Unique Requirements for Mono- and Polyubiquitination of the Peroxisomal Targeting Signal Co-receptor, Pex20

Xueqian Liu and Suresh Subramani

From the Section of Molecular Biology, Division of Biological Sciences, University of California, San Diego, La Jolla, California 92037-0322

In *Pichia pastoris*, the peroxisomal targeting signal 2 (PTS2)-dependent peroxisomal matrix protein import pathway requires the receptor, Pex7, and its co-receptor Pex20. A conserved lysine (Lys19 in *Pichia pastoris*) near the N terminus of Pex20 is required for its polyubiquitination and proteasomal degradation, whereas a conserved cysteine (Cys8) is essential for its recycling. In this study, we found that Cys8 is required for the DTT-sensitive mono- and diubiquitination of Pex20. We also show that the PTS2 cargo receptor, Pex7, is required for Pex20 polyubiquitination. Pex4, the E2 ubiquitin-conjugation enzyme, is required for monoubiquitination of Pex20. However, it is also necessary for polyubiquitination of Pex20, making its behavior distinct from the ubiquitination described for other PTS receptors. Unlike the roles of specific RING peroxins in Pex5 ubiquitination, we found that all the RING peroxins (Pex2, Pex10, and Pex12) are required as E3 ubiquitin ligases for Pex20 mono- and polyubiquitination. A model for Pex20 ubiquitination is proposed based on these observations. This is the first description of the complete ubiquitination pathway of Pex20, which provides a better understanding of the recycling and degradation of this PTS2 cargo co-receptor.

Peroxisomes are single membrane-enclosed organelles that house oxidative enzymes involved in a variety of metabolic pathways (1). Peroxisomal matrix proteins endowed with peroxisomal targeting signals (PTS),

3 PTS1 and/or PTS2, are imported into the organelle via the action of specific co-receptors and/or receptors. This theme is exemplified by the PTS1 receptor, Pex5 (2), and the PTS2 receptor, Pex7, with its co-receptors, such as Pex5L in higher eukaryotes (3, 4), or Pex18/Pex21 in *Saccharomyces cerevisiae* (5, 6), or Pex20 in other fungi (7–10), respectively.

After delivering the cargo into the peroxisomal matrix, the receptors/co-receptors are recycled for the next round of cargo import or degraded by the proteasome via the receptor accumulation and degradation in the absence of recycling (RADAR) pathway (7, 11) when the recycling is impaired. During the recycling and degradation, the receptors are modified via the ubiquitination pathway, in which the ubiquitin-activating enzyme (E1) transfers ubiquitin (Ub) to an ubiquitin-conjugating enzyme (E2), and a protein-Ub ligase (E3) binds both Ub-E2 and substrate and facilitates the direct or indirect transfer of the Ub moiety onto the substrate protein (12). The Ub is covalently linked via an isopeptide bond between its C-terminal glycine and an ε-amino group of a lysine on the substrate protein. However, Ub can also be linked to the ε-amino group of the N-terminal amino acid on the substrate protein or more rarely via a thioester bond to a cysteine or an oxyster bond to a serine or threonine on the substrate protein (13).

It is known that the recycling and degradation of Pex5 and Pex18 depend on mono- and polyubiquitination pathways, respectively. The N-terminal conserved cysteines of Pex5 (Cys11 in *Hansenula polymorpha* (11, 14) and Pex18 (Lys13/Lys20 in *Saccharomyces cerevisiae* (5) are required for polyubiquitination and degradation. The N-terminal conserved lysines of Pex5 (Lys18/Lys24 in *Saccharomyces cerevisiae* and Lys21 in *Hansenula polymorpha* (11, 14) and Pex18 (Lys13/Lys20 in *Saccharomyces cerevisiae* (5) are required for polyubiquitination and degradation. A conserved lysine near the N terminus of Pex20 (Lys19 in *Pichia pastoris*) is required for polyubiquitination and degradation (7), whereas an N-terminal conserved cysteine of *P. pastoris* Pex20 (Cys8) is essential for its recycling (15). However, it is unknown whether Cys8 is a site for monoubiquitination.

Pex4/Pex22 in yeast and plants (16) and UbcH5 in mammals (17) function as the E2 ubiquitin-conjugating enzymes in monoubiquitination of Pex5, whereas Ubc4 in *S. cerevisiae* (18) functions as the E2 enzyme in the polyubiquitination of Pex5.
The E2 enzymes involved in Pex20 mono/polyubiquitination are not known.

