68 kDa protein which is a member of the tetratricopeptide repeat (TPR) or snap helix protein family (Sikorski *et al.*, 1990; Goebl and Yanagida, 1991). Other TPR motif-containing proteins with which PAS8p shares homology include the *Saccharomyces cerevisiae* proteins PAS10p and MAS70p, as well as the *Neurospora crassa* protein MOM72p. Interestingly, PAS10p appears to be involved in peroxisomal polypeptide import (Van Der Leij *et al.*, 1993). MAS70p and MOM72p

are mitochondrial outer membrane proteins which are receptors for many, but not all, polypeptides imported into the mitochondria of these organisms (Hines *et al.*, 1990; Söllner *et al.*, 1990; Goebl and Yanagida, 1991; Hines, 1992). These results led us to consider that PAS8p might be a peroxisomal import receptor in *P.pastoris*. Indeed, our evidence presented here suggests that PAS8p is an important component of the PTS1 import machinery.

Results

PAS8p is tightly associated with peroxisomal membranes and faces the cytosol

We examined the intracellular localization of PAS8p by immunoblotting organellar and cytosolic fractions of oleate-induced *P.pastoris* cells with anti-PAS8p antibody. Figure 1A shows that >95% of the immunoreactive PAS8p (see immunoblot) was associated with the organellar fraction of wild-type cells; no immunoreactive PAS8p was associated with the organellar fraction of *pas8*Δ (null) mutant cells. When these fractions (rich in peroxisomes and mitochondria) were separated on Nycodenz gradients, PAS8p co-localized with the peroxisomal marker enzyme catalase (Figure 1B). (Some catalase and PAS8p found in fractions 11–14 corresponds to material presumably released by the rupture of peroxisomes and is variable from preparation to preparation.) Furthermore, PAS8p behaved like PER6p, a peroxisomal integral membrane protein (J.Cregg, personal communication), in that it largely remained associated with organelle membranes after extraction with sodium carbonate (Figure 1C). In contrast, the peroxisomal matrix protein catalase was released into the supernatant by such treatment (Figure 1C). To examine the orientation of PAS8p within the membrane, we treated the organelles with proteinase K (Figure 2). Whereas the peroxisomal matrix enzyme thiolase was resistant to proteolytic cleavage, PAS8p was susceptible. As expected, if the organelles were lysed with Triton X-100 prior to proteinase K treatment thiolase became a proteolytic substrate; Triton X-100 alone caused no degradation of either PAS8p or thiolase. Note that PAS8p displays an increased sensitivity to proteinase K in the presence of detergent, perhaps reflecting an enhanced accessibility of, or susceptibility to, the protease.

Specific binding of a PTS1-containing peptide to peroxisomes in vitro

If PAS8p is a membrane receptor, peroxisomes from wild-type, but not from *pas8*Δ, *P.pastoris* cells might be predicted to bind a PTS1-containing molecule. To examine this, we prepared organelles from both cell types and fractionated them on Nycodenz gradients. The resultant fractions were examined by anti-thiolase immunoblotting (since thiolase is a PTS2-containing molecule and is imported into the peroxisome ghosts present in *pas8*Δ cells) (Figure 3A; McCollum *et al.*, 1993). For reference, in gradients of the *pas8*Δ mutant cells the mitochondrial marker enzyme cytochrome c oxidase peaked in fractions 12 and 13 (data not shown). In gradients of the wild-type cells thiolase immunoreactivity peaked in the same fractions (numbers 4–6) as catalase and PAS8p (compare Figures 1B and 3A). Equivalent amounts of these fractions were incubated in an *in vitro* organelle binding assay with

