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# Identification of phosphorylation sites in *Hansenula polymorpha* Pex14p by mass spectrometry

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## ABSTRACT

**Pex14p is a peroxisomal membrane protein that is involved in both peroxisome biogenesis and selective peroxisome degradation. Previously, we showed that *Hansenula polymorpha* Pex14p was phosphorylated *in vivo*. In this study, we identified its phosphorylation site by mass spectrometry. Recombinant His-tagged Pex14p (H6-Pex14p) was overexpressed and purified from the yeast. The protein band corresponding to H6-Pex14p was in-gel digested with trypsin and subjected to LC/MS. As a result of LC/MS, Thr<sup>248</sup> and Ser<sup>258</sup> were identified as the phosphorylated sites. To confirm the phosphorylation sites and explore its functions, we made Ala mutants of the candidate amino acids. In the western blot analysis with anti-Pex14p, S258A mutant gave doublet bands while wild type (WT) and T248A mutants gave triplet bands. Moreover, the double mutant (T248A/S258A) gave a single band. WT and all mutant Pex14p labeled with [<sup>32</sup>P] orthophosphate were immunoprecipitated and analyzed by autoradiography. The phosphorylation of Pex14p was suppressed in S258A mutant, but enhanced in T248A mutant compared to WT. Moreover, the phosphorylated Pex14p was not detected in the T248A/S258A double mutant. All mutants were able to grow on methanol and their matrix proteins (alcohol oxidase and amine oxidase) were mostly localized in peroxisomes. Furthermore all mutants showed selective degradation of peroxisome like WT during the glucose-induced macropexophagy.**

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## 1. Introduction

The peroxisome is an essential organelle of eukaryotic cells. Peroxisomes function in a wide variety of metabolic pathways, including  $\beta$ -oxidation of very long chain fatty acids and biosynthesis of plasmalogens [1]. The molecular mechanism of peroxisome biogenesis is conserved among eukaryotic cells from yeasts to humans. However the defects in peroxisome biogenesis cause severe diseases in man that in some cases are lethal (e.g., Zellweger syndrome) [2,3].

In the methylotrophic yeast *Hansenula polymorpha*, peroxisomes play an indispensable role in the metabolism of methanol [4]. Thus peroxisomes are massively induced in this yeast grown on methanol. The peroxisome matrix proteins harbor the peroxisomal targeting signal-1 (PTS1) at the C terminus or the PTS2 at the N terminus region. In mammals most of the PTS2 proteins are synthesized as precursor proteins and cleaved to mature size after imported into peroxisomes. The PTS1 receptor, Pex5p, and the PTS2 receptor, Pex7p, specifically recognize these signals in cytosol. The soluble receptor–cargo protein

complexes bind to the docking complex, which consists of Pex13p and Pex14p and in yeast also Pex17p. The docking complex associates with the RING–finger complex (composed of Pex2p, Pex10p and Pex12p) to form the importmer, the assembled import-competent state of the peroxisomal protein import machinery [5,6]. Pex14p has a central role in both PTS1- and PTS2-dependent import pathways of peroxisomal matrix proteins.

When peroxisomes have become dysfunctional or redundant, they are selectively degraded by the autophagic pathway, called pexophagy. In *H. polymorpha*, massively induced peroxisomes of methanol-grown cells are selectively degraded by macropexophagy when cells are shifted to glucose medium [7]. HpPex14p is also essential for this selective peroxisome degradation besides the peroxisome proliferation [8,9]. But the regulatory mechanism of its multiple function is not clearly understood. Previously, we showed the direct evidence indicating that HpPex14p is phosphorylated *in vivo* by the incorporation of [<sup>32</sup>P] orthophosphate into HpPex14p [8,10]. In this paper, we investigated Pex14p phosphorylation sites by mass spectrometry and analyzed the role of phosphorylation in regulating Pex14p function.