The roles of the RING subcomplex in Pex5 ubiquitination were only determined in *S. cerevisiae in vitro*. The RING peroxins Pex2, Pex10, and Pex12 form a heteromeric complex and stabilize each other in vivo (19–21). In vitro data showed that ScPex12 functions as E3 ligase for monoubiquitination of Pex5 (18). ScPex2 (18) and ScPex10 (22) have been implicated as E3 enzymes for polyubiquitination of Pex5, because mutation or truncation of ScPex10 only reduces Pex5 polyubiquitination (22), whereas this receptor modification is completely absent when ScPex2 is mutated (18). ScPex10 functions as a central component and directly binds to ScPex2 and ScPex12 while bridging the indirect interaction between these two RING peroxins, and the ubiquitination activity of the Pex10/Pex12 RING domains is enhanced in the presence of Pex4 (16). However, the E2 and E3 ligases involved in Pex20 mono- and polyubiquitination have not been characterized.

Pex20 interacts indirectly with PTS2 cargo through Pex7 and functions in the translocation of the Pex7-cargo complexes, although Pex7 alone does not require Pex20 for translocation across the peroxosomal membrane into the matrix (7). However, no role has been described for Pex7 in Pex20 ubiquitination.

Most of these insights on the sites and enzymes involved in these ubiquitination steps and the biological role of mono- or polyubiquitination of PTS receptors have come from studies on the PTS1 receptor, Pex5, and to a far lesser extent from studies on the PTS2 pathway co-receptor Pex18/Pex20. Understanding these processes for the Pex20 family of proteins, the subject of this paper, is essential for a complete understanding of the PTS1 and PTS2 import pathways. Additionally, this information on the PTS2 pathway is relevant for disease evolution of the PTS1 and PTS2 import pathways. Furthermore, defects in this pathway impair fungal pathogenicity (24). The roles of the RING subcomplex in Pex5 ubiquitination were only determined in *S. cerevisiae in vitro*. The RING peroxins Pex2, Pex10, and Pex12 form a heteromeric complex and stabilize each other in vivo (19–21). In vitro data showed that ScPex12 functions as E3 ligase for monoubiquitination of Pex5 (18). ScPex2 (18) and ScPex10 (22) have been implicated as E3 enzymes for polyubiquitination of Pex5, because mutation or truncation of ScPex10 only reduces Pex5 polyubiquitination (22), whereas this receptor modification is completely absent when ScPex2 is mutated (18). ScPex10 functions as a central component and directly binds to ScPex2 and ScPex12 while bridging the indirect interaction between these two RING peroxins, and the ubiquitination activity of the Pex10/Pex12 RING domains is enhanced in the presence of Pex4 (16). However, the E2 and E3 ligases involved in Pex20 mono- and polyubiquitination have not been characterized.

In this study, we show for the first time that the Cys8 is required for the DTT-sensitive, mono/diubiquitination of Pex20 that relies on the E2 enzyme, Pex4. Pex7 and Pex4 were also found for the first time to affect polyubiquitination of Pex20. Unlike the roles of specific RING peroxins in Pex5 ubiquitination described in other studies, we found that all three RING peroxins (Pex2, Pex10, and Pex12) are required as E3 protein-Ub ligases for Pex20 mono- and polyubiquitination. A model for Pex20 ubiquitination is proposed based on these observations. This is the first description of the complete ubiquitination pathway of Pex20, which provides new roles of Pex4, Pex7, and RING peroxins in Pex20 mono/polyubiquitination, thereby providing a better understanding of the recycling and degradation of this PTS2 cargo co-receptor.

**E X P E R I M E N T A L  P R O C E D U R E S**

*Strains, Plasmids, and Culture Conditions—*Strains, plasmids, and oligonucleotides used are listed in supplemental Tables S1–S3, respectively. Growth media include rich medium YPD and oleate medium YNO (7). All of the cultures were grown at 30 °C in YPD to 1 A600/ml, washed with distilled H2O, and shifted to YNO for biochemical experiments. Oleate induction was overnight (16 h) unless otherwise indicated in the figures.

