The peroxisomal membrane protein Pex14p of *Hansenula polymorpha* is phosphorylated in vivo

Masayuki Komori[a,*], Jan A.K.W. Kiel[b], Marten Veenhuis[b]

[a] *Laboratory of Molecular Biology, Department of Veterinary Science, Osaka Prefecture University, 1-1 Gakuen-cho, Sakai, Osaka 599-8531, Japan*
[b] *Laboratory of Eukaryotic Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, Postbus 14, 9750 AA Haren, The Netherlands*

Received 29 June 1999; received in revised form 3 August 1999

Abstract ***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 *H. polymorpha* cells, grown in the presence of [^{32}P]orthophosphate, the ^{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 ^{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.**

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, [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 [3,4]. Recent reports suggest that vesicular transport systems may also participate in peroxisomal matrix protein import [5].

In the methylotrophic yeast *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 *H. polymorpha*. We have cloned nine *PEX* genes by functional complementation of *H. polymorpha* peroxisome-deficient (*pex*) mutants [6]. Among them, the *H. polymorpha PEX14* gene encodes a novel peroxisomal membrane protein (HpPex14p) essential for peroxisome biogenesis [7]. We demonstrated that not only deletion but also overexpression of the *PEX14* gene in wild-type (WT) *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 [7]. *PEX14* homologues have been isolated from other organisms [8–12]. The *Saccharomyces cerevisiae* Pex14p (ScPex14p) was shown to represent a component of the common translocation machinery for both PTS1 and PTS2 peroxisomal matrix proteins [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) [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 [^{32}P]orthophosphate into HpPex14p.

2. Materials and methods

2.1. Organisms and growth conditions

H. polymorpha NCYC495 (*leu1-1*) was grown in batch culture on mineral medium containing various carbon and nitrogen sources [14]. For the in vivo labelling experiments, *H. polymorpha* NCYC495 (*leu1-1*) and *Δpex14* strains transformed with pP_{AOX}-PEX14 [7] were grown on phosphate-depleted YPD [15] to an OD_{600} of approximately 1.5 and then shifted to phosphate-depleted YPM (0.5% methanol) containing 100 μCi/ml [^{32}P]orthophosphate. In the case of chase experiments, cells were subsequently shifted to normal YPD without [^{32}P]orthophosphate.

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 μg/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 ^{32}P-labelled HpPex14p was recovered by immunoprecipitation using the Cellular Labelling and Immunoprecipitation kit (Boehringer, Mannheim, Germany). Protein determination [16], sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [17] and Western blotting [18] were performed by established methods. Anti-HpPex14p antiserum has been described before [7].

2.3. Alkaline phosphatase treatment

^{32}P-Labelled HpPex14p was recovered from extracts of *Δpex14* cells overexpressing *PEX14* by immunoprecipitation. Protein A-agarose-bound ^{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

^{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 μl of 6 M HCl for 1 h at 110°C, dried and resuspended in 10 μl 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.

*Corresponding author. Fax: (81) (722) 54-9489.
E-mail: komori@vet.osakafu-u.ac.jp

0014-5793/99/$20.00
PII: S0014-5793(99)01087-X

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}P]orthophosphate 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}P]orthophosphate/methanol-containing media and thus, with the volume fraction of peroxisomes in the cells (Fig. 2b). Conversely, a chase experiment showed that the 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 pP$_{AOX}$-PEX14 [7] for this pur-

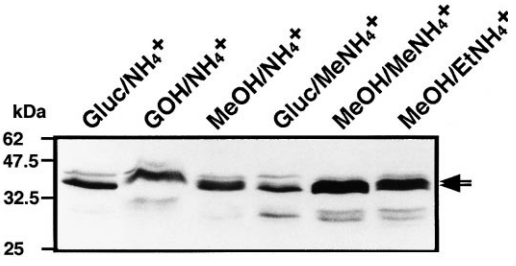

Fig. 1. Multiple forms of HpPex14p detected by Western blotting in variously grown WT cells. *H. polymorpha* WT cells were grown in mineral medium containing glucose and ammonium sulfate until the OD_{660} reached about 1.5. Subsequently, cells were harvested, resuspended in the indicated various media and grown for an additional period of 20 h. Crude extracts were prepared using glass beads. Equal amounts of protein (30 μg) were loaded per lane. Gluc, glucose; GOH, glycerol; MeOH, methanol; NH_4^+, ammonium sulfate; $MeNH_4^+$, methylamine; $EtNH_4^+$, ethylamine. Two arrows indicate the strong doublet bands recognized by anti-HpPex14p antibodies. Other bands seem to represent non-specific ones or degradation products.