**Abbreviations:** AMO, amine oxidase; AOX, alcohol oxidase; PNS, post nuclear supernatant; PTS, peroxisomal targeting signal; PVDF, polyvinylidene difluoride; SDS–PAGE, sodium dodecyl sulfate poly-acrylamide gel electrophoresis; TCA, trichloroacetic acid

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## 2. Materials and methods

### 2.1. Microorganisms and growth conditions

The yeast strain used in this study was *H. polymorpha*  $\Delta pex14$  (*leu1.1. ura3 pex14::URA3*) [11]. The cells were grown at 37°C in YPD media (1% yeast extract, 1% peptone, 1% glucose), selective media containing 0.67% Yeast Nitrogen Base without amino acids and 1% glucose (YND) or mineral media (MM; [12]) supplemented with 0.5% glucose or a mixture of 0.1% glycerol and 0.5% methanol as carbon source and 0.25% (w/v) ammonium sulfate or methylamine as nitrogen source. Cells were extensively precultivated and grown to the mid-exponential growth phase in MM supplemented with glucose and ammonium sulfate and subsequently shifted to MM supplemented with glycerol and methanol. For the *in vivo* labeling experiments,  $\Delta pex14$  strains transformed with pP<sub>AOX</sub>-PEX14 were grown on phosphate-depleted YPD [13] to an OD<sub>660</sub> of approximately 1.5 and then shifted to phosphate-depleted YPM (0.5% methanol) containing 3.7 MBq/ml [<sup>32</sup>P]orthophosphate [8,10]. For macropexophagy, the cells grown on 0.5% methanol containing media were resuspended in fresh 0.5% glucose-containing media. Samples were taken at appropriate time intervals after the shift of cells into the new environment [14]. When required, media were supplemented with 30 µg/ml leucine. For growth on plates 1.5% agar was added to the media. For construction of recombinant plasmids, *Escherichia coli* DH5 $\alpha$  (*supE44*  $\Delta lacU169$  ( $\phi 80 lacZ \Delta M15$ ) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*) and MV1184 (*ara*  $\Delta(lac-proAB)$  *rpsL thi* ( $\phi 80 lacZ \Delta M15$ )  $\Delta(srl-recA) 306::Tn10$  (tet<sup>r</sup>) / F' [*traD36 proAB<sup>+</sup> lacI<sup>q</sup> lacZ*  $\Delta M15$ ]) were used as host. The *E. coli* strains were grown at 37 °C in LB medium supplemented with 50 µg/ml kanamycin when required.

### 2.2. Preparation of organelle membrane fraction and purification of recombinant His-tagged Pex14p

The N-terminal His-tagged recombinant Pex14p (H6-Pex14p) was expressed in  $\Delta pex14$ . The cDNA for H6-Pex14p was isolated by PCR using a specific primer (5'-GGGATCCATGCATCACCATCACCATCACTCTCAACAGCCAGCAACG-3'). Obtained cDNA fragment was subcloned into pHIPX4 vector (pHIPX4-H6PEX14). After *H. polymorpha*  $\Delta pex14$  cells transformed with pHIPX4-H6PEX14 was grown on mineral medium containing glycerol/methanol (0.2%/0.3%) for 20 h, the cells were collected by centrifugation, resuspended in buffer (50 mM potassium phosphate pH 7.6 containing protease and phosphatase inhibitors), and then disrupted with glass beads at 4 °C. After removing nuclear and cell debris by centrifugation 2200  $\times g$  for 10min, the post nuclear supernatant (PNS) was further separated to organelle membrane pellet (P) and its supernatant (S) fraction by centrifugation 30,000  $\times g$  for 30min. The organelle membrane were solubilized with solubilization buffer (20 mM sodium phosphate pH 7.6, 500 mM NaCl, 20 mM imidazole, 0.1% Nonidet P-40, and inhibitors) for 30 min and then centrifuged again. The obtained solubilized supernatant was applied to Ni-NTA resin (QIAGEN). After washing the column with the solubilization buffer, the recombinant H6-Pex14p bound to the resin was eluted with a linear gradient from 20 mM to 500 mM imidazole. The eluate was subjected to SDS-PAGE, and protein bands were visualized by Coomassie Brilliant Blue staining.

