THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 280, No. 3, Issue of January 21, pp. 1921–1930, 2005
Printed in U.S.A.

Ubiquitination of the Peroxisomal Targeting Signal Type 1 Receptor, Pex5p, Suggests the Presence of a Quality Control Mechanism during Peroxisomal Matrix Protein Import*

Received for publication, April 1, 2004, and in revised form, October 27, 2004
Published, JBC Papers in Press, November 9, 2004, DOI 10.1074/jbc.M403632200

Jan A. K. W. Kiel‡§, Kerstin Emmrich‡¶, Helmut E. Meyer‖, and Wolf-H. Kunau‡¶

From the ‡Abteilung für Zellbiochemie and the ‖Medical Proteom Center, Medizinische Fakultät der Ruhr-Universität Bochum, D-44780 Bochum, Germany

***PEX* genes encode proteins (peroxins) that are required for the biogenesis of peroxisomes. One of these peroxins, Pex5p, is the receptor for matrix proteins with a type 1 peroxisomal targeting signal (PTS1), which shuttles newly synthesized proteins from the cytosol into the peroxisome matrix. We observed that in various *Saccharomyces cerevisiae pex* mutants disturbed in the early stages of PTS1 import, the steady-state levels of Pex5p are enhanced relative to wild type controls. Furthermore, we identified ubiquitinated forms of Pex5p in deletion mutants of those *PEX* genes that have been implicated in recycling of Pex5p from the peroxisomal membrane into the cytosol. Pex5p ubiquitination required the presence of the ubiquitin-conjugating enzyme Ubc4p and the peroxins that are required during early stages of PTS1 protein import. Finally, we provide evidence that the proteasome is involved in the turnover of Pex5p in wild type yeast cells, a process that requires Ubc4p and occurs at the peroxisomal membrane. Our data suggest that during receptor recycling a portion of Pex5p becomes ubiquitinated and degraded by the proteasome. We propose that this process represents a conserved quality control mechanism in peroxisome biogenesis.**

Peroxisomes are vital cell organelles and may contain highly variable sets of enzymes that control many important cellular processes. Their importance is demonstrated by the discovery of a number of inherited human metabolic disorders, with the prototype being Zellweger Syndrome, that have been associated with peroxisomal defects, varying from the non-functioning of a single peroxisomal enzyme to complete absence of the organelle (reviewed in Ref. 1). Peroxisomal enzymes are synthesized in the cytosol and delivered post-translationally to their target organelle. To enable this sorting, these enzymes contain specific peroxisomal targeting signals (PTS),[1] most of which fall into two categories (reviewed in Ref. 2). The vast majority of proteins contains a signal (PTS1) that is located at the carboxyl terminus and has a consensus sequence related to the canonical -S-K-L-COOH sequence observed in firefly luciferase (3). So far, only a few proteins have been discovered that utilize a PTS2 to enter peroxisomes. This signal, first described for rat 3-ketoacyl-CoA thiolase (4), is located at the amino terminus of proteins and has the consensus sequence (R/K)(L/V/I)X_5(H/Q)(L/A). Additionally, certain proteins contain neither a PTS1 nor a PTS2, but are sorted via still unidentified signals. Finally, because folded and multimeric proteins are also imported into peroxisomes, an alternative strategy that is used by certain proteins is to hitch-hike into the organelle by binding to a protein containing a PTS (see *e.g.* Refs. 5 and 6).

For both peroxisomal targeting signals, separate cytosolic receptor molecules have been discovered, Pex5p for the PTS1, and Pex7p for the PTS2 (reviewed in Ref. 2), which appear to have a shuttling function. During the receptor cycle, Pex5p binds cargo proteins in the cytosol, sorts these to the surface of the organelle, and, subsequently, assists in transporting them across the membrane in a hitherto unknown fashion. Recent evidence suggests that the PTS1 receptor may actually accompany the cargo protein into the peroxisome, prior to its release into the lumen of the organelle (7). Finally, the receptor is brought back to the cytosol for a new import cycle, a step that has been demonstrated to require ATP hydrolysis (8).

