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The *Hansenula polymorpha* peroxisomal targeting signal 1 receptor, Pex5p, functions as a tetramer

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Abstract **We have studied *Hansenula polymorpha* Pex5p and Pex20p, peroxins involved in peroxisomal matrix protein import. In vitro binding experiments suggested that *H. polymorpha* Pex5p and Pex20p physically interact. We used single particle electron microscopy (EM) to analyze the structure of purified Pex5p and its possible association with Pex20p. Upon addition of Pex20p, a multimeric Pex20p complex was observed to be associated to the periphery of the Pex5p tetramer. In this Pex5p–Pex20p complex, the conformation of tetrameric Pex5p had changed from a closed conformation with a diameter of 115 Å into an open conformation of 134 Å. EM also indicated that the Pex5p–Pex20p complex was capable to bind native, folded catalase, a peroxisomal PTS1 protein. This suggests that the Pex5p–Pex20p complex may be functional as receptor complex.**

Keywords: Pex5p; Peroxisome; Membrane transport; Electron microscopy; *Hansenula polymorpha*

1. Introduction

Peroxisomal matrix proteins are synthesized on free polyribosomes and directed to the organelle by specific peroxisomal targeting signals (PTSs). The routing of most matrix proteins depends on one of the two conserved PTSs, designated PTS1 and PTS2, which are recognized by the receptor proteins Pex5p or Pex7p, respectively.

The *PEX5* gene encodes the PTS1 receptor, Pex5p, which interacts with the PTS1 signal via a series of tetratricopeptide repeats (TPRs) within its C terminus. A crystal structure has been determined of a 41 kDa fragment of human Pex5p that includes six TPR motifs in complex with a small peptide containing a PTS1 sequence [1,2] or the sterol carrier protein [3]. This structure reveals the molecular basis for PTS1 recognition which is mostly formed by two clusters of three TPRs almost completely surrounding the PTS1-peptide.

However, whether or not Pex5p functions as an oligomer, is still a matter of debate. Gel filtration chromatography and electron microscopy studies indicated that human Pex5p (HsPex5p) is a homotetramer [4]. Fluorescence spectroscopy studies on Pex5p of the yeast *Hansenula polymorpha* (HpPex5p) indicated that HpPex5p also forms oligomers [5]. Moreover, HpPex5p oligomers were shown to bind PTS1 containing synthetic peptides, suggesting that this is indeed the conformation of a functional PTS1 receptor [6]. On the other hand, recent studies on human Pex5p (HsPex5p) using sucrose density centrifugation revealed that HsPex5p is monomeric [7]. The behavior of HsPex5p in gel filtration chromatography, which indicated a high molecular weight of the native protein, was suggested to result from a non-globular shape of the protein. Studies on the N-terminal domain of HsPex5p pointed to an unfolded pre-molten globule-like structure, which may contribute to a non-globular shape of full length HsPex5p [8].

Pex7p is the receptor for the PTS2 signal. In higher eukaryotes (plants, mammals), Pex7p associates with Pex5p during peroxisomal protein import. In lower eukaryotes (yeasts, fungi) Pex7p binds to Pex20p (or Pex18p and Pex21p in *Saaccharomyces cerevisiae*; for a review see [9]). For PTS2 protein import Pex20p's most likely fulfill a similar function as Pex5p in higher eukaryotes. Indeed, the N-terminal half of Pex5p's and Pex20p's share a few conserved domains and show similar dynamics during import [9,10]. Like Pex5p also Pex20p has been reported to form oligomers [11].

Our understanding of the structure of the peroxins involved in peroxisomal protein translocation is still limited. For initial characterization of such complexes single particle electron microscopy (EM) is a well-established technique to obtain information at a resolution of 10–20 Å [12,13]. It is attractive for samples of mixed complexes with a mass above about 200 kDa, because the statistical analysis and classification procedures used are effective in sorting of (slightly) different projection views originating from different conformations or subunit compositions. In this study, the projection structures of HpPex5p and HpPex5p–HpPex20p complexes were investigated by single particle electron microscopy. The analysis shows that HpPex5p is a tetramer and that HpPex20p is able to induce a major conformational change leading to a rather open space in the centre of the HpPex5p tetramer. In a successive set of experiments, we show that HpPex5p–HpPex20p complexes are able to bind folded copies of tetrameric catalase at the periphery. Since catalase is one of the major peroxisomal proteins this indicates that such HpPex5p–HpPex20p-catalase complexes are functional as receptor complex.