### 2.3. In-gel digestion and mass spectrometry

The in-gel digestion using trypsin and peptide extraction was performed following a protocol from Shevchenko et al. [15,16]. Mass spectrometric analysis was performed in a data-dependent manner on a hybrid ion-trap time-of-flight mass spectrometer (LCMS-IT-TOF; Shimadzu) equipped with monolithic silica C18 nano electrosprayer

(GL Science) in positive ion mode. All the tandem mass spectra obtained were used to search against the Swiss-Prot and NCBI nr protein database using the MASCOT Ion search engine. Putative phosphorylated peptides mass fragmentation data were confirmed by manually.

### 2.4. Expression of mutant Pex14p

The Thr<sup>248</sup>, Ser<sup>258</sup>, and both residues of Pex14p were replaced with Ala using the Mutan-Super Express Km system (TAKARA BIO) according to the manufacturer's instruction. Oligonucleotides used for site-directed mutagenesis were T248A (5'-GCTCCGAGCTAAGCGCGCTCCAAGTGAGTC-3') and S258A (5'-TCGACGTCTAGGCGAGGCGCTGCTGCGGAAG-3'). These PEX14 mutants (T248A, S258A, and T248A/S258A double mutant) were confirmed by DNA sequencing (GenomeLab GeXP; Beckman Coulter). Finally, mutant PEX14 genes were ligated behind the AOX promoter of pHIPX4 or the PEX14 promoter of pHIPX10 [8]. The resulting plasmids were used to transform  $\Delta pex14$  cells.

### 2.5. In vivo <sup>32</sup>P-labeling of HpPex14p

Cells were cultured for 20 h in phosphate-depleted YPM (0.5% methanol) containing [<sup>32</sup>P]orthophosphate as described above. <sup>32</sup>P-labeled HpPex14p was recovered from crude extracts by immunoprecipitation as previously described [10]. The bound proteins to Protein A/G PLUS-Agarose resin (Santa Cruz Biotechnology) were eluted with Laemmli sample buffer and subjected to SDS-PAGE. Following PAGE, proteins were transferred to PVDF membranes and visualized by western blot using  $\alpha$ -Pex14p with a LAS-4000 (GE Healthcare) after enhancement with chemiluminescence. Autoradiography of the same membrane was carried out with a FLA-7000 image analyzer (GE Healthcare).

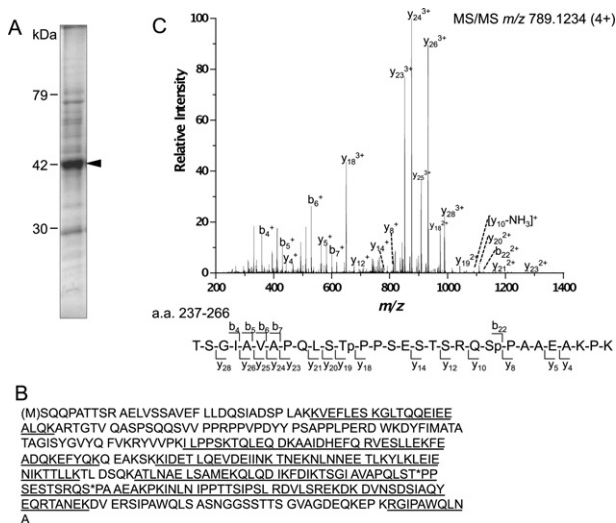
### 2.6. Molecular and biochemical techniques

Standard recombinant DNA techniques were performed as described [17]. *H. polymorpha*  $\Delta pex14$  cells were electrotransformed with pHIPX4- or pHIPX10-derived plasmid [18]. Crude cell extracts of TCA-precipitated yeast cells were prepared as described previously [19]. Protoplast was prepared with zymolyase as previously described [20,21]. Western blots were probed with polyclonal antibodies raised in rabbit against various *H. polymorpha* proteins. A rabbit anti-actin affinity purified polyclonal antibody (A2066) was purchased from sigma.