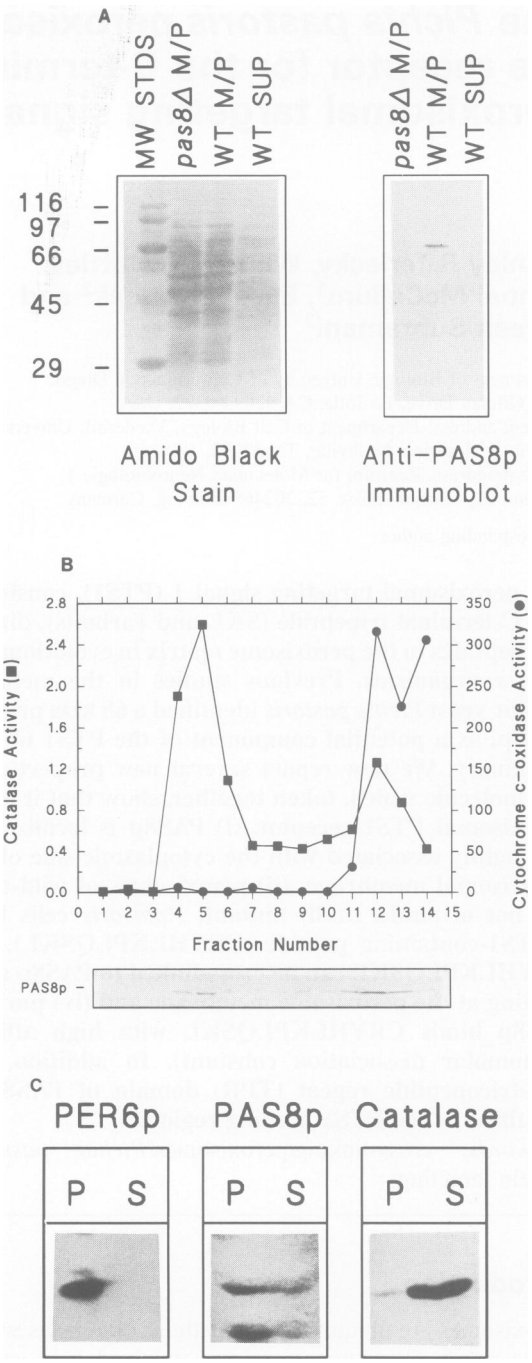

Fig. 1. PAS8p is tightly associated with peroxisomal membranes. (**A**) Wild-type and *pas8*Δ *P.pastoris* cells were cultured in rich oleate medium and fractionated into a cytosolic supernatant (SUP) and an organellar pellet (M/P) as described under Materials and methods. Proteins (50 μg) were examined after SDS–PAGE by amido black staining or anti-PAS8p immunoblotting. (**B**) Organelles from oleate-induced wild-type *P.pastoris* cells were separated on Nycodenz gradients as described (Nuttley *et al.*, 1990) and equivalent portions of the (14) resultant fractions assayed for catalase activity (a peroxisomal marker) and cytochrome c oxidase activity (a mitochondrial marker) (top panel) and for PAS8p immunoreactivity (bottom panel). (**C**) As described in Materials and methods, peroxisomes from oleate-induced, wild-type *P.pastoris* cells were separated into pellet (P) and supernatant (S) fractions after carbonate extraction. These fractions were examined after SDS–PAGE by anti-PER6p, anti-PAS8p and anti-catalase immunoblotting. Only the relevant portions of the immunoblots are shown.

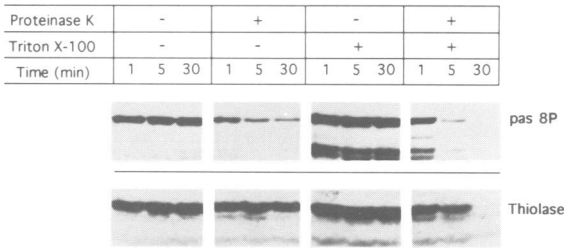

Fig. 2. PAS8p is exposed on the peroxisome surface. As described in Materials and methods, organelles from oleate-induced, wild-type *P.pastoris* cells were treated (+) or not (–) with proteinase K in the presence (+) or absence (–) of Triton X-100 for the times indicated. The resultant fractions were examined after SDS–PAGE by anti-PAS8p and anti-thiolase immunoblotting.

an iodinated PTS1-containing peptide ([^{125}I]CRYHLKPLQSKL) under conditions known to allow binding, but suppress peroxisomal import (i.e. incubation at low temperature and without ATP; Imanaka *et al.*, 1987). Figure 3B shows that only peroxisomes from wild-type cells displayed significant specific binding. Little or no binding was observed to organelles in fractions from the *pas8*Δ gradient.

Specific and saturable cross-linking of a PTS1-containing peptide to PAS8p

In an attempt to identify the peroxisomal protein responsible for binding [^{125}I]CRYHLKPLQSKL in the *in vitro* assay above, we performed a cross-linking reaction. Peroxisomes from wild-type cells (i.e. fractions 4–6 from the Nycodenz gradients; see Figures 1B and 3A) were incubated with [^{125}I]CRYHLKPLQSKL and the peroxisomes and bound peptide were then cross-linked using the primary amine-reactive cross-linker bis(sulfosuccinimidyl)suberate (BS^3). Cross-linked proteins were identified after SDS–PAGE by autoradiography (Figure 4A). With wild-type peroxisomes a 68 kDa protein was cross-linked to [^{125}I]CRYHLKPLQSKL in a reaction that was dependent on the addition of cross-linker and competed with unlabeled (CRYHLKPLQSKL) peptide. No cross-linked species were observed using peroxisomes from the *pas8*Δ mutant cells (i.e. the thiolase-positive, cytochrome c oxidase-negative fractions 7–9 from the Nycodenz gradients; see Figure 3A) or in reactions conducted without organelles (Figure 4A). Also, no cross-linking was observed using an iodinated peptide ([^{125}I] CRYHLKPLQ) lacking a PTS1 signal (data not shown). Importantly, cross-linking of [^{125}I]CRYHLKPLQSKL to the 68 kDa protein of wild-type *P.pastoris* peroxisomes was saturable, with the peptide concentration required for half maximal cross-linking being ~500 nM (Figure 4B). Additional minor cross-linked proteins at 80 and 95 kDa were also sometimes observed (Figures 4 and 5). The identity of these proteins is under investigation.

The anti-PAS8p antibodies do not recognize the native molecule well, making it difficult to use immunoprecipitation as a means of identifying the cross-linked 68 kDa protein as PAS8p. As an alternative approach, we transformed *pas8.1* mutant *P.pastoris* cells with a gene encoding a polyhistidine-tagged PAS8p molecule [$(His)_6$–PAS8p]. This protein, modified at its N-terminus, fully complemented the mutant phenotype, restoring the ability of the