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2. Materials and methods

2.1. Organisms and growth

The *Hansenula polymorpha* strains used in this study are wild type (WT), *pex5*, *pex7* and *pex20*. Yeast cells were grown in a carbon-limited chemostat culture at 37 °C using 0.25% glucose as carbon source and 0.2% choline as nitrogen source at a dilution rate of 0.1 h^{-1}. Cells were harvested from steady state cultures. The levels of Pex5p and Pex20p were analyzed by western blotting using total cell lysates.

2.2. Expression and purification

Escherichia coli M15 cells containing plasmids pREP4 and pQE60–Pex20p-His_8 [11] or pQE60–Pex5p-His_6 [6] were grown as described before. Expression of the *PEX5* or *PEX20* genes was induced by the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside as detailed before [6,11]. Cells were harvested by centrifugation. All subsequent steps were carried out at 4 °C. Pex5p was purified as detailed [6]. To isolate the Pex5p–Pex20p complex, Pex5p-His_6 and Pex20p-His_8 were isolated by affinity chromatography using Ni-NTA (Qiagen). Cell pellets were resuspended in 40 ml buffer A (50 mM phosphate buffer, pH 7.4, 300 mM NaCl, 1% Tween 20, 10% glycerol, 0.2 mM β-mercaptoethanol, 1 mM phenylmethyl sulfonyl fluoride, 1 mM sodium azide, 5 mM sodium fluoride, and Complete™ (Roche, Almere, The Netherlands). Cells were disrupted using a French Press. The lysates were centrifuged (20 min, $10\,000 \times g$), and supernatants incubated for 1 h with Ni-NTA (Qiagen, 500 mg protein/ml resin). Subsequently, the resin was extensively washed with buffer B (50 mM phosphate buffer, pH 7.4, 100 mM NaCl) containing increasing concentrations of imidazole (up to 40 mM). Bound proteins were eluted with buffer B containing 200 mM imidazole. Elution fractions were analyzed by SDS–PAGE followed by Coomassie brilliant blue staining and Western blotting using antibodies raised against Pex5p or Pex20p. Fractions highly enriched in Pex5p-His_6or Pex20p-His_8, respectively were pooled. Equal portions of these fractions were mixed and incubated for one hour on ice. Then, the Pex5p-His_6–Pex20p-His_8 mixture was subjected to gel filtration chromatography (Superose 12) using 50 mM potassium phosphate buffer, pH 7.4 as running buffer. The elution fractions were analyzed by SDS–PAGE followed by Coomassie brilliant blue staining and Western blotting using anti-Pex5p and anti-Pex20p antibodies. The first fractions that eluted from the column and contained both Pex5p-His_6 and Pex20p-His_8 were used for electron microscopy.

Gel filtration experiments to estimate the mass of Pex5p and Pex5p–Pex20p complexes were performed on a Superose 6 size exclusion column on an AKTA FPLC system, equipped with a UV detector with catalase, ferritin, thyroglobulin as marker proteins.

2.3. In vitro binding studies

Pex5p-His_6 and Pex20p-His_8 were produced in *E. coli* and purified using Ni-NTA agarose as detailed above. Fractions containing Pex20p or Pex5p were diluted 10 times using buffer A and incubated with Ni-NTA agarose for 1 h at 4 °C. Methanol grown *H. polymorpha* wild type or *pex7* cells were disrupted using glass beads. Cell homogenates were centrifuged at 4 °C for 5 min at $20\,000 \times g$ to remove unbroken cells and cell debris. Supernatants were loaded onto the Ni-NTA agarose containing either Pex20p-His_8 or Pex5p-His_6 and incubated for 1 h at 4 °C with continuous rotation. After extensive washing with buffer B containing increasing concentrations of imidazole (from 10 mM up to 40 mM), Pex20p-His_8 or Pex5p-His_6 together with the bound proteins were eluted with buffer B containing 250 mM imidazole. The elution fractions were analyzed by Western blotting.