Previous investigations have identified a variety of other proteins directly involved in the biogenesis of peroxisomes (termed peroxins; see Ref. 9; reviewed in Ref. 2). Based on these data, specific peroxins have been suggested to function at distinct steps during peroxisome biogenesis (*cf.* 10). Peroxins that are required for the formation/maintenance of the peroxisomal membrane are Pex3p and Pex19p (11). Furthermore, a docking/translocation complex at the peroxisomal membrane has been proposed that initiates binding of cargo-loaded Pex5p molecules and, subsequently, facilitates translocation of the cargo into the peroxisomal matrix (1, 2). This large complex has been shown to consist of two subcomplexes: a docking complex, comprising Pex13p, Pex14p, and Pex17p, and a putative translocation complex consisting of Pex2p, Pex10p, and Pex12p. These complexes are presumed to be held together by protein-protein interactions via the peroxins Pex3p or Pex8p (12, 13). The intraperoxisomal peroxin Pex8p, which has so far only been discovered in yeast species, appears also to have a more direct role in matrix protein import by releasing PTS1-carrying cargo from Pex5p molecules (14, 15). Finally, a number of interacting proteins have been suggested to play a role in recycling of the empty receptor to the cytosol. These include two interacting ATPases of the AAA family, Pex1p and Pex6p. In *Saccharomyces cerevisiae*, these ATPases are bound to the peroxisomal membrane by a

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported by the Sonderforschungsbereiche (Grants SFB394 and SFB480). To whom correspondence should be addressed (present address): Eukaryotic Microbiology, GBB, University of Groningen, Kerklaan 30 NL-9751 NN Haren, The Netherlands. Tel.: 31-50-363-2218; Fax: 31-50-363-2154; E-mail: J.A.K.W.Kiel@Biol.RUG.nl.

¶ Supported by the Sonderforschungsbereiche (Grant SFB480/Teilprojekt B1) and by the Deutsche Forschungs Gemeinschaft (DFG Grant Ku 329/17-5).

[1] The abbreviations used are: PTS, peroxisomal targeting signal; protA, protein A; MALDI, matrix-assisted laser desorption ionization.

physical interaction between Pex6p and the peroxisomal membrane protein Pex15p (see Refs. 16 and references therein). In humans, a protein only structurally related to Pex15p, which was designated Pex26p, appears to perform a similar function (17). Additionally, two physically interacting peroxins that have so far only been found in yeast species, the ubiquitin-conjugating enzyme Pex4p/Ubc10p and the integral membrane protein Pex22p (18), are thought to play a role in Pex5p recycling (10, 19). However, so far the target protein to which Pex4p actually conjugates ubiquitin has remained elusive.

Despite a wealth of knowledge about which factors are involved in PTS1 matrix protein import, little is actually known what happens to the receptor Pex5p during this process. Recently, it was observed in the yeast *Pichia pastoris*, in human cell lines, and in plant cells that the loss of one of the peroxins proposed to function in receptor recycling, Pex1p, Pex4p, Pex6p, or Pex22p, leads to strongly reduced steady-state levels of Pex5p (18, 20, 21). This phenomenon has actually been used to determine the order in which a number of peroxins supposedly act in matrix protein import (10). To investigate whether this process also occurs in *S. cerevisiae*, we determined the steady-state levels of Pex5p in various *pex* mutant strains of this yeast species. Surprisingly, we observed that in mutants of those *PEX* genes that are directly implicated in PTS1 import, steady-state Pex5p levels are enhanced as compared with wild type cells. Furthermore, we found that the PTS1 receptor is ubiquitinated when one of the peroxins proposed to be required for Pex5p recycling was absent. Our data suggest the presence of a possible quality control mechanism at the Pex5p export site that determines whether the receptor will be recycled to the cytosol to perform another round of import or will be turned over by the proteasome.

EXPERIMENTAL PROCEDURES

Strains, Media, and Growth Conditions—Yeast strains used in this study are listed in Table I, and are derivatives of *S. cerevisiae* wild type UTL7A unless indicated otherwise (22). Deletion mutants were constructed using the KanMX-Marker (23). Strains in which the genomic copy of the *PEX5* gene was replaced by a *PEX5-ProtA* fusion gene were obtained by transforming haploid yeast cells with a PCR product according to Knop *et al.* (24). The sequences of oligonucleotide primers used for the construction of these strains are available upon request.

Complete (YPD) and glucose minimal media (SD) used for yeast culturing have been described previously (25). 0.1 mM $CuSO_4$ was added to SD medium in all experiments that required induction of the *CUP1* promoter. In galactose minimal medium, 2% galactose replaced the glucose in SD medium. Oleate induction medium contained 0.67% yeast nitrogen base without amino acids, 0.1% glucose, 0.1% oleic acid, 0.05% Tween 40, and 0.1% yeast extract, adjusted to pH 6.0. Oleate plates contained 0.67% yeast nitrogen base without amino acids, 0.1% oleic acid, 0.5% Tween 40, 0.1% yeast extract, adjusted to pH 6.0, and supplemented with 2.4% granulated agar.