*Immunofluorescence Microscopy—*Samples were prepared as described previously (25). Anti-thiolase antibody (1:40,000 dilution), anti-Pex12 antibody (1:20,000 dilution), or anti-HA antibody (1:100 dilution) was used as primary antibody. Alexa Fluor 488 goat anti-rabbit IgG antibody (Invitrogen) and/or Alexa Fluor 568 goat anti-rat IgG antibody (Invitrogen) was used as secondary antibody.

*Subcellular Fractionation—*Oleate-grown cells were grown overnight and homogenized as described previously (26), except that the last centrifugation was performed at 200,000 × g to ensure pelleting of peroxisome remnants in pex mutants (27).

*Protease Protection Assay—*The cells were broken as for subcellular fractionation but without protease inhibitors. Pellets of a 200,000 × g centrifugation (see previous section) were resuspended in ice-cold Dounce buffer (26) to a protein concentration of 1 mg/ml. Freshly prepared protease K (40 µg) and trypsin (40 µg) were added to 200 µg of pellet fraction in the absence or presence of 0.5% Triton X-100, respectively. Aliquots were taken after incubation at room temperature for the indicated times. Trichloroacetic acid (final concentration, 12.5%) was added to terminate the reactions. Proteins were precipitated overnight on ice, washed three times with ice-cold acetone, and resuspended in lysis buffer. Equal amounts of samples were loaded to SDS-PAGE and subjected to immunoblot analysis. Anti-HA, anti-Pex8, anti-Pex17, anti-catalase, anti-Pex5, anti-thiolase, anti-Pex2, anti-Pex10, anti-Pex12, and anti-G6PDH antibodies were used as primary antibodies.

*In Vivo Ubiquitination Assay for Pex20—*Oleate-grown cells (8 ODs) were collected and resuspended in 200 µl of ice-cold lysis buffer (20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.5% Nonidet P-40, 10% glycerol, 1 mM EDTA) containing the following inhibitors (Sigma): yeast protease inhibitor mixture, NaF (10 mM), leupeptin (1 µg/ml), aprotinin (1 µg/ml), PMSE (1 mM), N-ethylmaleimide (20 mM), and MG-132 (100 µM). The cells were broken with glass beads for 10 min at 4 °C and pelleted at 4,000 × g for 5 min. SDS sample buffer was then added, and samples were boiled for 5 min. 20 µl were loaded to SDS-PAGE and subjected to immunoblot analysis. Anti-HA and anti-F1β antibodies were used as primary antibodies.

*In Vivo Ubiquitination Assay for Pex5—*Oleate-grown cells (8 ODs) were collected, and crude extracts were prepared in the presence of TCA (28). Anti-HA and anti-F1β were used as primary antibodies for immunoblotting.

*Protein Stability Assay—*Oleate-grown cells (5 ODs) were collected and resuspended in 200 µl of ice-cold lysis buffer as described above. Cell lysates were made using glass beads. 10 µl were loaded to SDS-PAGE and subjected to immunoblot analysis. Anti-HA and anti-F1β antibodies were used as primary antibodies.