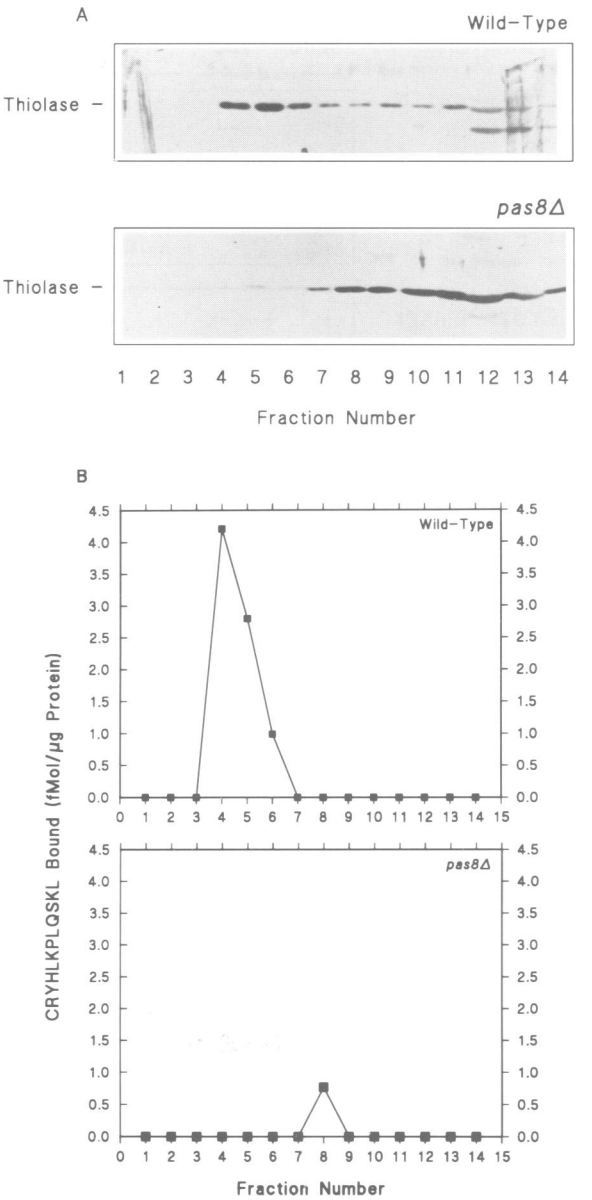

Fig. 3. Binding of [^{125}I]CRYHLKPLQSKL to peroxisomes. (**A**) Organelles from oleate-induced wild-type or *pas8*Δ *P.pastoris* cells were separated on Nycodenz gradients and the (14) resultant fractions assayed for thiolase immunoreactivity. (**B**) Binding of [^{125}I]CRYHLKPLQSKL to equal amounts of the fractions described in (A) was assayed as described under Materials and methods.

cells to grow on oleate and to import catalase into their peroxisomes (data not shown). When we isolated peroxisomes from these transformants and performed a cross-linking reaction with [^{125}I]CRYHLKPLQSKL we observed a labeled 68 kDa band (Figure 5). (Note that addition of the polyhistidine tag to PAS8p resulted in no discernible change in the apparent molecular weight of the purified protein, as judged by SDS–PAGE.) Importantly, after detergent solubilization of the organelles nearly two thirds of this cross-linked protein bound to a nickel–nitrilotriacetic acid (Ni–NTA) (histidine affinity) resin (Figure 5, bottom panel). In contrast, the 68 kDa protein cross-linked from wild-type *P.pastoris* (lacking a polyhistidine tag on PAS8p) did not bind to the Ni–NTA resin (Figure 5). These results confirm that PAS8p is the 68 kDa

