Antibodies against Pex14p block ATP-independent binding of matrix proteins to peroxisomes in vitro

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Abstract The membrane protein Pex14p is a key component of the protein import machinery of peroxisomes. Antibodies raised against human Pex14p recognise a 66 kDa protein in sunflower glyoxysomes (HaPex14p) and immunoprecipitate in vitro-translated *Arabidopsis* Pex14p (AtPex14p). These antibodies inhibit the ATP-independent binding to sunflower peroxisome membranes of peroxisome targeting signal type (PTS) 1- and PTS2-targeted matrix proteins, but not an integral membrane protein. These results suggest that Pex14p functions before the ATP-dependent step of peroxisome assembly.

Key words: Peroxisomal protein import; Peroxin; Pex14p; Peroxisome biogenesis; In vitro protein translocation assay; Sunflower glyoxysome

1. Introduction

Of all the cellular organelles, the biogenesis of peroxisomes is least well understood. Genetic studies carried out with yeasts and Chinese hamster ovary (CHO) cells have led to the identification of about 20 proteins (peroxins) required for organelle biogenesis and the import of proteins [1,2]. Orthologues of many of these have now been discovered in organisms as diverse as humans and plants and mutations in these genes have devastating consequences for multicellular development [3,4]. Despite these advances, a biochemical description of the function of these proteins is in most cases superficial or non-existent.

Matrix proteins are targeted to peroxisomes by one of two pathways dictated by the type of targeting signal which the protein possesses [1,2]. The majority of peroxisomal matrix proteins is targeted by a carboxy-terminal tripeptide signal (peroxisome targeting signal type (PTS) 1). This interacts with its receptor, the product of the PEX5 gene, resulting in targeting of the complex to the peroxisome membrane where it interacts with (at least) two membrane proteins, Pex13p and Pex14p. A subset of peroxisomal proteins have an amino-terminally located nonapeptide signal (PTS2). The PEX7 gene encodes the PTS2 receptor which binds PTS2-targeted proteins in the cytosol and delivers them to the membrane via interactions with Pex14p and Pex13p. Yeast mutants defective in Pex13p or Pex14p fail to import matrix proteins, but correctly localise membrane proteins, suggesting that Pex13p and Pex14p are the point of convergence of the PTS1 and PTS2 import pathways but are not involved in the insertion of peroxisomal membrane proteins [5,6]. The mechanism of transmembrane translocation remains unknown. Peroxisomes are unusual in that they can import folded, even oligomeric proteins. There is some circumstantial evidence to suggest that the PTS1 and PTS2 receptors may be co-imported with their cargo and presumably recycled [7]. How the import machinery can accommodate such large structures is unknown. ATP is needed for import but its role is presently undefined.

To understand the mechanism of protein import into peroxisomes better, we developed an in vitro protein translocation system using sunflower glyoxysomes, a developmental stage specific type of plant peroxisome [8], and have used this to identify inhibitors [9] and translocation intermediates [10]. These studies allow us to break the import process down into stages which can be analysed in more detail. In this study, we have used antibodies to human Pex14 protein (HsPex14p), a peroxin known from in vivo studies to be a crucial component of the import machinery, and studied their effect on peroxisomal protein import in vitro.

2. Materials and methods

2.1. Isolation of glyoxysomes and preparation of carbonate-washed membranes

Glyoxysomes were isolated from sunflower cotyledons 3 days post-imbibition as described in [11], except that additional Complete protease inhibitors cocktail (Boehringer Mannheim) was added to the homogenising buffer. Carbonate-washed membranes were obtained by osmotic shock of the organelles with 25 mM HEPES-KOH (pH 7.2) and further homogenisation (Jencons homogeniser) for 30 min on ice. Then, membranes were pelleted by ultracentrifugation at $200\,000 \times g$ for 30 min in a Beckman 100.4 rotor at 4°C and the pellets were washed with Na_2CO_3 (pH 11.5) in the homogeniser for 30 min on ice. The process was repeated twice and the final membrane pellets were resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer.