**R E S U L T S**

Cys8 Is Required for Pex20 Mono/Diubiquitination and Recycling—Pex20-HA was expressed in a pex20Δ mutant strain from its endogenous promoter (1.5 kb), which was almost fully...
functional as determined by growth curves on oleate (Fig. 1A), subcellular fractionation assays (Fig. 1B), and import of peroxisomal matrix markers (Fig. 1C). Pex20 (C8S)-HA (incapable of recycling), Pex20 (K19R)-HA (incapable of polyubiquitination on Lys19), or Pex20 (C8S/K19R)-HA (potentially incapable of mono- or polyubiquitination on Cys8 or Lys19) were expressed from their endogenous promoters in the pex20/H9004 mutant strain. The Pex20 (C8S)-HA strain grew partially, and the Pex20 (C8S/K19R)-HA strain had a strong growth defect, whereas the Pex20 (K19R)-HA strain grew almost as well as the wild-type Pex20-HA strain (Fig. 2A). After overnight induction in oleate, Pex20 (C8S)-HA was unstable compared with the Pex20 (K19R)-HA, Pex20 (C8S/K19R)-HA and wild-type Pex20-HA fusion proteins (Fig. 2B). These results suggested that the mutation of C8S, which cannot be recycled, might render Pex20 (C8S)-HA unstable by making it available for polyubiquitination on Lys19 and proteasomal degradation via the RADAR pathway. Consistent with a role for Cys8 in Pex20 recycling, Pex20 (K19R)-HA was stable and functional because it can be recycled. However, Pex20 (C8S/K19R)-HA, which cannot be recycled or degraded by the RADAR pathway, is stable but non-functional. These results, regarding the roles of N-terminal Cys and Lys residues of the PTS receptors in receptor recycling and RADAR, are consistent with previous findings for Pex20-GFP (7, 15), Pex5 (2, 14), and Pex18 (5). The polyubiquitination of Pex20 on Lys19 was first observed in the Pex20-GFP strain when His6-Myc-tagged Ub (K48R) was constitutively overexpressed to block Lys48-branched poly-
ubiquitination (7). However, the monoubiquitination of Pex20 was not detected (7, 15). To detect the ubiquitination status of Pex20-HA, His6-Myc-tagged Ub (K48R) was overexpressed in Pex20-HA and its mutants. In the in vivo ubiquitination assay, we found for the first time the DTT-sensitive mono/diubiquitination of Pex20 (K19R)-HA (Fig. 3A). Pex20-HA was found mostly to be mono- and also polyubiquitinated (DTT-resistant) (Fig. 3A) and stable (Fig. 2B), whereas Pex20 (C8S)-HA was robustly polyubiquitinated (DTT-resistant) (Fig. 3A) and unstable, as expected (Fig. 2A) (7, 15). The ubiquitinated band shifted to a lower molecular weight (from 10.9 kDa to 8.8 kDa) when the N-terminal His6-Myc tag was removed (Fig. 3B), indicating that the modification is ubiquitination. Therefore, these results strongly suggest that Cys8 acquires the DTT-sensitive, mono/diubiquitination in Pex20 (K19R)-HA.

Notably, Pex20 (C8S/K19R)-HA, which is missing both the normal mono- and polyubiquitination sites on Pex20, was stable (Fig. 2B) and gave an aberrant DTT-resistant monoubiquitination band, which could not be on Cys8 or Lys19 (Fig. 3A). The deletion of the first 6 amino acids or mutation of Pex20 (C8A/K19R) or each of the lysines in Pex20 (C8S/K19R)-HA singly or doubly, did not affect the DTT-resistant monoubiquitination (Fig. 3C), indicating that neither these N-terminal residues nor the serine 8 in C8S or any lysine is involved in the aberrant monoubiquitination. However, the aberrant monoubiquitination band of Pex20 (C8S/K19R)-HA was sensitive to mild alkaline conditions, such as hydroxylamine and NaOH (Fig. 3D), indicating that the ubiquitination is by esterification on an unknown serine or threonine (13). Because Pex20 (C8S/K19R)-HA fails to be recycled and degraded and is not functional (Fig. 2), this aberrant monoubiquitination on the Ser/Thr is of minor importance for Pex20 physiological function in peroxisomes.

RING Subcomplex Components Are Required for Monoubiquitination/Recycling and Polyubiquitination/Degradation of Pex20—The prototypic RING domain coordinates two zinc atoms in a cross-brace fashion through one histidine and seven cysteine residues and obeys the consensus $^{36}CX_{3}CX_{4}CXHX_{2}C_{5}$ (referred to as the C3HC4 finger) (29, 30). Among the peroxins, Pex2 and Pex10 possess a canonical RING domain in most species (31–34), whereas in Pex12, only five of the eight conserved residues are present (35–37), suggesting that Pex12 can bind only one zinc atom per monomer. The RING peroxins are required for each other’s stability (19, 20, 38), whereas the first two Cys residues of the RING domain are known to be essential for their E3 ligase activity (39).

We created double-point mutations at the first two Cys of the RING domains in Pex2, Pex10, and Pex12, to inactivate their E3 ligase without destabilization of the RING subcomplex.
P. pastoris Pex20 Ubiquitination

FIGURE 4. The majority of peroxins (Pex20-HA, Pex2, Pex10, and Pex12), PTS2 cargo thiolase and G6PDH (cytosolic marker) are mostly correctly localized in RING domain point mutants. The strains used were skxq167, skxq168, skxq169, and skxq63.

subcellular fractionation assay showed that the majority of Pex20-HA, RING peroxins, and peroxisomal membrane protein Pex17 were localized correctly in the RING domain point mutants in comparison to the wild-type strain (Fig. 4), even though less thiolase is imported in the RING mutants, and the recovery of Pex2 (especially in Pex12 C/S) is poor. Therefore, for further studies, Pex2, Pex10, and Pex12 RING domain point mutations were used to maintain their stabilities and that of the RING subcomplex.