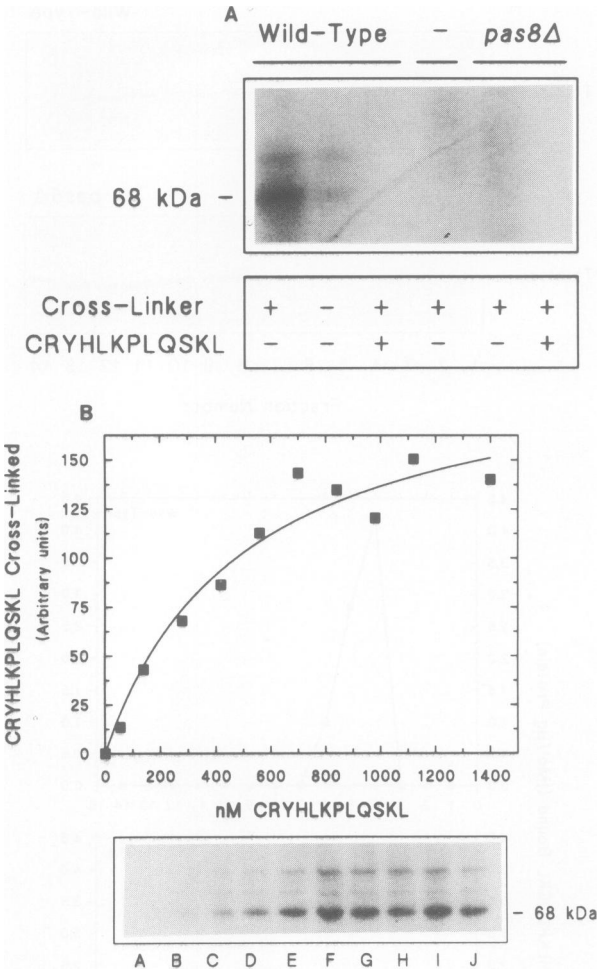

Fig. 4. Cross-linking of [^{125}I]CRYHLKPLQSKL to a 68 kDa peroxisomal protein. (**A**) Peroxisomes from wild-type *P.pastoris* cells (fractions 4–6 from Figures 1B and 3 above) (wild-type) and peroxisomes from *pas8Δ P.pastoris* cells (fractions 7–9 from Figure 3A above) (*pas8Δ*) or no organelles (–) were incubated with and cross-linked to [^{125}I]CRYHLKPLQSKL as described under Materials and methods. After cross-linking, proteins were separated by SDS–PAGE and cross-linked species identified by autoradiography. 'CRYHLKPLQSKL' indicates the presence of an excess (2 mM) of unlabeled peptide during the binding reaction. (**B**) Increasing concentrations of [^{125}I]CRYHLKPLQSKL were incubated with a fixed amount of wild-type *P.pastoris* peroxisomes and cross-linked as described under Materials and methods. After cross-linking, proteins were separated by SDS–PAGE and the cross-linked species identified by autoradiography (lower panel). The concentrations of [^{125}I]CRYHLKPLQSKL used were: A, 56 nM; B, 140 nM; C, 280 nM; D, 420 nM; E, 560 nM; F, 700 nM; G, 840 nM; H, 980 nM; I, 1120 μM; J, 1400 μM. The amount of radioactivity in the 68 kDa band was determined using a Molecular Dynamics Phosphorimager and is presented in the upper panel.

peroxisomal protein which binds and can be cross-linked to the PTS1-containing peptide CRYHLKPLQSKL.

Direct and high affinity binding of a PTS1-containing peptide to PAS8p

In order to measure the affinity of PAS8p for CRYHLKPLQSKL and to demonstrate that it binds the PTS1 sequence directly, we employed the $(His)_6$–PAS8p molecule which, when overexpressed in *Escherichia coli*, can be isolated in a single step chromatographic purification on Ni–NTA resin (Figure 6). We determined the affinity by incubating

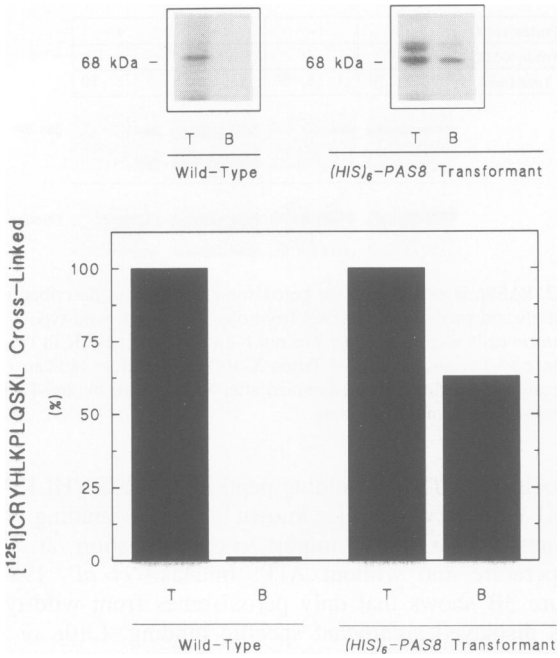

Fig. 5. Identification of PAS8p as the 68 kDa peroxisomal protein cross-linked to [^{125}I]CRYHLKPLQSKL. Peroxisomes were prepared from wild-type and *(HIS)$_6$–PAS8*-transformed *pas8.1 arg4* cells, respectively, cultured in rich oleate medium. These organelles were incubated with [^{125}I]CRYHLKPLQSKL and cross-linked as described under Materials and methods. Proteins were separated by SDS–PAGE and total cross-linked species (T) identified by autoradiography. Duplicate samples were solubilized with Triton X-100 and incubated with Ni–NTA–agarose. Cross-linked proteins remaining bound to the Ni–NTA–agarose after washing (B) were eluted by boiling for 2 min in SDS–PAGE sample buffer. These molecules were separated by SDS–PAGE and identified by autoradiography. The amount of radioactivity in the 68 kDa band was quantitated with a phosphorimager and the relative amounts in the T and B samples are presented in the lower panel.

$(His)_6$–PAS8p with [^{125}I]CRYHLKPLQSKL in an *in vitro* binding assay. As shown in Figure 6, this binding is saturable, with an apparent dissociation constant of ~460 nM. In control experiments [^{125}I]CRYHLKPLQSKL showed no specific binding to an unrelated polyhistidine-tagged protein or to no protein at all (i.e. Ni–NTA resin alone) (data not shown).

PAS8p binds PTS1 via its TPR domain

As previously reported, PAS8p contains seven TPR motifs, regions to which several biochemical properties, including protein binding, have been ascribed (Hirano *et al.*, 1990; Sikorski *et al.*, 1990; McCollum *et al.*, 1993; Lamb *et al.*, 1994). In order to determine whether or not these TPR domains are involved in the actual binding of PTS1, we constructed several truncated versions of PAS8p and examined their binding to CRYHLKPLQSKL in an *in vitro* binding assay described previously (McCollum *et al.*, 1993). Various PAS8p constructs were synthesized *in vitro*, labeled with [^{35}S]methionine and analyzed for their ability to bind CRYHLKPLQSKL [coupled to human serum albumin (HSA)–agarose]. Figure 7 shows that PAS8p constructs lacking the N-terminal region of the wild-type protein (*Bam*HI and TPR) were still capable of binding CRYHLKPLQSKL. Similarly, deletion of the 3′-end of the *PAS8* gene to the *Xho*I site resulted in a protein that

