Studies on peroxisome biogenesis
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Citation for published version (APA):

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Download date: 09 Jan 2019
ADDENDUM TO CHAPTER 4

EFFECT OF MUTATIONS IN SACCHAROMYCES CEREVISIAE PEX6P ON PEROXISOMAL PROTEIN IMPORT

INTRODUCTION

The function of Pex6p in peroxisome biogenesis is discussed in chapter 4. Here we present several additional observations made with Pex6p containing mutations in the second AAA domain. Such mutants affect growth of wild type cells. They also affect the import of PTS1 proteins. Taking into account that ATP hydrolysis by the second AAA domain is required for dissociation of Pex6p from Pex15p we explain these results by assuming that all functional Pex15p will eventually become locked by mutant Pex6p despite the presence of wild type Pex6p.

MATERIALS AND METHODS

See materials and methods in chapter 4. For fractionation and floatation gradients, oleate induced cells were subcellularly fractionated as described previously (Hetteema et al., 2000). For stepwise fractionations in addition to the 25,000 xg pellet, a 2,500 x g pellet was made, and the resulting 2,500 x g supernatant was subjected to 100,000 x g centrifugation for 1 hour at 4°C. Samples were adjusted to the same volume by resuspending the pellets in fractionation buffer. For floatation gradients 25,000 x g pellets of spheroplasts were resuspended in 1 ml 60% sucrose (w/v), both containing 5 mM MES pH 6, 1 mM EDTA, 1 mM KCl and protease inhibitors. Samples were loaded at the bottom of a centrifuge tube, on top of these a sucrose step gradient consisting of 2 ml fractions of 46, 42, 38, 30 and 25% sucrose in the same buffer was built. The gradients were centrifuged for 18 hours at 100,000 x g in an SW41
rotor at 4°C. 1ml fractions were collected, proteins were TCA precipitated (with 10% TCA, 4°C for 1 hour, followed by centrifugation at 11,000 x g at 4°C for 30 minutes), resuspension in Laemli sample buffer supplemented with 4 mM basic Tris and analysis by SDS-PAGE and Western blotting. Antibodies used were anti-Pex15p (Elgersma et al., 1997), anti-Pex5p, anti-catalase and anti-thiolase (Fox3p) (Erdmann and Kunau, 1994). Assays for enzyme activity of catalase (Lücke, 1963) and 3-HAD (Wanders et al., 1990) were descibed before.

RESULTS

Dominant negative effect of Pex6pA2 and B2.

GFP-Pex6p can complement the pex6Δ phenotype, when it is expressed behind the PEX5 promoter and grown on selective oleate plates. When GFP-Pex6p is expressed at the same level in wild type cells, this does not influence their ability to grow on an oleate based medium either. However, expression of GFP-pex6 mutants A2 or B2 in wild type cells has a dominant negative effect (figure 1). Even though the wild type form of Pex6p is present, the mutated pex6 prevents the cells from growing on oleate medium. This effect is also observed in another strain (UTH7A, not shown). On the other hand, no negative effect occurs when mutated Pex6p is untagged and expressed under control of its own promoter. Apparently at somewhat higher expression levels (possibly in combination with the presence of a tag), mutated Pex6p titrates away all functional Pex15p.

The experiments described below are performed with transformants expressing wild type or mutated Pex6p. The cells are precultured on glucose and then shifted to oleate medium. On this latter medium, the cells with Pex6pA2 or B2 stop growing.

Localisation of Pex6p and its mutant derivatives

A portion of the Pex6p molecules present in a cell is probably bound to peroxisomes via Pex15p, while another portion is either cytosolic, bound to membrane structures distinct from peroxisomes or maybe both. Flotation experiments on the 25,000 x g organellar pellet fraction of cells expressing GFP-Pex6p show that GFP-Pex6p floats to the same density as peroxisomal membrane proteins, like Pat1p, Pex11p, Pex15p and Pex13p (figure 2). This is in line with the idea that Pex6p is partially bound to peroxisomes. GFP-
**Figure 1:** Dominant negative effect of Pex6pA2 and Pex6pB2 on growth of yeast on oleate medium. Wild type and mutant derivatives of Pex6p were N-terminally tagged with GFP and were expressed in wild type and pex6Δ cells controlled by the PEX5 promoter (upper panel) or the PEX6 promoter (lower panel).

pex6pA1 (pex6K489A) which is mutated in the first AAA domain and has a weak interaction with Pex15p, floats to the same density. This can be interpreted in two ways: either this protein is peroxisome associated (via Pex15p), or it is bound to other membrane particles which have the same density as peroxisomes. GFP-pex6pA2 and B2 were shown (figure 2) to be partially membrane bound to structures that have a density higher than that of ghosts and lower than that of mature peroxisomes. At this density also (other) peroxisomal membrane proteins were found in these mutants. These floating particles are most likely peroxisomal ghosts, which are more dense than pex6Δ ghosts, since in pex6Δ mutants still some import of matrix proteins can take place (see further on).
Two arguments favour the idea that Pex6p is partially membrane bound to structures distinct from peroxisomes: (1) The capacity of GFP-Pex6p in pex3Δ cells to float in a floatation gradient (not shown), since pex3Δ cells lack peroxisomal ghosts. (2) The floatation of Pex6p in pex3Δ cells is comparable to that in wild type cells.