Pex2 (C281S/C284S), Pex10 (C313S/C316S), and Pex12 (C339S/C342S) had growth defects in oleate (Fig. 5A) (as well as in methanol, data not shown), indicating that Pex2, Pex10, and Pex12 are required for peroxisome biogenesis. After overnight induction in oleate, the Pex2, Pex10, and Pex12 RING domain point mutants were more stable than their expressed counterparts in the pex2 deletion strain and in vivo ubiquitination assay, in the absence of Pex4 (Fig. 5C). Note in particular the disappearance of the di-Ub band for Pex20 (K19R)-HA, which is typical of Cys8 ubiquitination (because it is DTT-sensitive) (Fig. 5D), as well as the disappearance of Pex20 (C8S)-HA polyubiquitination (Fig. 5E). However, the RING peroxin mutants produced the aberrant DTT-resistant mono ubiquitination band (Fig. 5D, right panel with DTT). Therefore, all three RING peroxins are essential for Pex20 monoubiquitination during recycling and for polyubiquitination during degradation.

Pex4 Is Required for Pex20 Mono- and Diubiquitination on Cys8—To determine the roles of E2 enzymes (i.e., Pex4 or Ubc4) in Pex20 ubiquitination, the PEX4 or UBC4 gene was deleted in the strains expressing Pex20 (C8S)-HA and Pex20 (K19R)-HA, respectively, in which His6-Myc-tagged Ub (K48R) was overexpressed. In the in vitro ubiquitination assay, in the pex4Δ deletion, Pex20 (K19R)-HA only showed a monoubiquitination band, which was likely to be on Cys8, because most, but not all, of this band was DTT-sensitive (Fig. 6A, compare the second and fourth lanes). The small amount of DTT-resistant monoubiquitination of the Pex20 (K19R)-HA remaining in the pex4Δ deletion (Fig. 6A, fourth lane) corresponds to the aberrant ubiquitination described earlier using Pex20 (C8S/K19R)-HA and is produced only when both the receptor recycling and RADAR pathways are nonfunctional. Although the loss of the diubiquitinated Pex20 (K19R)-HA in the pex4Δ deletion (Fig. 6A, first and second lanes), suggested that Pex4 is necessary for the mono- and diubiquitination at Cys8 on Pex20, we were surprised by the appearance of monoubiquitination (Fig. 6A, second lane) on Cys8 (suggested by DTT sensitivity of most of this species, Fig. 6A, fourth lane), in the absence of Pex4. This must mean that some other Ubc is capable of monoubiquitination of Cys8 when Pex4 is absent. Our experiments suggest that this monoubiquitination on Cys8 by an alternative Ubc is a peculiar situation that we have uncovered only when the RADAR pathway, which is the normal preferred pathway that is activated in pex4Δ cells, is crippled by mutation of the polyubiquitination site (Lys19) necessary for the RADAR pathway, and this monoubiquitination is seen only after overnight growth of the cells on oleate (i.e., it is a slow process). This result suggests that in wild-type cells, Pex4 is necessary for the mono- and diubiquitination on Cys8 (Fig. 6A); when Pex4 is absent (or when C8S is used), the preferred pathway is that Pex20 is polyubiquitinated on Lys19 and degraded rapidly by the RADAR pathway (Fig. 3A), but when Pex4 is absent and the RADAR pathway is inactivated, only then is it possible for some other redundant Ubc to substitute for Pex4, to put just a mono-Ub on Cys8, and with much lower efficiency on another DTT-resistant site (Fig. 6A, second and fourth lanes).

Because the overexpression of Ub (K48R) slows down the proteasomal degradation and causes the accumulation of polyubiquitination of Pex20 (C8S)-HA (7), we further checked the effect of Pex4 on Pex20 (C8S)-HA polyubiquitination at shorter time points (from 1 to 3 h) (Fig. 6B) and found that Pex20 (C8S)-HA polyubiquitination was delayed at a shorter time point (3 h) in the pex4Δ strain and that there was more of the DTT-resistant mono- and diubiquitated species, relative to the wild-type strain (Fig. 6B), indicating that although Pex4 is not essential, the pex4Δ deletion does slow the Pex20 polyubiquitination.