## 3. Results

### 3.1. Identification of in vivo phosphorylation sites on HpPex14p

To identify *in vivo* Pex14p phosphorylation sites, a MS-based analysis was carried out. The N-terminal His tagged recombinant Pex14p was expressed in  $\Delta pex14$  *H. polymorpha* induced by media containing glycerol/methanol (0.2/0.3%) as a carbon source for 20 h. Ni-NTA affinity purified H6-Pex14p was further separated by one dimensional SDS-PAGE. The H6-Pex14p band at 42 kDa, stained with Coomassie Brilliant Blue, was excised and subjected to in-gel trypsin digestion. A representative result of SDS-PAGE is shown in Fig. 1A. Pex14p peptides derived from tryptic digestion were analyzed by LC-MS/MS and identified by using the Mascot search engine toward the database, allowing protein modifications, such as oxidation of methionine, and specifically phosphorylation. In total, sequence coverage of 59.7% of the Pex14p peptides was obtained (Fig. 1B). Fig. 1C shows a representative MS/MS spectrum of a peptide obtained from a tryptic digest of H6-Pex14p. This quadruple charged peptide *m/z* 789.1234 corresponds to double phosphorylation on a single peptide (a.a. 237–266). A series of phosphate containing fragment ions ( $b_{22}+P$ ,  $y_{19}+2P$ ,



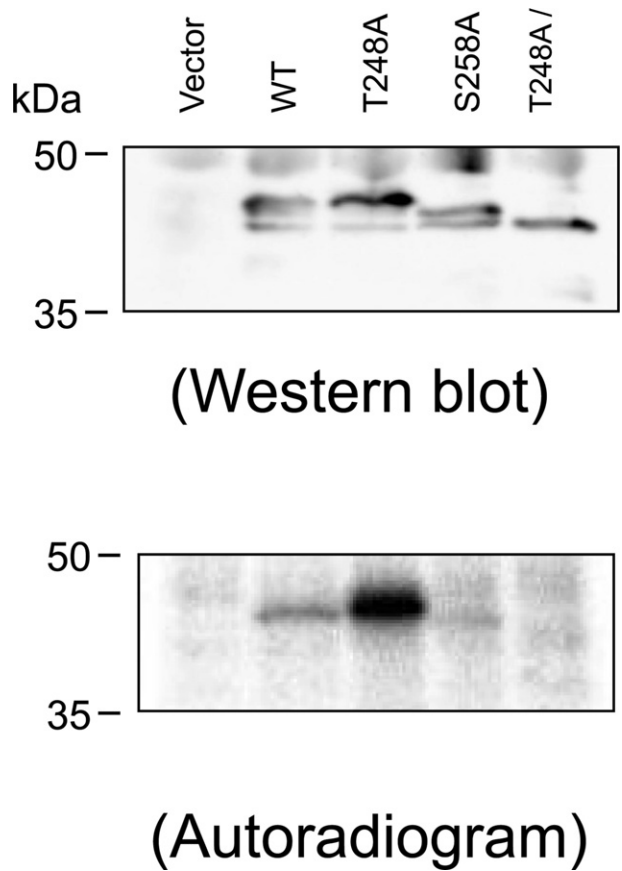
**Fig. 1.** Purification and mass spectrometry of phosphorylated H6-Pex14p. (A) H6-Pex14p was purified from an overexpression *H. polymorpha* strain grown on methanol by Ni-NTA affinity resin, separated by SDS-PAGE, and visualized with Coomassie staining. The molecular mass (kDa) of marker proteins is indicated. (B) Observed trypsin digested peptides of the *HpPex14p* are underlined (amino acid sequence coverage is 59.7%). Asterisk (\*) shows the phosphorylation sites predicted by mass spectrometry. (C) MS/MS spectra of diphosphorylated  $MH^{4+}$  peptide ion ( $m/z$  789.1234) corresponding to tryptic peptide TSGIAVAPQLSTPPSESTSRQPAAEAKPK (a.a. 237–266). Produced y and b fragment ion series were described in the sequence. The phosphorylation sites were unambiguously assigned to Thr<sup>248</sup> and Ser<sup>258</sup> by the detection of phosphate containing fragment ions ( $b_{22}+P$ ,  $y_{19}+2P$ ,  $y_{10}+P-NH_3$ ).