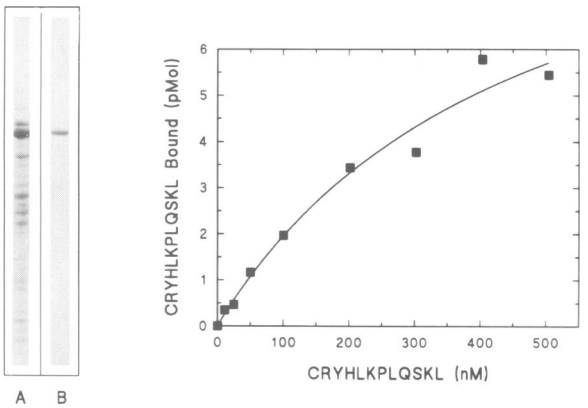

Fig. 6. Binding of [^{125}I]CRYHLKPLQSKL to PAS8p. PAS8p was modified at its N-terminus by the addition of a $(His)_6$ affinity tag using the Qiagen QIAexpress system as described under Materials and methods. This protein, overexpressed in *E.coli*, was isolated in a single step chromatographic purification on Ni–NTA–agarose. The left panel shows a Coomassie stain of the *E.coli* extract (A) and the $(His)_6$–PAS8p purified on Ni–NTA–agarose (B). In the right panel increasing concentrations of [^{125}I]CRYHLKPLQSKL were incubated with $(His)_6$–PAS8p in an *in vitro* binding assay, as described under Materials and methods.

still bound CRYHLKPLQSKL. In contrast, constructs missing the 3′-end of the gene from either the *Eco*RV site or the beginning of the TPR region (labeled Amino-term. in Figure 7) lost their binding capacity. These results indicate that the PTS1 binding region of PAS8p resides in the first three TPR domains. To test this directly, the appropriate construct, consisting of the region from the first TPR to the *Xho*I site, was synthesized and assayed for binding. Figure 7 shows this construct is clearly capable of binding CRYHLKPLQSKL.

Discussion

The C-terminal tripeptide SKL or a biochemically related variant targets polypeptides to the peroxisome in a variety of organisms (Subramani, 1993). A potential mechanism for effecting such selectivity would be through the existence of a cellular protein which binds the PTS1 peptide motif and commits substrates to import. Such receptor-based systems are the well-established mechanisms of import into such organelles as mitochondria (reviewed in Schwarz and Neupert, 1994) and the endoplasmic reticulum (reviewed in Gilmore, 1993). A candidate for the PTS1 receptor was first identified in the yeast *P.pastoris* through a screen for mutants of peroxisome assembly (Gould *et al.*, 1992; McCollum *et al.*, 1993). This mutant, called *pas8*, was unable to import PTS1-containing polypeptides, but was fully competent to import the PTS2-containing polypeptide thiolase (McCollum *et al.*, 1993). Furthermore, whereas these cells induced peroxisomal polypeptides normally, they were deficient for growth of the organelle. The *PAS8* gene was cloned and the encoded protein identified as a member of the TPR or snap helix family. Such proteins have been implicated in a number of cellular processes (Goebl and Yanagida, 1991) and, in some cases, are thought to be involved in membrane association and protein–protein interactions (Hirano *et al.*, 1990; Sikorski *et al.*, 1990; Lamb *et al.*, 1994). Also, PAS8p shares homology with the *S.cerevisiae* and *N.crassa* mitochondrial import receptors MAS70p and MOM72p respectively, heightening our suspicion that PAS8p was directly involved in peroxisomal polypeptide import. Our results presented in this study provide the biochemical evidence necessary to support this conclusion.

PAS8p localizes to the peroxisome membrane and faces the cytoplasm

We analyzed the subcellular distribution of PAS8p in oleate-grown *P.pastoris* cells and found most of the molecule associated with peroxisomes (Figure 1). Little, if any, of the protein was found in the soluble (cytosolic) fraction of cells. Furthermore, PAS8p is largely resistant to extraction with sodium carbonate (Figure 1C), indicative of a very tight association with the peroxisome membrane. Based on its primary sequence, PAS8p lacks an obvious hydrophobic transmembrane domain, therefore, the basis of this association is unclear. Perhaps PAS8p is tightly associated with an as yet unidentified peroxisomal integral membrane protein; this possibility is currently being examined using peroxisomes containing polyhistidine-tagged PAS8p and reversible cross-linkers.

In order for PAS8p to act as a membrane-bound receptor, it should be accessible to the cytoplasm. Our results (Figure 2) suggest this is indeed the case; proteinase K treatment of an organelle fraction shows that PAS8p is susceptible to cleavage under conditions in which a matrix protein, thiolase, is resistant.

PAS8p on peroxisomes binds the PTS1 sequence specifically

The *in vitro* assay used to demonstrate binding of the PTS1-containing peptide to peroxisomes is very similar to one used previously to identify a protease-sensitive, saturable binding site on the surface of human fibroblast lysosomes (Terlecky and Dice, 1993). The organelles capable of binding [^{125}I]CRYHLKPLQSKL are in the catalase- and PAS8p-positive fractions (4–6) of wild-type *P.pastoris* cells (Figure 3B). It is interesting to note that although there are other catalase- and PAS8p-positive fractions, specifically wild-type fractions 11–13, these do not show any binding of [^{125}I]CRYHLKPLQSKL, an apparent discrepancy if PAS8p is thought to be the binding molecule. However, although we might expect some binding of [^{125}I]CRYHLKPLQSKL to the PAS8p present, if these fractions do represent ruptured peroxisomes, these complexes would not be retained on the filter membranes and binding would not be detected.