To understand the delay of Pex20 (C8S)-HA polyubiquitination in the pex4Δ deletion, we checked its stability in the pex4Δ deletion strain without overexpressing Ub (K48R). Interestingly, after overnight induction in oleate, Pex20 (C8S)-HA was stabilized in the pex4Δ cells and gave the DTT-resistant mono/ diubiquitination bands, whereas Pex20 (C8S)-HA was completely degraded by the RADAR pathway in the presence of Pex4 (Fig. 6C). This mono/diubiquitination is likely on Lys19 because these DTT-resistant bands disappear in Pex20 (K19R)-HA (Fig. 6A, fourth lane). Thus, Pex4 is required for efficient polyubiquitination of Pex20 (C8S)-HA after some Ubc, other than Pex4, has initiated mono- and diubiquitina-
tion at Lys19. The action of redundant cellular Ubcs that perform the DTT-resistant mono- and di-Ub additions on Pex20 (C8S)-HA and the nonessential role of Pex4 in polyubiquitination account for the stabilization of the Pex20 (C8S)-HA (Fig. 6C) and its slower polyubiquitination in the pex4/-H9004 strain (Fig. 6B). Additionally, the ubc4/- deletion did not affect Pex20 (K19R)-HA monoubiquitination or Pex20 (C8S)-HA polyubiquitination (data not shown), indicating either that Ubc4 is not required for Pex20 mono/polyubiquitination or that other substitute/redundant E2 enzymes might be involved.

Overall, Pex4 is required not only for Pex20 mono/diubiquitination on its Cys8 during its recycling, but also for a step in the polyubiquitination of Pex20 at Lys19. However, Pex4 is not obligatory for Pex20 polyubiquitination, because other Ubcs can substitute for it, albeit less efficiently. This role of Pex4 in polyubiquitination of other receptors, such as Pex5 and Pex18, may have been missed or alternatively may be peculiar to Pex20.

Pex7 Is Only Required for Pex20 Polyubiquitination—To check the requirement of Pex7 in Pex20 mono/polyubiquitination, the PEX7 gene was deleted in the strains expressing Pex20 (C8S)-HA and Pex20 (K19R)-HA, in which His6-Myc-tagged Ub (K48R) was overexpressed. In the in vivo ubiquitination assay, the pex7/- deletion did not affect the DTT-sensitive mono- or diubiquitination of Pex20 (K19R)-HA (Fig. 7A) but affected Pex20 (C8S)-HA polyubiquitination (Fig. 7B), indicating that Pex7 is only required for Pex20 polyubiquitination. In a previous study, mutation of the conserved Ser residue (S280F) in Pex20 disrupted the interaction with Pex7 (7). We also checked the ubiquitination of Pex20 (C8S/S280F)-HA in which His6-Myc-tagged Ub (K48R) was overexpressed,
which showed the same loss of polyubiquitination (Fig. 7B), indicating that the interaction of Pex7 and Pex20 is required for Pex20 polyubiquitination.

After overnight induction in oleate, Pex20 (C8S)-HA was stabilized in the pex7Δ strain (Fig. 7C), whereas Pex20 (C8S/S280F)-HA was also more stable as compared with Pex20 (C8S)-HA (Fig. 7C), which is consistent with the finding that Pex7 is essential for Pex20 polyubiquitination during its degradation. The protease protein assay performed on the P200 fraction from Pex20 (C8S)-HA/Δpex7Δ strain showed that the import of Pex20 (C8S)-HA is not impacted by the Δpex7Δ deletion (Fig. 7D).

Overall, we conclude that Pex7 itself and the interaction of Pex7 with Ser280 on Pex20 are not required for Pex20 import into peroxisomes but are indeed necessary either for Pex20 export or polyubiquitination. However, because Pex20 mono-ubiquitination, which also requires Pex20 import and export, does not require Pex7, it is most likely that Pex7 is required specifically for Pex20 polyubiquitination and degradation by the RADAR pathway, which is a novel finding.