$y_{10}+P-NH_3$ ) were also observed (Supplemental Table). In addition, the monophosphorylated triple charged peptide (a.a. 237–256) and double charged peptide (a.a. 237–256) were also observed at  $m/z$  1025.1740 and at  $m/z$  1033.4872, respectively. The parent ions  $m/z$  1025.1740 and  $m/z$  1033.4872 produced the phosphate containing fragment ions ( $y_9+P$ ) and ( $b_{12}+P$ ,  $y_9+P$ ), respectively (Supplemental Fig. S1). These results indicate that the phosphorylation sites are Thr<sup>248</sup> and Ser<sup>258</sup>.

### 3.2. *In vivo* labeling of *HpPex14p* with [<sup>32</sup>P]orthophosphate

To confirm whether Thr<sup>248</sup> and Ser<sup>258</sup> can be phosphorylated *in vivo*, mutant *PEX14* cDNAs were made by site-directed mutagenesis. Obtained mutant *Pex14p*, namely Ala substitution of Thr<sup>248</sup> (T248A) and Ser<sup>258</sup> (S258A), and both residues (T248A/S258A), were expressed in  $\Delta pex14$  cells using pHIPX4 vector system. <sup>32</sup>P-labeled mutant *Pex14p* proteins were immune-precipitated with  $\alpha$ -*Pex14p* and were loaded to one-dimensional SDS-PAGE. S258A mutant and T248A/S258A double mutant showed doublet bands and a single band, respectively, as compared to cells expressing WT, which showed triplet bands on western blot (Fig. 2, upper panel). This band shift of S258A mutant might be a result of blocking the Ser<sup>258</sup> phosphorylation site with Ala mutation.

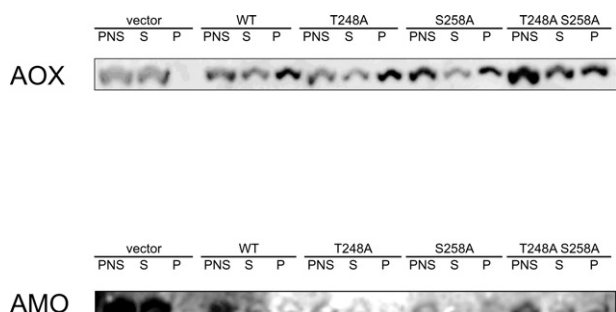
*In vivo* phosphorylation of *Pex14p* was reduced in S258A mutant, and undetectable in T248A/S258A mutant (Fig. 2, lower panel). Interestingly, the phosphorylation of *Pex14p* was stimulated in T248A mutant. At moment the reason of this phenomenon is unknown. We also analyzed point mutants of another putative S/T phosphorylation sites in the region of a.a. 237–266. However, none of them altered the phosphorylation state as compared with that of WT (data not shown). These results suggested that Thr<sup>248</sup> and Ser<sup>258</sup> were the major phosphorylation sites of *Pex14p*.