Electrophoresis and blotting were performed as described in [10], except that samples were solubilised in SDS sample buffer for 45 min at 37°C prior to loading on the gel.

2.2. In vitro translation and immunoprecipitation

Glycolate oxidase (GO) [12], malate dehydrogenase (MDH) [13], 22 kDa peroxisomal membrane protein (PMP22) [14] and *Arabidopsis thaliana* Pex14 (AtPEX14) (manuscript in preparation) were transcribed and translated in vitro for 90 min at 30°C by using the TNT-coupled wheat germ lysate system (Promega). All proteins were labelled with [^{35}S]methionine (1175 Ci/mmol) (ICN pharmaceuticals).

Anti-HsPex14p antibody was used to immunoprecipitate ^{35}S-labelled AtPex14 protein as follows: 20 μl translated protein was incubated with an equal volume of 25 mM Tris pH 7.6, 2% (w/v) SDS for 45 min at 37°C. The SDS concentration was then lowered to less than

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Abbreviations: GO, glycolate oxidase; MDH, malate dehydrogenase; PMP22, 22 kDa peroxisomal membrane protein; AtPex14, *Arabidopsis thaliana* Pex14; HsPex14, human Pex14; PTS1 and PTS2, peroxisome targeting signal types 1 and 2

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0.1% by dilution with 50 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% (v/v) Triton X-100 (IP buffer) containing Complete protease inhibitors cocktail (Boehringer Mannheim). The sample was precleared with 50 μl of IP-washed protein A coupled to Sepharose beads (Sigma) for 1 h at 4°C with gentle agitation and then spun for 1 min in a microfuge. Supernatants were transferred to clean tubes and incubated with 4 μl anti-HsPex14 or non-immune serum for 1 h at room temperature and incubated further with protein A as above. The beads were spun again and washed several times with IP buffer and finally resuspended in SDS sample buffer and heated at 37°C for 45 min prior to electrophoresis.

2.3. Antibody inhibition experiments

Isolated glyoxysomes (70–85% intact organelles based on latency of malate synthase) and in vitro translated proteins were incubated separately with 20 U/ml apyrase in import buffer for 15 min at 26°C to deplete ATP. Four μl of the appropriate antiserum (anti-HsPex14p, anti-*A. thaliana* PMP22 (AtPMP22) or non-immune serum) was added to 30 μg of apyrase-treated glyoxysomes and the incubation continued for a further 15 min at 26°C. The samples were transferred to ice, in vitro translated proteins were added (5 μl GO and PMP22, 2.5 μl MDH) and binding was allowed to occur for 5 min at 26°C prior to re-isolation of the organelles and bound translation products through a 0.7 M sucrose cushion as described in [8,11]. Samples were then analysed by SDS-PAGE and phosphoimaging. Incubations with anti-HsPex14p were performed in duplicate.

3. Results

3.1. Anti-HsPex14 recognises a protein of 66 kDa in carbonate-washed glyoxysome membranes

Glyoxysomes were prepared from sunflower cotyledons and treated to obtain carbonate-washed membranes as described in Section 2. Carbonate-washed membranes were separated by SDS-PAGE and immunoblotted with antisera raised against the N-terminal 134 amino acids of HsPex14p [15] and as a control, AtPMP22 [14] (Fig. 1). As expected, the anti-PMP22 antibody detected an integral membrane protein of 22 kDa (lane 4). HsPex14p recognised a single protein band of ca. 66 kDa in the carbonate-washed membranes (Fig. 1, lane 3). HsPex14p has a reported molecular weight of 55 000–57 000 and behaves as an integral membrane protein, as it is resistant

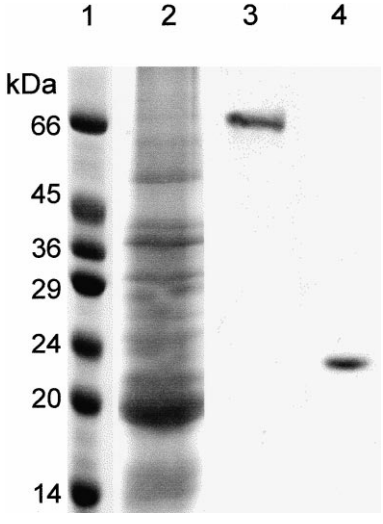

Fig. 1. Anti-HsPex14p recognises a 66 kDa protein in sunflower glyoxysomes. Lane 1, molecular mass markers; lane 2, carbonate-washed membranes from sunflower peroxisomes stained with Coomassie; lanes 3 and 4, immunoblot of glyoxysomal carbonate-washed membranes (20 μg of protein) probed with anti-HsPex14p and anti-AtPMP22, respectively.