**Effect of Pex6p mutations A2 and B2 on protein import**

We noticed that pex6pA2 and B2 floated to a density in between that of peroxisomal ghosts and mature peroxisomes. We know from electron microscopy studies in these mutants that the peroxisomal particles look like ghosts. Here the possibility that import of proteins can take place in these ghosts is studied, which might explain their higher density. The pelletability of proteins in a fractionation experiment shows that they are bound to or inside organelles. To study the import of PTS1 and PTS2 proteins, their pelletability was analysed by Western blotting (figure 3). Pex15p pelletability was used as a positive control for the presence of peroxisomes and ghosts. The PTS2 protein thiolase is completely in the 25,000 x g pellet, indicating that import of matrix proteins into organelles via the PTS2 pathway is still possible when the second ATPase domain of Pex6p is mutated. The PTS1 protein catalase on the other hand is not completely pelletable, approximately 55 % of the total catalase is found in the pellet fraction when pex6A2 is expressed instead of Pex6p. In the pex6pB2 mutant approximately 40 % is pelletable. The pellet/supernatant ratio of another PTS1 protein, 3-HAD, as measured by an enzyme assay indicates...
Figure 3: Effect of mutations in Pex6p on peroxisomal protein import. Fractionation of wild type cells, complemented pex6Δ cells, and pex6Δ cells with expression of Pex6pA2 or Pex6pB2, showing the pelletability (peroxisome association) of the PMP Pex15p, the PTS2 protein thiolase, the PTS1 protein catalase and the PTS1 import receptor Pex5p. H = homogenate, P1 = 2,500 x g, P2 = 25,000 x g, P3 = 100,000 x g pellet, S = 100,000 xg supernatant.

that import of this protein is slightly affected; in wild type cells 100 % is pelletable, in pex6pA2 85 % and in pex6pB2 also 85 %. These results suggest that there is a partial import defect of PTS1 proteins into organelles when Pex6p is mutated (A2 or B2).

Since PTS1 protein import is mediated by Pex5p we also studied the behaviour of this protein in a fractionation experiment. The pellet/supernatant ratio of Pex5p in oleate induced wild type cells is usually about 40:60 %. When pex6pA2 is fractionated this ratio is approximately 60:40 % and for pex6pB2 this ratio is even more affected to approximately 80:20 % (figure 3). This means that a larger amount of Pex5p is organelle bound when the second ATPase domain of Pex6p is mutated.

**DISCUSSION**

The dominant negative effect of pex6 ATPase mutations is an additional indication for the ideas that Pex6p by binding to Pex15p either temporarily inhibits the function of Pex15p or that the release of Pex6p from Pex15p is important for peroxisome maintenance. Mutated Pex6p
(A2 and B2) interacts with Pex15p and is probably unable to dissociate from it. So, eventhough endogenous Pex6p is present all Pex15p will eventually end up with mutated Pex6p bound to it with as a consequence no more peroxisome biogenesis and no more growth of cells on oleate. When a lower amount of mutated Pex6p is present, the effect is not seen probably because there are still Pex15p molecules which are not blocked. In most experiments Pex6p or its mutant derivatives were expressed at the normal physiological level of Pex6p or were shown to give the same results at different expression levels. For detection with immuno EM, the higher expression was essential for detection. Whatever the process is which is regulated by the binding and release of Pex6p from Pex15p, the relative levels of these two peroxins seem to be important.

The pelletable fraction (at 25,000 x g) of Pex6p is most likely peroxisome bound. Pex6pA2 and B2 are probably bound to peroxisomal ghosts, which in this situation are slightly more dense than complete pexΔ ghosts since import of matrix proteins is only partially disturbed. When a peroxisomal protein is pelletable at 25,000 x g most probably that protein is (under the conditions used) peroxisome associated. Pelletable does not necessarily mean that the protein is imported, it might also be bound to the surface of peroxisomes, to components of the import complex. PTS2 proteins are pelletable, while PTS1 proteins are only partially pelletable. This indicates that Pex6p mutations cause a differential defect on matrix protein import. These mutations also appeared to have an effect on Pex5p, the PTS1 receptor. It is not the first time that an effect of Pex6p on Pex5p is observed, in human and Pichia pastoris Pex6p mutants Pex5p is instable (Yahraus et al., 1996) which indicates that somehow Pex5p depends on the presence of functional Pex6p. Here we noticed that Pex5p became pelletable for a larger fraction than it is in wild type cells. One explanation for this effect might lie in the fact that these cells containing the mutated versions of Pex6p do not survive on oleate medium.

Recently observations were made which indicate that Pex5p is peroxisome bound in wild type cells which are grown on glucose medium, while they become partially cytosolic when the cells are shifted to oleate medium (G. Bottger, unpublished results). Cells with mutations in Pex6p do not survive on oleate medium and it is possible that Pex5p does not get the opportunity to leave the import complex. If this is not the cause for the larger pelletability of Pex5p than a more interesting
possibility is the one that ATP hydrolysis by Pex6p is necessary for release of Pex5p from the peroxisomal membrane. An ATP dependence in matrix protein import was already reported years ago (Bellion and Goodman, 1987). The ATPases which might be acting here are Pex1p, Pex6p (since these are the only known ATP binding and hydrolysing peroxins) or a cytosolic Hsp70 protein (Walton et al., 1994). Because we observed an import defect when the ATP binding to and hydrolysis by Pex6p was prevented, Pex6p remains a good candidate.

REFERENCES