2.4. Preparation Pex5p-Pex20p complexes with catalase

Crystalline catalase from bovine liver (from Sigma-Aldrich) at a stock concentration of 20 mg/ml was diluted with water to the final concentration of 33 μg/ml. Pex5p–Pex20p (5 μl) complexes from the best fractions were incubated with 1 μl catalase for 1 h at room temperature and immediately prepared for EM.

2.5. Electron microscopy and single-particle analysis

Negatively stained Pex5p, Pex5p–Pex20p and Pex5p–Pex20p complexes mixed with catalase were prepared with 2% uranyl acetate on glow-discharged carbon-coated copper grids. Electron microscopy was performed on a Philips CM12 FEG electron microscope operated at 120 kV. Images were recorded with a Gatan 4 K slow-scan CCD camera at 80 000× magnification with a pixel size (after binning the images) of 3.75 Å at the specimen level, with "GRACE" software for semi-automated specimen selection and data acquisition. About 6000 images were recorded and about 7520 single particle projections from Pex5p, 6071 projections from Pex5-20 complexes, 1300 from Pex5-20p-catalase and 5800 single catalase projections were selected, respectively. Single-particle analysis was performed with the Groningen Image Processing ("GRIP") software package on a PC cluster. Selected single-particle projections (128×128 pixel frame) were aligned by a multireference alignment and reference-free alignment procedures according to [13,14]. Next, particles were subjected to multivariate statistical analysis, followed by hierarchical ascendant classification (HAC) [13]. The resolution of the class averages was measured by Fourier Ring Correlation according to [15]. After several cycles of multireference alignments, statistical analysis and classification the best projections from each set were averaged.

3. Results and discussion

We recently performed a detailed comparative study on *H. polymorpha pex* mutants, which were identically grown in chemostat cultures [16] (Koek and Van der Klei, unpublished results). Unexpectedly, in samples of steady state cultures we observed that the levels of HpPex5p were reduced in cells lacking Pex20p (*H. polymorpha pex20* cells), whereas HpPex20p levels were lower in the absence of Pex5p (in *H. polymorpha pex5*), as shown in Fig. 1. This indicates that HpPex5p and HpPex20p may stabilize each other in vivo. To analyze whether this apparent stabilization is related to a physical interaction between both proteins, we performed in vitro interaction studies. Crude extracts of *H. polymorpha* wild type cells were loaded onto columns containing either immobilized His-tagged HpPex5p or HpPex20p, purified from *E. coli*. As shown in Fig. 2A (lane 2), Pex20p from the *H. polymorpha* extract co-eluted with Pex5p-His_6. The cytosolic enzyme pyruvate carboxylase (Pyc1p), used as a negative control, did not co-elute with Pex5p-His_6. Moreover, Pex20p was not detected in the eluate when an empty column was used (Fig. 2A, lane 1). In a reverse approach, using a column containing Pex20p-His_8, Pex5p from the *H. polymorpha* extract co-eluted with Pex20p-His_8 (Fig. 2B, Lane 2). Again, the Pyc1p control protein did not co-elute with Pex20p-His_8 and no Pex5p was present in the elution fraction when an empty column was used

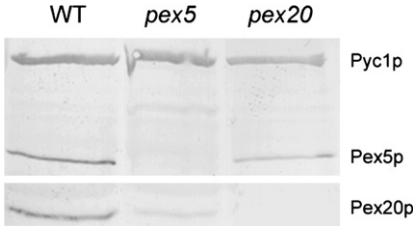

Fig. 1. Pex5p and Pex20p levels in cells of *H. polymorpha* WT and *pex* mutants. Shown are steady-state levels of Pex5p and Pex20p in WT and *pex5* and *pex20* mutants grown in chemostat cultures on glucose/choline. Pex5p and Pex20p levels were analyzed by Western blotting using specific antibodies against Pex5p and Pex20p, respectively. Pex5p and Pex20p are both evident in WT cells. In *pex5* cells Pex5p is absent as expected. However, in addition the level of Pex20p is strongly reduced in *pex5* cells. Similarly, Pex20p is absent in *pex20* cells, but Pex5p levels are slightly reduced. Equal amounts of protein were loaded per lane. The cytosolic protein pyruvate carboxylase (Pyc1p) was included as a loading control.