Cross-linking experiments show directly that PAS8p is the peroxisomal protein binding [^{125}I]CRYHLKPLQSKL. Cross-linking has been instrumental in identifying membrane receptors, constituents of translocation channels and other components of polypeptide import machineries (Gillespie, 1987; Vestweber *et al.*, 1989; Scherer *et al.*, 1990; Görlich *et al.*, 1992; Musch *et al.*, 1992; Sanders *et al.*, 1992; Söllner *et al.*, 1992). The experiments described here show that [^{125}I]CRYHLKPLQSKL specifically interacts with a 68 kDa protein on the surface of peroxisomes from wild-type *P.pastoris* cells (Figure 4A). Furthermore, no cross-linking was observed with peroxisomes from *pas8*Δ cells, further supporting the notion

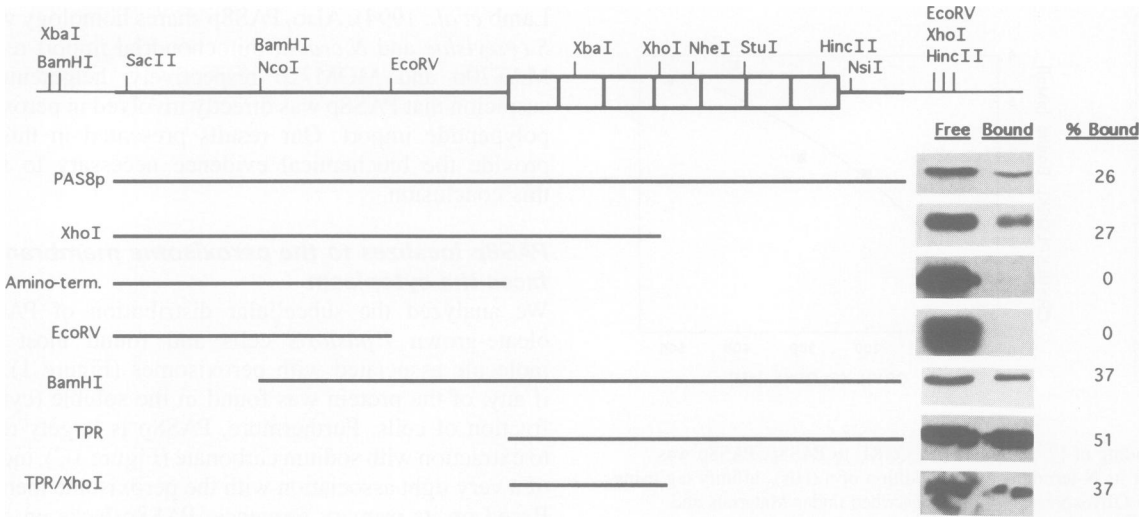

Fig. 7. Localization of the PAS8p PTS1 binding domain. Wild-type and various truncated PAS8p molecules were synthesized *in vitro* as described under Materials and methods and the resultant proteins assayed for their ability to bind CRYHLKPLQSKL coupled to HSA–agarose, as described (McCollum *et al.*, 1993). Proteins that bound the PTS1 affinity resin (Bound) as well as those that did not (Free) are shown to the right of each construct. Protein levels were quantitated using the Is-1000 Digital Imaging System (Alpha Innotech, San Leandro, CA). The values for '% Bound' were determined by dividing the amount bound by the total (Bound + Free).

that the 68 kDa protein was the molecule binding [^{125}I]CRYHLKPLQSKL in the organelle binding assay. By using a polyhistidine affinity tag we were able to identify the 68 kDa cross-linked species as PAS8p (Figure 5).

Recombinant PAS8p binds the PTS1 sequence directly with the same affinity as peroxisomal PAS8p

Purified PAS8p binds [^{125}I]CRYHLKPLQSKL directly (Figure 6) with relatively high affinity ($K_d \approx 460$ nM). This affinity very closely approximates the concentration at which [^{125}I]CRYHLKPLQSKL cross-linking to the peroxisome membrane is half maximal (Figure 4B), suggesting that we are measuring similar processes.

The TPR domain of PAS8p binds the PTS1 sequence

We further characterized PAS8p and showed that the C-terminal TPR domains mediate PTS1 binding (Figure 7). Indeed, the first three of the seven TPR domains alone are capable of binding SKL. Perhaps the N-terminus of the protein targets the molecule to the peroxisome membrane; this possibility is currently being examined.

Taken together, these results show that PAS8p serves as the peroxisomal PTS1 receptor. It is also worth noting that PAS8p is induced on both methanol and oleic acid (McCollum *et al.*, 1993). This would serve to facilitate import of PTS1-containing polypeptides into peroxisomes under these induction conditions. Although genes for several proteins involved in peroxisomal biogenesis have been cloned and characterized to date, PAS8p is the only protein for which there is a clear and definitive role in peroxisomal polypeptide import. The function described for PAS8p in this paper provides a satisfying explanation for the PTS1-specific import defect in *pas8Δ* mutant cells. Importantly, we and others have cloned the human homolog of PAS8p and shown that the human PTS1 receptor gene can complement the PTS1 polypeptide import defect in cells from human patients (belonging to complement group 2) with fatal peroxisomal disorders (Dodt *et al.*, 1995; Wiemer *et al.*, 1995). Thus elucidation of the role of PAS8p in peroxisomal biogenesis is clearly relevant to understanding human disorders.