**Fig. 2.** *In vivo* labeling of *HpPex14p* with [<sup>32</sup>P]orthophosphate. WT, T248A, S258A, and T248A/S258A *Pex14p* were expressed in  $\Delta pex14$  cells using pHIPX4 system. PNS fraction was prepared from the glass-beads disrupted cells grown on phosphate depleted YPM medium containing [<sup>32</sup>P]orthophosphate for 20 h. <sup>32</sup>P-labeled *HpPex14p* was recovered from the solubilized supernatant by immunoprecipitation, separated by SDS-PAGE, transferred onto a PVDF membrane, and detected by autoradiography. Subsequently, western blotting was carried out using the same membrane.

### 3.3. Subcellular distribution of peroxisomal matrix proteins in *Ala* mutants of *HpPex14p* phosphorylation sites

To study the localization of peroxisomal matrix proteins in phosphorylation site mutants, *Ala* mutants of *Pex14p* were expressed in  $\Delta pex14$  cells using pHIPX10 vector system under the control of *PEX14* promoter. All mutant cells could grow in YNM medium (0.5% methanol as a sole carbon source) like WT cells (Supplement Fig. S2). Furthermore all mutant *Pex14ps* were recovered in the 30,000 x g organelle pellet fraction like WT after differential centrifugation (Supplement Fig. S3). To assess the localization of the peroxisomal matrix proteins, alcohol oxidase (AOX) and amine oxidase (AMO), each containing PTS1 and PTS2, respectively, we cultured the cells according to the previous report with slight modifications [19]. Briefly, these mutant cells were pre-grown on YND medium containing 1% glucose as carbon source and 0.25% ammonium sulfate as nitrogen source, and then transferred to mineral medium containing 0.3% methanol/0.2% glycerol as carbon source and 0.25% methylamine as nitrogen source for 20 h. Differential centrifugation of the homogenized protoplasts of mutant cells revealed that both of AOX and AMO were predominantly present in the organelle pellet fraction, suggesting that these matrix proteins in mutant cells were localized in peroxisome like WT cells (Fig. 3). The AOX and AMO detected in the soluble fractions of all cells including WT were most likely due to leakage of these proteins as a result of the fractionation procedure. From these



**Fig. 3.** Subcellular distribution of peroxisomal matrix proteins in Ala mutants of *HpPex14p* phosphorylation sites. PNS prepared from methanol/methylamine-grown cells of the indicated strains were subjected to differential centrifugation. Obtained organelle membrane pellet and supernatant were analyzed by western blotting with antibodies against the *H. polymorpha* peroxisome matrix proteins: AOX (PTS1 matrix protein) and AMO (PTS2 matrix protein). Equivalent volumes of each fraction were loaded per lane.

results we concluded that the phosphorylation of Pex14p at Thr<sup>248</sup> and Ser<sup>258</sup> do not affect the matrix protein import into peroxisome under the peroxisome inducible conditions.

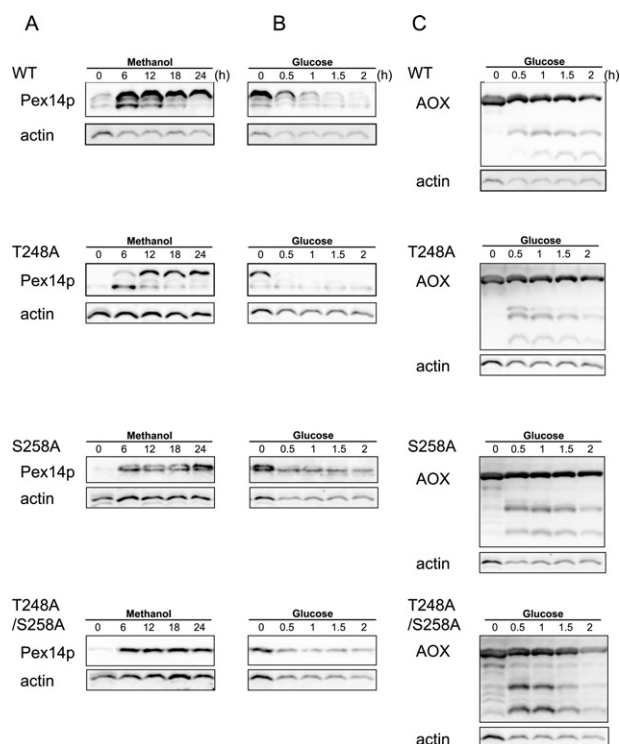
#### 3.4. Pex14p phosphorylation dynamics during peroxisome proliferation and degradation