**DISCUSSION**

**Pex20 Mono/Diubiquitination and Recycling Depend on Cys8**—Pex20 acts as a co-receptor for Pex7 and is required for the import of PTS2 cargos into the peroxisomal matrix. Ubiquitination of Pex20 was first observed in the P. pastoris pex4Δ deletion strain or in cells overexpressing Ub (K48R) (7). At the N terminus of Pex20, the conserved Lys19 is required for polyubiquitination and degradation via the RADAR pathway, whereas Cys8 is required for recycling (7). However, in this earlier study, monoubiquitination was not found (15). Pex20-GFP, used in this previous study and driven by a 0.5-kb endogenous promoter, only partially complements the pex20Δ deletion strain and did not allow us to find the monoubiquitination site (7). In this study, we expressed Pex20-HA and its mutants under the control of a longer endogenous promoter (1.5 kb), which allowed us for the first time to detect the DTT-sensitive mono/diubiquitination of Pex20 on Cys8 (Fig. 3A). The two types of ubiquitination of Pex20, dependent on the conserved Cys8 (for DTT-
sensitive mono/diubiquitination) and Lys19 (for DTT-resistant polyubiquitination) (Fig. 3A) are consistent with what was found in Pex5 and Pex18 ubiquitination, suggesting that the N-terminal conserved cysteine and lysine(s) have the same function in Pex20, Pex5, and Pex18 ubiquitination during the recycling and degradation. These receptors/co-receptors therefore appear to have co-evolved similar strategies for the recycling and degradation.

**Pex2, Pex10, and Pex12 Function Nonredundantly as E3 Ub Ligases for Both Mono- and Polyubiquitination of Pex20**—The roles of RING peroxins in ubiquitination of Pex5 were first determined in *S. cerevisiae* in vitro. In this study, we analyzed the stabilities of RING peroxins in strains deleted for the corresponding genes or with point mutations in their RING domains, as well as their effect on Pex20 ubiquitination (Fig. 5). Pex2, Pex10, and Pex12 RING domain double point mutants all have strong growth defects on oleate and impair the mono/polyubiquitination of Pex20. We conclude that in *P. pastoris*, Pex2, Pex10, and Pex12 are all required for Pex20 mono/polyubiquitination during its recycling and degradation. The same requirement of RING peroxins in Pex5 ubiquitination was also found in vivo (Fig. 8). This is the first in vivo finding of the requirements of three RING peroxins in mono/polyubiquitination of the PTS receptors/co-receptors. This is different from the previous *in vitro* studies, which showed that ScPex12 is required for ScPex5 monoubiquitination (18), whereas ScPex10 (22) or ScPex2 (18) is required for ScPex5 polyubiquitination.

**Pex4 Facilitates Both Mono- and Polyubiquitination of Pex20 but Is Not Essential for the Latter**—In *S. cerevisiae*, monoubiquitination of the receptor on a cysteine is facilitated by the E2 protein Pex4, whereas the Pex4-like UbcH5a/b/c does this in humans (11, 14, 18). In *S. cerevisiae*, polyubiquitination of the receptor on lysine(s) is not Pex4-dependent (18). Interestingly, we demonstrated here that Pex4 not only facilitates the Pex20 mono/diubiquitination on Cys8 but also affects the efficiency of Pex20 polyubiquitination on Lys19 (Fig. 6). Another E2 enzyme, Ubc4, was required for polyubiquitination of Pex5 in *S. cerevisiae* (14, 18, 40); however, the deletion of the *P. pastoris* UBC4 gene did not abolish the mono- or polyubiquitination of Pex20 (data not shown). A role for Ubc4 would be missed in our analysis if a redundant protein fulfilled its function.

**Pex7 Is Only Required for Pex20 Polyubiquitination**—Pex7, the PTS2 cargo receptor, interacting with Pex20 during cargo import, as well as its interaction with Pex20 through Ser280, are required for Pex20 polyubiquitination (Fig. 7), indicating a new role for Pex7 in Pex20 degradation via the RADAR pathway. Because Pex7 is not necessary for Pex20 mono/diubiquitination on Cys8, which requires Pex20 import into and export from peroxisomes during the import cycle, we conclude that Pex7 is required for the actual polyubiquitination of Pex20 and its subsequent degradation by the RADAR pathway. Pex7 is believed to shuttle between the cytosol and peroxisomal lumen in an “extended shuttle” model (41), like Pex5. However, no evidence

