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The peroxisomal membrane protein Pex14p of \textit{Hansenula polymorpha} is phosphorylated in vivo

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Abstract \textit{Hansenula polymorpha} Pex14p (HpPex14p) is a component of the peroxisomal membrane essential for peroxisome biogenesis. Here, we show that HpPex14p is phosphorylated in vivo. In wild-type \textit{H. polymorpha} cells, grown in the presence of \textsuperscript{32}Porthophosphate, the \textsuperscript{32}P label was incorporated into HpPex14p. Labelled HpPex14p was induced after a shift of cells to methanol-containing media and rapidly disappeared after a shift to glucose medium, which induces specific peroxisome degradation. Alkaline phosphatase treatment of labelled HpPex14p resulted in the release of \textsuperscript{32}P and a minor shift of the HpPex14p band on Western blots. Phosphoamino acid analysis by two dimensional silica gel thin layer chromatography suggested that the major phosphoamino acid in phosphorylated HpPex14p was acid-labile.

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Key words: Methylotrophic yeast; Peroxin; Phosphoprotein; Protein translocation; Peroxisome biogenesis

1. Introduction

Peroxisomes are ubiquitous organelles of eukaryotic cells. In man, malfunction of these organelles leads to severe abnormalities which are often lethal (e.g. Zellweger syndrome, \textsuperscript{1,2}). Peroxisomal proteins are generally synthesized in the cytosol and post-translationally imported into the organelle. At least two import pathways for peroxisomal matrix proteins have been shown to exist, specified by their specific targeting signals, namely the C-terminal PTS1 and the N-terminal PTS2 \textsuperscript{3,4}. Recent reports suggest that vesicular transport systems may also participate in peroxisomal matrix protein import \textsuperscript{5}.

In the methylotrophic yeast \textit{Hansenula polymorpha}, peroxisomes play an indispensable role in the metabolism of methanol. Both alcohol oxidase and dihydroxyacetone synthase, which are involved in the methanol metabolism, are localized in peroxisomes of this yeast. Thus, peroxisomes are strongly induced during methylotrophic growth of \textit{H. polymorpha}. We have cloned nine \textit{PEX} genes by functional complementation of \textit{H. polymorpha} peroxisome-deficient (pex) mutants \textsuperscript{6}. Among them, the \textit{H. polymorpha} \textit{PEX14} gene encodes a novel peroxisomal membrane protein (HpPex14p) essential for peroxisome biogenesis \textsuperscript{7}. We demonstrated that not only deletion but also overexpression of the \textit{PEX14} gene in wild-type (WT) \textit{H. polymorpha} cells resulted in a peroxisome-deficient phenotype, suggesting that the stoichiometry of HpPex14p relative to one or more other components of the peroxisome biogenesis machinery might be critical \textsuperscript{7}. \textit{PEX14} homologues have been isolated from other organisms \textsuperscript{8-12}. The \textit{Saccharomyces cerevisiae} Pex14p (ScPex14p) was shown to represent a component of the common translocation machinery for both PTS1 and PTS2 peroxisomal matrix proteins \textsuperscript{8}. It was demonstrated that ScPex14p interacted with two other membrane-bound peroxins (ScPex13p and ScPex17p) as well as the two PTS receptor proteins (ScPex5p and ScPex7p) \textsuperscript{8,13}. However, nothing is known about the regulation of these interactions between Pex14p and other peroxins in yeast.

In this paper, we show direct evidence indicating that HpPex14p is phosphorylated in vivo by the incorporation of \textsuperscript{32}Porthophosphate into HpPex14p.

2. Materials and methods

2.1. Organisms and growth conditions \textit{H. polymorpha} NCYC495 (leu1-1) was grown in batch culture on mineral medium containing various carbon and nitrogen sources \textsuperscript{14}. For the in vivo labelling experiments, \textit{H. polymorpha} NCYC495 (leu1-1) and \textit{pex14} strains transformed with \textit{pPAX-PEX14} \textsuperscript{7} were grown on phosphate-depleted YPD \textsuperscript{15} to an OD\textsubscript{600} of approximately 1.5 and then shifted to phosphate-depleted YPM (0.5% methanol) containing 100 \textmu{}Ci/ml \textsuperscript{32}Porthophosphate. In the case of chase experiments, cells were subsequently shifted to normal YPD without \textsuperscript{32}Porthophosphate.

2.2. In vivo labelling of HpPex14p Cells were harvested from 1 ml of culture, washed three times with 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM PMSF and 2.5 \textmu{}g/ml leupeptin and then disrupted with glass beads. Subsequently, the crude extracts were lysed with 1% Nonidet P-40 and 0.5% sodium deoxycholate and \textsuperscript{32}P-labelled HpPex14p was recovered by immunoprecipitation using the Cellular Labelling and Immunoprecipitation kit (Boehringer, Mannheim, Germany). Protein determination \textsuperscript{16}, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) \textsuperscript{17} and Western blotting \textsuperscript{18} were performed by established methods. Anti-HpPex14p antiserum has been described before \textsuperscript{7}.

2.3. Alkaline phosphatase treatment \textsuperscript{32}P-labelled HpPex14p was recovered from extracts of \textit{pex14} cells overexpressing \textit{PEX14} by immunoprecipitation. Protein A-agarose-bound \textsuperscript{32}P-labelled HpPex14p was treated with bacterial alkaline phosphatase for 1 h at 37°C and analyzed by Western blotting.