With the peroxisomal binding assays described herein it should now be possible to address several important questions, including what drives the release of PTS1 from PAS8p. Also, is release followed by interaction of PTS1 with other components of the translocation machinery or does it lead directly to entry into the organelle matrix? Is there a receptor for PTS2? If so, do the PTS1 and PTS2 receptors interact or share import machineries? Answers to these and other questions should shed further light on the mechanistic details of peroxisomal polypeptide import.

Materials and methods

Yeast strains and culture conditions

The *P.pastoris* wild-type strain (21-1) was obtained from the Northern Regional Research Laboratories (Peoria, IL). The *pas8Δ* (null) mutant strain (*arg4 his4 pas8Δ::ARG4*) and *pas8.1* (point) mutant strain (*pas8.1 arg4*) were generated as described previously (McCollum *et al.*, 1993). Cells were grown in medium containing 1% (w/v) yeast extract and 2% bacto-peptone, supplemented with the carbon sources dextrose (2% final concentration) or oleic acid:Tween 40 [0.2%:0.02% (v/v) final concentrations respectively] as described (McCollum *et al.*, 1993). Electroporation was used for yeast transformations (Rickey, 1990) and the recombinant DNA techniques employed were as described (Sambrook *et al.*, 1989). *pas8.1*, which expressed no detectable PAS8p, was transformed with the *(HIS)$_6$–PAS8*-containing plasmid pAM-HIS-PAS8. This plasmid was constructed by cloning the *Eco*RI-partial–*Pst*I fragment from pQE9-PAS8 (see below) into the *P.pastoris* expression vector pAM2F (a kind gift of J.Heyman), digested with *Eco*RI and *Pst*I. (His)$_6$-PAS8p expression from this plasmid was driven by the *P.pastoris* methanol oxidase promoter. Transformants were selected for arginine prototrophy.

Cell fractionation

The preparation of organellar and cytosolic fractions from oleate-induced *P.pastoris* cells using differential centrifugation was as previously described (Gould *et al.*, 1992), except that the protease inhibitors used were phenylmethylsulfonyl fluoride (PMSF) (1 mM), sodium fluoride (5 mM) and pepstatin A (1 μM).

Peroxisomes isolated on Nycodenz gradients as described (Nuttley *et al.*, 1990) were extracted with 100 mM Na_2CO_3, pH 11.5, for 60 min on ice and centrifuged at 100 000 *g* for 1.5 h, essentially as described (Fujiki *et al.*, 1982). Resultant supernatants and resuspended pellets were precipitated with trichloroacetic acid (12.5% final concentration) for 30 min on ice and centrifuged. The resultant pellets were resuspended in SDS–PAGE sample buffer and the pH adjusted with 25% NH_4OH.

For the protease protection assays, organelles (100 μg protein) were treated with 25 mg/ml agarose-coupled proteinase K in the presence or absence of 0.1% Triton X-100 at 30°C. After 1, 5 and 30 min sample aliquots were treated with 2 mM PMSF to inhibit proteinase K, centrifuged to remove the beads and the final supernatants loaded on SDS–PAGE gels.

Protein and enzyme assays

Protein concentrations were determined by the Lowry (Lowry *et al.*, 1951) or BioRad (Hercules, CA) procedures. Assays for the organellar marker enzymes catalase (EC 1.11.1.6) and cytochrome c oxidase (EC 1.9.3.1) were as described (Storrie and Madden, 1990). Catalase activity is expressed as the change in absorbance units (at 405 nm) per 20 min. Cytochrome c oxidase activity is expressed as the change in absorbance units (at 550 nm) per min $\times 10^{-4}$.

Western blotting

SDS–PAGE was carried out as described (Laemmli and Favre, 1973). Protein transfer to nitrocellulose was performed in a Trans-Blot electrophoretic cell (BioRad, Hercules, CA). Blocking (with 5% non-fat milk) and immunoblotting incubations were performed in a Tris-buffered saline solution (20 mM Tris–HCl, pH 7.5, 500 mM NaCl) containing 0.05% Tween-20. Antibodies to PAS8p were raised against $(His)_6$–PAS8p. This fusion protein was expressed under control of the inducible T5 promoter in *Escherichia coli* after cloning the PAS8-containing *Bgl*II–*Hin*dIII fragment from pSP72-PAS8 (McCollum *et al.*, 1993) into pQE9 (Qiagen, Chatsworth, CA) digested with *Bam*HI and *Hin*dIII. The resultant protein was purified from *E.coli* lysates on Ni–NTA–Sepharose CL-6B (Qiagen) and used to immunize rabbits. The antiserum was used at a 1:10 000 dilution. Anti-(*S.cerevisiae*) thiolase antibodies were a kind gift of Dr W.Kunau (Ruhr University, Bochum, Germany) and were used at a 1:500 dilution. Anti-(*P.pastoris*) PER6p antibodies were a gift of Dr J.Cregg (Oregon Graduate Institute of Science and Technology, Portland, OR) and were used at a 1:500 dilution. Anti-(*S.cerevisiae*) catalase antibodies were also used at a 1:500 dilution. Antibodies were visualized with goat anti-rabbit IgGs conjugated to alkaline phosphatase (Kirkegaard and Perry Laboratories, Gaithersburg, MD) or with protein A conjugated to horseradish peroxidase and Enhanced Chemiluminescence (Amersham, Arlington Heights, IL), as recommended by the manufacturers.

Radiolabeling of peptides

Iodination of CRYHLKPLQSKL and CRYHLKPLQ was carried out using the IODO-BEADS™ reagent as suggested by the manufacturer (Pierce Chemical, Rockford, IL).