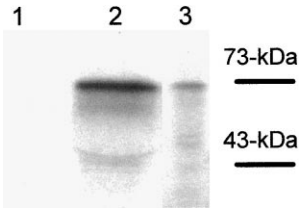

Fig. 2. Immunoprecipitation of in vitro translated AtPex14p with anti-HsPex14p. Lane 1, immunoprecipitation of AtPex14p with non-immune serum. Lane 2, immunoprecipitation of AtPex14p with anti-HsPex14p antiserum. Lane 3, in vitro translation of At Pex14p.

to extraction from the peroxisome membrane by sodium carbonate [15,16]. In contrast, *Saccharomyces cerevisiae* Pex14p behaves as a tightly bound peripheral membrane protein of 38 kDa [5].

3.2. Evidence that the 66 kDa polypeptide is sunflower Pex14p

To confirm that the anti-HsPex14 antisera recognised Pex14 from higher plants, an immunoprecipitation of an in vitro translated AtPex14 cDNA clone (Oh, Lopez-Huertas, Charlton and Baker, manuscript in preparation) was carried out. In vitro translated AtPex14p has a molecular weight of 73 000 (Fig. 2, lane 3) and is precipitated by anti-HsPex14p (lane 2). No Pex14p was immunoprecipitated by a non-immune serum (Fig. 2, lane 1), demonstrating the specificity of the antisera. Therefore, the anti-HsPex14p antiserum recognises plant Pex14p, so the 66 kDa protein in carbonate-washed sunflower glyoxysome membranes is immuno-related to Pex14 and is most likely sunflower (*Helinathus annus*) Pex14p (HaPex14p).

3.3. Anti-HsPex14 inhibits ATP-independent binding of PTS1- and PTS2-targeted matrix proteins but not the integral membrane protein PMP22

Anti-HsPex14p was used to probe the requirement for Pex14p in an in vitro import assay with sunflower glyoxysomes [8,11]. Glyoxysomes were treated with apyrase to deplete ATP, then pre-incubated with either non-immune serum, anti-AtPMP22 serum or anti-HsPex14p serum prior to the addition of apyrase-treated in vitro translation products. Binding was allowed to occur for 5 min before the organelles were re-isolated and analysed by SDS-PAGE and phosphoimaging (Fig. 3). In the case of GO (top panel), a PTS1-targeted matrix protein [17] pre-incubation with anti-HsPex14p

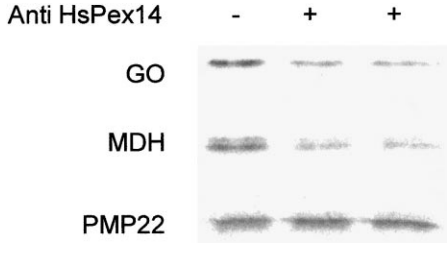

Fig. 3. Effect of anti-HsPex14p antiserum on peroxisomal protein binding. Top panel, GO and middle panel, pre-MDH in vitro translation products incubated with apyrase and antibody-treated glyoxysomes as follows. Lane 1, anti-PMP22 antiserum. Lanes 2 and 3, duplicate incubations with anti-HsPex14p antiserum. Bottom panel, in vitro translated PMP22 incubated with apyrase and antibody-treated glyoxysomes. Lane 1, non-immune serum. Lanes 2 and 3, duplicate incubations with anti-HsPex14 antiserum.

antisera decreased binding (lanes 2 and 3, duplicate samples) by 30–50% ($n = 5$) relative to the control in which the glyoxysomes were pre-incubated with an equivalent amount of anti-PMP22 antisera. Binding of pre-glyoxysomal MDH (middle panel), a PTS2-targeted matrix protein [18], was inhibited by anti-HsPex14p to a similar degree (compare lanes 2 and 3 with lane 1). In contrast, the binding of the integral membrane protein PMP22 was not inhibited by anti-HsPex14p serum (PMP22, lower panel). In the case of the PMP22 experiment, the control incubation was performed with non-immune serum rather than anti-PMP22.