We first examined the dynamics of Pex14p induction during adaptation of cells to peroxisome-inducing growth conditions on glycerol/methanol (0.1/0.5%) medium. As shown in Fig. 4A, WT cells gave three bands around 42 kDa on western blot. The phosphorylated Pex14p (highest band) was rapidly induced after the shift to methanol-containing media that strongly induced peroxisome proliferation. On the other hand, the non-phosphorylated Pex14p level (lowest band) in WT cells decreased under the limit of detection at 24 h after the shift to methanol medium. T248A mutant cells gave three bands on western blot, and showed similar pattern of Pex14p dynamics like WT. S258A mutant cells gave two bands on western blot showing similar dynamics of Pex14p except for remaining non-phosphorylated Pex14p (lower band) even at 24 h after the shift to methanol medium. T248A/S258A double mutant showed only one band corresponding non-phosphorylated Pex14p on western blot.

Subsequently, macropexophagy was studied in the mutant cells by monitoring the AOX and Pex14p levels on western blot. Cells induced for 18 h on methanol were shifted to fresh glucose medium (0.5%). In WT cells, macropexophagy normally occurred judging by the degradation of AOX protein on western blot (Fig. 4B and C). Western blot with  $\alpha$ -Pex14p antiserum revealed that the phosphorylated Pex14p (highest band) of WT cells diminished rapidly in the first 30 minutes during the glucose-induced pexophagy, whereas the non-phosphorylated Pex14p displayed a relatively slow reduction according to the typical peroxisome degradation by pexophagy. All mutants showed similar dynamics of Pex14p degradation. Similarly AOX band of all mutants decreased with time like WT cells. Furthermore the lower molecular weight bands characteristic AOX degradation products were also observed in all mutant cells.

#### 4. Discussion

Biological systems use regulatory networks, such as kinase/phosphatase signaling, to rapidly integrate a multitude of individual sensory input events into a coordinated biological response [22–24]. We previously reported *H. polymorpha* Pex14p is phosphorylated *in vivo* [10]. However, its phosphorylation site(s) and physiological role of phosphorylated Pex14p are still unknown. Here we identified the phosphorylation sites using mass spectrometry and analyzed the role



**Fig. 4.** Pex14p phosphorylation dynamics during peroxisome proliferation and macropexophagy. (A) Each strain grown to the mid-exponential phase in glucose medium was shifted to methanol medium. At the indicated time points, equal amounts of whole-cell extracts were loaded per lane and analyzed the level of Pex14p on western blot. Actin was monitored as a loading control. (B) After the cells were grown for 18 h in methanol medium, subsequently shifted to fresh glucose medium. At the indicated time points after the shift to glucose medium, whole-cell extracts were analyzed the level of Pex14p on western blot like (A). (C) The same membranes in (B) were reused for analysis of AOX by western blotting.

of the phosphorylation of Pex14p in peroxisome proliferation and macropexophagy.

In mass spectrometry, we discovered that the peptide a.a. 237–266 contains two phosphorylation sites and identified these sites as Thr<sup>248</sup> and Ser<sup>258</sup> by analyzing the phosphate containing fragment ions. These sites are located between a.a. 227 and 293, consistent with our previous result of *in vivo* phosphorylation of N- or C-terminal truncated mutants, showing that  $\Delta$ C58 (1–293 a.a.) was phosphorylated but  $\Delta$ C124 (1–227 a.a.) was not [8]. To confirm the phosphorylation sites, we made Ala mutants of these sites, namely T248A, S258A, and T248A/S258A double mutant. From the western blot analysis of these mutants, T248A showed triplet bands, although S258A showed doublet and T248A/S258A double mutant showed a single band. Moreover *in vivo* phosphorylation experiment of these mutants showed that the band intensity of S258A was reduced and that of T248A/S258A double mutant was almost undetectable. Unexpectedly, the T248A mutation resulted in enhancement of *in vivo* phosphorylation, suggesting that the phosphorylation of another site(s) including Ser<sup>258</sup> might be enhanced.