In vitro organelle binding

Organelle binding assays were performed using the Multiscreen Assay System (Millipore Corp., Bedford, MA; Terlecky and Dice, 1993), with the following changes. The filter membranes were blocked with 10 mM 4-morpholine ethanesulfonic acid (MES), pH 6.0, 2 mM EDTA, 2 mM KCl, 0.2% ethanol, 1% bovine serum albumin (BSA) (buffer A) prior to the addition of organelles and washed twice afterward with the same solution. Trial experiments showed >95% of the organelles (mitochondria and peroxisomes) were retained on the filter membranes. Organelles were incubated with [^{125}I]CRYHLKPLQSKL (50 nM) and buffer A in a final volume of 100 μl for 1 h at 4°C. Radioactivity bound to organelles on filter membranes was determined in a Traco Analytic model 1197 gamma counter. Non-specific binding, measured in the presence of excess (2 mM) unlabeled CRYHLKPLQSKL, was subtracted from all data points.

Cross-linking

Chemical cross-linking of [^{125}I]CRYHLKPLQSKL to peroxisomes was carried out largely as described (Gillespie, 1987). Briefly, peroxisomes (15 μg protein) were incubated with [^{125}I]CRYHLKPLQSKL (560 nM) in 10 mM MES, pH 6.5, 3 mM EDTA, 2 mM KCl, 0.2% ethanol, 1% ovalbumin (buffer B) in a final reaction volume of 200 μl for 1 h at 4°C. The organelles were pelleted by centrifugation and resuspended in (75 μl) cross-linking buffer (buffer B without ovalbumin) containing 50 μg/ml BS^3 (Pierce Chemical, Rockford, IL). After 1 h at 23°C, SDS–PAGE sample buffer was added to the peroxisomes and the samples loaded on SDS–PAGE gels.

$(His)_6$–PAS8p binding

For each condition, $(His)_6$–PAS8p (20 μg) was coupled to 25 μl Ni–NTA–Sepharose CL-6B by incubation for 30 min at 37°C in 100 μl 10 mM MES, pH 6.5, 1 mM EDTA (buffer C). The affinity resin was blocked with 10 mg/ml BSA for 30 min at 37°C and washed thoroughly. Increasing amounts of [^{125}I]CRYHLKPLQSKL were incubated with the $(His)_6$–PAS8p resin in a final volume of 100 μl with gentle shaking for 90 min at 37°C. The solution was lightly centrifuged, the pellet washed with buffer C and the radioactivity associated with the final pellet determined. Non-specific binding, measured in the presence of excess (2 mM) unlabeled CRYHLKPLQSKL, was subtracted from all data points.

Construction of plasmids expressing truncated forms of PAS8p

A plasmid to express full-length wild-type PAS8p *in vitro* (pKSPAS8) was constructed by cloning the *PAS8*-containing *Pst*I–*Eco*RI fragment from p2-18 (McCollum *et al.*, 1993) into pKS2 (Stratagene, La Jolla, CA) digested with *Pst*I and *Eco*RI. *PAS8* and various deletion constructs were transcribed from this vector using T7 polymerase (Promega, Madison, WI), translated *in vitro* and the resultant proteins assayed for peptide binding. Deletions from the 3′-end of *PAS8* were made by linearizing pKSPAS8 with either *Xho*I or *Eco*RV as shown in Figure 7. The 5′-deletion construct, pBamHI, was made by cutting pKSPAS8 with *Bam*HI to delete the 5′-fragment and then the vector was religated. PCR was used to construct a truncated from of PAS8p that contained the N-terminal half of PAS8p (pAmino-term.), beginning at the initiator methionine and continuing to residue 277 at the beginning of the first TPR motif. Plasmid pKSPAS8 was used as a template and oligonucleotides with the sequences 5′-ATCTAAAGATCTACCATGTCGCTTA-TTGGCGG-3′ and 5′-TTCATGAATTCATTAATCGTTACGGAATTGA-TTC-3′ were used to prime at the 5′- and 3′-ends of the mutant gene respectively. The 5′ oligonucleotide added a *Bgl*II site (underlined) and an optimal translation initiation sequence (Kozak, 1986) in front of the ATG. The 3′ oligonucleotide introduced two stop codons after residue 277, followed by an *Eco*RI site (underlined). The resulting PCR fragment was digested with *Bgl*II and *Eco*RI and cloned into *Bgl*II/*Eco*RI-cut pSP72 (Promega). PCR was also used to construct a mutant form of PAS8p that contained the C-terminal TPR-containing half of the protein (pTPR), beginning at an added initiator methionine upstream of residue 277 at the beginning of the first TPR repeat and continuing to the end of the protein. Again, plasmid pKSPAS8 was used as a template and the oligonucleotide 5′-AAGAATAGATCTACCATGGATCCAGATGC-CTATG-3′ was used to prime at the 5′-end. This oligonucleotide introduced a *Bgl*II site (underlined), followed by a start methionine in front of residue 277. PCR was then carried out using this oligonucleotide at the 5′-end and the pKS2 reverse sequencing primer at the 3′-end of the gene. The resulting PCR fragment was cut with *Bgl*II and *Eco*RI and cloned into *Bgl*II/*Eco*RI-cut pSP72. A version of PAS8p containing the first three TPR repeats was generated by linearizing pTPR with *Xho*I. A schematic diagram of these truncated forms of PAS8p is shown in Figure 7.

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