4. Discussion

Pex14 has been characterised in *S. cerevisiae* [5], *Hansenula polymorpha* [19], human [15,16] and *Arabidopsis* (Oh, Lopez-Huertas, Charlton and Baker, in preparation). The calculated molecular masses range from 38 kDa for ScPex14p to 55.6 kDa for AtPex14p. However, the protein runs aberrantly on SDS-PAGE gels, which seems to be due to the low pI reported for this protein [15], as highly acidic or basic proteins do not react very well with SDS. HsPex14p has a predicted molecular weight of 41 kDa but migrates at 57 kDa [16]. In vitro translated AtPex14p migrates at 73 kDa (Fig. 2), but the protein detected in sunflower is only 66 kDa (Fig. 1). This could be the result of a species difference in the proteins or possibly due to proteolysis. HsPex14p is highly protease sensitive, giving rise to a 35 kDa fragment [16]. In the absence of protease inhibitors, sunflower Pex14p is clipped to a 51 kDa species which remains tightly associated with the glyoxysome membrane (data not shown) and the 66 kDa species in Fig. 1 could conceivably be less than full length. However, even if this is the case, this portion of HaPex14p is sufficient to mediate binding and import of matrix proteins.

The antibody inhibition experiments demonstrate that HaPex14p is required for import of PTS1- and PTS2-targeted matrix proteins in vitro but not for insertion of (at least one) integral membrane protein(s). These results are in complete agreement with the phenotype of *S. cerevisiae pex14* mutants, where PTS1- and PTS2-dependent matrix import is abolished but the integral membrane protein Pex11p is inserted into peroxisomal 'ghosts' [5]. Antibodies against HsPex14p inhibited PTS1-dependent import as shown by the blocking of uptake of the artificial substrate HSA-SKL in permeabilised CHO cells [16], but the effects on PTS2 and membrane protein import were not investigated. Our results are also in agreement with our earlier finding that a translocation intermediate formed under conditions of ATP depletion also blocks binding of GO and MDH but not PMP22 [10]. Pex14p was proposed as a likely candidate for interaction with this intermediate. Our results permit us to extend our understanding of the role of Pex14p in peroxisomal import. As the antibodies block the binding of substrates under conditions of ATP depletion, this indicates that docking of cargo proteins via Pex14p at the peroxisomal membrane occurs before the ATP requiring step of protein import. In vivo studies have not permitted this distinction to be made. Also consistent with this observation, amino-terminal truncations of hsPex14p have been reported to form stable complexes in vitro with recombinant human Pex5p in the absence of ATP [20]. The only peroxins with a known ATP-dependent activity are two ATPases associated with diverse cellular functions, Pex1p and Pex6p. However, the localisation of these proteins is predominantly cytosolic and associated with small vesicles [21]. The other known peroxisomal ATP binding proteins are ScPxa1p and 2p (= Pat1p and 2p) [22], involved in transport of fatty acids into peroxisomes, and the PMP70, PMP70R, ALD and ALDR proteins of mammalian peroxisomes. Their function is less clear. ALDp, when mutated, gives rise to X-linked adrenoleukodystrophy, a peroxisome biogenesis disorder where peroxisomal very long chain acyl CoA synthetase is absent, and PMP70 is mutated in a small number of Zellweger syndrome patients [23]. However, recent data implicate PMP70 in transport of VLCF acyl CoA into peroxisomes [24]. None of these proteins is an obvious candidate to energise the translocation step of peroxisomal protein import, raising the possibility that there are as yet unknown peroxins to be discovered.

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