2.4. Phosphoamino acid analysis \textsuperscript{32}P-labelled HpPex14p was separated by SDS-PAGE and blotted onto a PVDF membrane (Immobilon, Millipore, Bedford, MA, USA). Membrane-bound HpPex14p was subsequently hydrolyzed in 200 \mu{}l of 6 M HCl for 1 h at 110°C, dried and resuspended in 10 \mu{}1 of 0.1 M HCl. The hydrolysate was developed on two dimensional (2D) silica gel thin layer chromatography (TLC) (Silica gel 60, 10 × 10 cm, Merck) using 50% n-buthanol/20% acetic acid as the first solvent and 10% acetic acid/50% phenol as the second solvent. Phosphoserine, phosphothreonine and phosphotyrosine were used as standards and were detected by ninhydrin reagent.

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3. Results and discussion

In *H. polymorpha*, peroxisomes are mainly involved in the metabolism of specific carbon and/or nitrogen sources used for growth and are strongly induced during methylotrophic growth conditions. Previously, we have observed multiple bands near 42 kDa on Western blots, prepared from crude extracts of methanol-grown *H. polymorpha* cells overexpressing PEX14 [7]. Hence, we examined whether multiple forms of HpPex14p were also present in crude extracts from *H. polymorpha* WT cells grown on various substrates. As shown in Fig. 1, the highest induction levels of HpPex14p were detected in cells grown on methanol/methylamine. Under these growth conditions, strong doublet bands recognized by anti-HpPex14p antibodies were clearly detected near 42 kDa. Furthermore, the relative amounts of these forms of Pex14p seemed to vary depending on the growth conditions.

To examine possible modification of HpPex14p by phosphorylation, *H. polymorpha* WT cells were incubated with $^{32}$Porthophosphate in vivo and HpPex14p was recovered from crude extracts by immunoprecipitation. As shown in Fig. 2a, the $^{32}$P-labelled form of HpPex14p could easily be detected, indicating that HpPex14p is indeed modified by phosphorylation in vivo. When glucose-grown WT *H. polymorpha* cells were shifted to methanol-containing medium, conditions that induce peroxisome biogenesis as well as the synthesis of HpPex14p, $^{32}$P-labelled HpPex14p was induced. Also, in cells grown on glucose, weak signals of $^{32}$P-labelled HpPex14p were detectable, which were absent in controls using pre-immune serum. A time course experiment showed that the amount of labelled HpPex14p increased with the incubation time of the cells in the $^{32}$Porthophosphate/methanol-containing media and thus, with the volume fraction of peroxisomes in the cells (Fig. 2b). Conversely, a chase experiment showed that the amount of labelled HpPex14p rapidly decreased after a shift of cells from methanol- into fresh glucose-containing media, conditions that are known to induce a rapid and selective degradation of peroxisomes present in the cells (Fig. 2c, [19]). Within a 2 h period after the shift, the amount of labelled HpPex14p was already reduced to the level observed in cells grown on glucose. This suggests that the labelled HpPex14p is either dephosphorylated or degraded together with the peroxisomes after the shift to glucose-containing medium.

To seek further confirmation that the modification of HpPex14p indeed represents active phosphorylation, we incubated labelled HpPex14p with alkaline phosphatase. We used Δpex14 cells transformed with pPAOX-PEX14 [7] for this pur-
pose in order to get enough amounts of $^{32}$P-labelled HpPex14p. As depicted in Fig. 3, this treatment resulted in the release of $^{32}$P. Additionally, on Western blots, the upper HpPex14p band shifted to the position of the lower band. These results indicate that HpPex14p is indeed phosphorylated in vivo and that, on Western blots, the HpPex14p band of lower mobility represents the phosphorylated form of the peroxin. So far, no reports on the phosphorylation of peroxins are available, except for ScPex15p [20]. However, the phosphorylation of ScPex15p was only suggested by the mobility shift after alkaline phosphatase treatment and not demonstrated by direct incorporation of $^{32}$P orthophosphate.

To analyze the phosphoamino acid in phosphorylated HpPex14p, we performed an acid hydrolysis of phosphoproteins immobilized on PVDF membranes, followed by separation of phosphoamino acids by 2D TLC. As a control, a mixture of bovine serum albumin (100 µg) and $[^{32}$P]orthophosphate was also hydrolyzed and analyzed by TLC in the same way. Standard phosphoamino acids used were phosphoserine (P-S), phosphothreonine (P-T) and phosphotyrosine (P-Y).

Fig. 4. Phosphoamino acid analysis of phosphorylated HpPex14p. Acid hydrolysate of $^{32}$P-labelled Pex14p was separated by 2D silica gel TLC. As a control, a mixture of bovine serum albumin (100 µg) and $[^{32}$P]orthophosphate was also hydrolyzed and analyzed by TLC in the same way. Standard phosphoamino acids used were phosphoserine (P-S), phosphothreonine (P-T) and phosphotyrosine (P-Y).

ponent of the matrix protein translocation machinery. Hence, phosphorylation of Pex14p, as described here for HpPex14p, might be essential for the regulation of its function, e.g. to mediate the interaction between Pex14p and the other peroxins such as Pex5p, Pex7p, Pex13p and Pex17p. Clearly, determination of the phosphorylation site of HpPex14p and an analysis of its effect on the interaction of HpPex14p with other peroxins are required to better understand the role of phosphorylation of HpPex14p in peroxisome biogenesis. These experiments are currently under way.

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