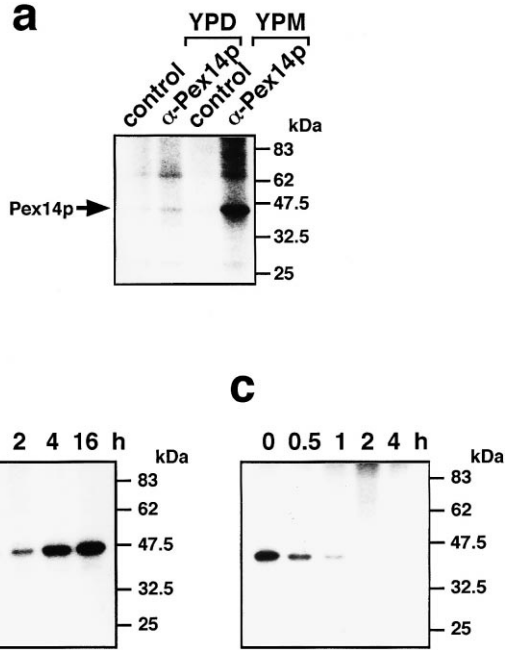

Fig. 2. In vivo labelling of HpPex14p in *H. polymorpha* WT cells with [^{32}P]orthophosphate. Cells were grown on phosphate-depleted YPD, harvested and resuspended in the same volume of phosphate-depleted YPM (or YPD) containing [^{32}P]orthophosphate for 16 h. In crude extracts, prepared from 1 ml of the culture, ^{32}P-labelled HpPex14p was recovered by immunoprecipitation using anti-HpPex14p antiserum (α-Pex14p) or pre-immune serum as control (a). Induction of ^{32}P-labelling of HpPex14p in WT cells, grown on methanol (b), and the subsequent fate of the labelled protein after a shift of such cells, incubated for 16 h, to fresh glucose media (c). Samples were taken at the indicated time points.

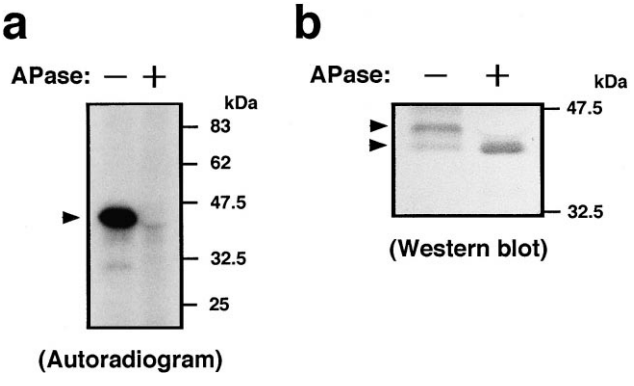

Fig. 3. Alkaline phosphatase treatment of ^{32}P-labelled HpPex14p. ^{32}P-labelled HpPex14p was recovered by immunoprecipitation from crude extracts of *Δpex14* cells overexpressing *PEX14*, grown in the same way as shown in Fig. 2a. Labelled HpPex14p, bound to protein A-agarose, was treated with bacterial alkaline phosphatase. After SDS-PAGE, radioactivity and HpPex14p protein were detected by autoradiography (a) and Western blotting using anti-HpPex14p antibodies (b), respectively. Two arrowheads in Fig. 2b correspond to the two arrows in Fig. 1.

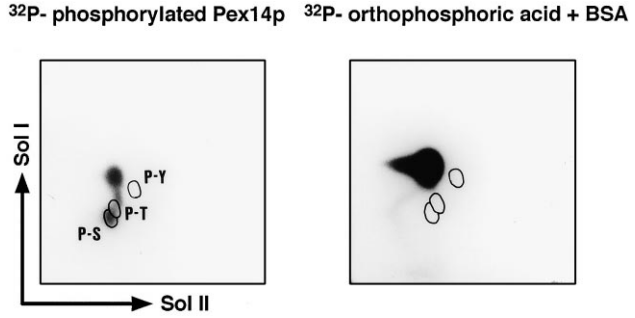

Fig. 4. Phosphoamino acid analysis of phosphorylated HpPex14p. Acid hydrolysate of ^{32}P-labelled Pex14p was separated by 2D silica gel TLC. As a control, a mixture of bovine serum albumin (100 μg) and [^{32}P]orthophosphate was also hydrolyzed and analyzed by TLC in the same way. Standard phosphoamino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