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**FIGURE 7. Pex7 is only required for Pex20 polyubiquitination.** A, the pex7Δ deletion did not affect Pex20 (K19R)-HA mono/diubiquitination. The strains used were slxq73 and slxq140. B, the S280F mutation or pex7Δ deletion affected Pex20 (C8S)-HA polyubiquitination. The strains used were slxq68, slxq57, and slxq139. C, the S280F mutation or the pex7Δ deletion stabilized Pex20 (C8S)-HA. The strains used were slxq53, slxq59, slxq66, and slxq63. D, subcellular fractionation and protease protection assay of the P200 fraction isolated from a PNS of Pex20 (C8S)-HA + pex7Δ and Pex20-HA. The strains used were slxq59 and slxq63.
exists to show that Pex7 recycling and degradation are dependent on ubiquitination.

A Model for the Pex20 Ubiquitination Pathway—We propose a schematic model for Pex20 ubiquitination during the peroxisomal matrix protein import cycle based on our findings (Fig. 9). As a PTS2 cargo co-receptor, Pex20, interacts with Pex7 during PTS2 cargo import. After cargo release, Pex20 would be exported back to the cytosol for the next round of import. The mono/diubiquitination of Pex20 occurs on Cys8, and this, without requiring interaction with Pex7, facilitates receptor recycling. We do not know whether the mono/diubiquitination of Pex20 precludes its association with Pex7 or whether recycling is simply the kinetically favored pathway under these circumstances. Alternatively, when the receptor recycling machinery is impaired, Pex20 binding to Pex7 is necessary for its polyubiquitination on Lys19 and subsequent degradation by the RADAR pathway.

Pex2, Pex10, and Pex12 function as E3 Ub ligases, which are all required for both mono- and polyubiquitination of Pex20. Pex4 functions as E2 ubiquitin-conjugating enzyme, which facilitates the first (and second) Ub to Pex20 during its mono/ diubiquitination, as well as for the initiation or elongation step, during the polyubiquitination process. However, whereas Pex4 is required in an obligatory fashion for Pex20 monoubiquitination, in its absence other Ubcs can assume its role, albeit less efficiently, to allow Pex20 polyubiquitination. The Ub hydrolase, Ubp15 from S. cerevisiae and USP9X from mammalian cells, are capable of removing Ub from ubiquitinated Pex5 (42, 43). However, the deletion of the P. pastoris UBP15 gene did not impair mono- or polyubiquitination of Pex20 (data not shown).

FIGURE 8. All three RING peroxins are required for both mono- and polyubiquitination of Pex5. A, the ubiquitination of Pex5-HA and its mutants in His6-Myc-Ub (K48R) overexpression strains. The strains used were slxq122, slxq123, slxq124, and slxq125. B, RING domain point mutations affect the ubiquitination of Pex5-HA and its mutants. The strains used were slxq122, slxq189, slxq190, slxq191, slxq124, slxq144, slxq145, slxq146, slxq123, slxq141, slxq142, and slxq143.

FIGURE 9. Model for Pex20 ubiquitination during its recycling and RADAR. The numbers denote Pex proteins assigned these designated numbers. C8 and K19 represent Cys8 and Lys19 on Pex20, respectively. Single or multiple circles with Ub denote mono- or poly-Ub on Cys8 or Lys19 of Pex20, respectively. Pex20 interacts with Pex7 during PTS2 cargo import. After cargo release, Pex20 is recycled back to the cytosol for the next round of import. The mono- and diubiquitination of Pex20 occurs on Cys8, and its recycling does not require Pex7 or interaction between Pex20 and Pex7. When the recycling machinery is impaired by mutation, Pex20 interaction with Pex7 and its subsequent polyubiquitination on Lys19 are necessary for Pex20 degradation (Pex20 cloud) by the ubiquitin proteasome system (UPS). Pex2, Pex10, and Pex12 function as E3 ubiquitin ligases, which are required for both mono- and polyubiquitination of Pex20. Pex4 functions as the E2 ubiquitin-conjugating enzyme, which is not only required for Pex20 mono- and diubiquitination on Cys8 during its recycling but also as a nonessential kinetic component for a step in the polyubiquitination of Pex20 on Lys19.
shown), suggesting that other deubiquitinating enzymes may perform this function in *P. pastoris* cells.

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