All mutant cells expressing mutant Pex14ps under the control of its own promoter could grow on methanol medium like WT, suggesting that the functions of all mutant Pex14ps were normal in peroxisome proliferation. Actually major part of PTS1 peroxisomal matrix protein (AOX) and PTS2 peroxisomal matrix protein (AMO) were recovered in organelle membrane fractions of these mutant cells. From these data, we conclude that Thr<sup>248</sup> and Ser<sup>258</sup> phosphorylation do not affect the peroxisomal matrix protein import and peroxisome proliferation.

It is also known that *HpPex14p* is involved in pexophagy, and its N-terminus 31 amino acids are especially essential for the selective peroxisome degradation [8]. However the glucose-induced pexophagy in T248A, S258A, and T248A/S258A mutants were not affected as compared to that of WT cells. These results are consistent with the result that glucose-induced pexophagy was normally observed even in  $\Delta$ C124 mutant cells [8]. Additionally, the band of phosphorylated *HpPex14p* was rapidly diminished (within the first 30 min) during the glucose-induced macropexophagy [25], suggesting that the dephosphorylation of *Pex14p* by phosphatase might be occurred. Another peroxin *Pex3p* also plays a role in macropexophagy in *H. polymorpha*, and its degradation is an essential initial stage of this selective peroxisome degradation [26]. It is also known that the methylotrophic yeast *Pichia pastoris* *Pex14p* is phosphorylated *in vivo* [27], and *PpPex14p* and *PpPex3p* interacted *in vivo* with *PpAtg30* in pexophagy [28]. Thus *Pex14p* may be involved in the recognition step by pexophagy-related sequestering membrane [28]. However the physiological function of phosphorylated *Pex14p* during the glucose-induced pexophagy is still unclear. Anyway further experiments are needed to elucidate the role of phosphorylation of *Pex14p* on the interaction with *Atg30p* during the process of pexophagy.

To date large scale phosphoproteome analysis using mass spectrometry revealed many protein phosphorylation sites in various species [29–32]. Ser<sup>232</sup>, Ser<sup>234</sup>, Ser<sup>335</sup>, Thr<sup>287</sup> and Thr<sup>289</sup> of *Homo sapiens* *Pex14p* [30,33] and Ser<sup>266</sup> and Ser<sup>313</sup> of *Saccharomyces cerevisiae* *Pex14p* [29] were assigned as phosphorylation sites by such phosphoproteomics. These sites are closely located with the identified two *HpPex14p* phosphorylation sites (Thr<sup>248</sup> and Ser<sup>258</sup>), but are not so highly conserved. Previously we showed that the major phosphoamino acid in phosphorylated *HpPex14p* was acid-labile [10]. Thus it cannot be excluded the possibility that the acid-labile residue(s) of *Pex14p* might be phosphorylated like the His-Asp phosphotransfer signaling system of two-component regulator [34]. Furthermore, in order to get enough amounts of phosphorylated *Pex14p*, we used overexpression system that caused peroxisome deficient phenotype, which is not physiological state [11].

In conclusion, we could identify the phosphorylation sites of *HpPex14p* as Thr<sup>248</sup> and Ser<sup>258</sup> using MS, although there were not so clear phenotypes in Ala mutants of these sites during peroxisome proliferation and selective degradation. Clearly much further experiments are required to elucidate the role of *Pex14p* phosphorylation.

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fob.2012.11.001.

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