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. After separation, a major signal, corresponding to free phosphate, and a weak signal, corresponding to phosphoserine, were detected (Fig. 4). These results suggest that the major phosphoamino acid in phosphorylated HpPex14p was acid-labile. However, phosphorylation at multiple sites of HpPex14p including serine residues cannot be excluded. Phosphorylated forms of acidic and basic amino acids have been demonstrated to be acid-labile [21]. Also, phosphocysteine is acid-labile but, since the primary sequence of HpPex14p lacks a cysteine residue, this possibility is unlikely in the case of HpPex14p.

Recently, it was demonstrated by two-hybrid analysis and co-immunoprecipitations that ScPex14p interacts with ScPex5p, ScPex7p, ScPex13p and ScPex17p [8,13]. These results have led to the view that Pex14p may serve as a docking site for Pex5p and Pex7p to guide their cargo molecules to the peroxisomal surface. Moreover, the three membrane proteins Pex13p, Pex14p and Pex17p are suggested to represent components 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.

Acknowledgements: M.K. is supported by JSPS (Japan Society for the Promotion Science) Programs.

References

[1] Lazarow, P.B. and Moser, H.W. (1989) in: Disorders of Peroxisome Biogenesis. The Metabolic Basis of Inherited Disease (Scriver, S.R., Beaudet, A.L., Sly, W.S. and Valle, D., Eds.), Vol. 2, pp. 1470–1509, McGraw-Hill, New York.
[2] Subramani, S. (1997) Nat. Genet. 15, 331–333.
[3] Subramani, S. (1996) J. Biol. Chem. 271, 32483–32486.
[4] Erdmann, R., Veenhuis, M. and Kunau, W.-H. (1997) Trends Cell Biol. 7, 400–407.
[5] Titorenko, V.I. and Rachubinski, R.A. (1998) Trends Biochem. Sci. 23, 231–233.
[6] van der Klei, I.J. and Veenhuis, M. (1996) Ann. N.Y. Acad. Sci. 804, 47–59.
[7] Komori, M., Rasmussen, S.W., Kiel, J.A.K.W., Baerends, R.J.S., Cregg, J.M., van der Klei, I.J. and Veenhuis, M. (1997) EMBO J. 16, 44–53.
[8] Albertini, M., Rehling, P., Erdmann, R., Girzalsky, W., Kiel, J.A.K.W., Veenhuis, M. and Kunau, W.H. (1997) Cell 89, 83–92.
[9] Brocard, C., Lametschwandtner, G., Koudelka, R. and Hartig, A. (1997) EMBO J. 16, 5491–5500.
[10] Fransen, M., Terlecky, S.R. and Subramani, S. (1998) Proc. Natl. Acad. Sci. USA 95, 8087–8092.
[11] Will, G.K., Soukupova, M., Hong, X., Erdmann, K.S., Kiel, J.A., Dodt, G., Kunau, W.H. and Erdmann, R. (1999) Mol. Cell. Biol. 19, 2265–2277.
[12] Shimizu, N., Itoh, R., Hirono, Y., Otera, H., Ghaedi, K., Tateishi, K., Tamura, S., Okumoto, K., Harano, T., Mukai, S. and Fujiki, Y. (1999) J. Biol. Chem. 274, 12593–12604.
[13] Huhse, B., Rehling, P., Albertini, M., Blank, L., Meller, K. and Kunau, W.H. (1998) J. Cell Biol. 140, 49–60.
[14] Van Dijken, J.P., Otto, R. and Harder, W. (1976) Arch. Microbiol. 111, 137–144.
[15] Warner, J.R. (1991) Methods Enzymol. 194, 423–428.
[16] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[17] Laemmli, U.K. (1970) Nature 227, 680–685.
[18] Kyhse-Andersen, J. (1984) J. Biochem. Biophys. Methods 10, 203–209.
[19] Veenhuis, M., Douma, A., Harder, W. and Osumi, M. (1983) Arch. Microbiol. 134, 193–203.
[20] Elgersma, Y., Kwast, L., van den Berg, M., Snyder, W.B., Distel, B., Subramani, S. and Tabak, H.F. (1997) EMBO J. 16, 7326–7341.
[21] Duclos, B., Marcandier, S. and Cozzone, A.J. (1991) Methods Enzymol. 201, 10–21.