Plasmid Constructions—The plasmids used in this study are listed in Table II. For the construction of plasmids pBKK8 and pBKK9, the 1.1-kb BamHI-(partial EcoRI) fragments from YEp105 and pUB203, respectively, were inserted between the BamHI and EcoRI sites of the polylinker of YEp352.

Biochemical Methods—Cell extracts were prepared as follows: Cells were grown to the end of the logarithmic growth phase on glucose minimal medium, galactose minimal medium, or oleate induction medium. Subsequently, the OD_{660} was determined and 3 OD_{660} units of cells were precipitated with 12.5% trichloroacetic acid, a treatment that completely destroys all proteolytic activity. After at least 30-min incubation at −80 °C, cells were precipitated and the cell pellet was washed twice with 80% acetone of −20 °C. Subsequently, the pellets were dried at room temperature and suspended in 80 μl of 1% SDS/0.1 M NaOH. Finally, after 30 min, 20 μl of 5× SDS-PAGE loading buffer (250 mM Tris-Cl, pH 6.8, 10% SDS, 25% β-mercaptoethanol, 50% glycerol, and 0.1% bromphenol blue) was added, and the samples were boiled for 5 min at 100 °C.

SDS-PAGE and Western blotting were carried out using established procedures. For Western blot analysis, equal amounts of protein were loaded per lane, which was controlled by Ponceau S staining of total protein on blots. Western blots were decorated with specific polyclonal antibodies against Pex5p, or monoclonal antibodies against ubiquitin (clone P4D1; a gift of T. Sommer, Max Delbrück Center, Berlin, Germany). Detection of proteins on Western blots was performed using the Enhanced Chemiluminescence system (Amersham Biosciences). Relative Pex5p levels were determined by laser densitometric scanning of Western blots decorated with specific antibodies against Pex5p.

Immunoprecipitation of Denatured Pex5p—Immunoprecipitation was carried out using acetone-washed and dried pellets of 3 OD_{660} units of trichloroacetic acid precipitated yeast cells for each reaction. The pellet was suspended vigorously in 100 μl of urea cracking buffer (50 mM Tris-Cl, pH 7.5, 6 M urea, 1% SDS) and incubated for 10 min at 65 °C. Subsequently, 1 ml of Tween 20-IP buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.5% Tween 20, 0.1 mM EDTA) and 10 μl of 100 mg/ml bovine serum albumin were added. After pelletting undissolved material, α-Pex5p antiserum was added, and the mixture was incubated under continuous swirling for 4 h at 4 °C. Subsequently, 75 μl of pre-swollen Protein A-Sepharose beads were added, and the mixture was further incubated for 1 h at 4 °C. The immunoprecipitated material was subsequently pelleted, washed two times with Tween 20-IP buffer, once with Tween 20-urea buffer (100 mM Tris-Cl, pH 7.5, 200 mM NaCl, 2 M urea, 0.5% Tween 20), and once with Tris-buffered saline buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl). Finally, the beads were boiled in 50 μl of IP-sample buffer (125 mM Tris-Cl, pH 6.8, 6% SDS, 10% β-mercaptoethanol, 20% glycerol, 0.1% bromphenol blue) and prepared for Western blotting.

Differential Centrifugation—Spheroplasting of yeast cells, homogenization, and differential centrifugation at 100,000 × *g* of homogenates to separate organelles and other membranous structures from cytosolic proteins was performed essentially according to Erdmann *et al.* (25).

Purification of Pex5p-protA from S. cerevisiae pex4 PEX5-protA—1 liter of yeast cells, cultivated to the end of the exponential growth phase on glucose minimal medium, was used for each experiment. The cells were pelleted by centrifugation, washed with MilliQ water, and suspended in 20 ml of 12.5% trichloroacetic acid. After overnight precipitation at −80 °C, the cells were pelleted by centrifugation and washed twice with 80% acetone of −20 °C. After thorough drying, the pellet was solubilized in 4 ml of solubilization buffer (1% SDS, 0.1 M NaOH) by vigorous mixing with the aid of glass beads. Subsequently, the mixture was neutralized by the addition of 1 ml of 5× boiling buffer (250 mM Tris-Cl, pH 7.5, 10% SDS) and incubated for 5 min at 99 °C. After cooling to room temperature, 25 ml of the Tween 20-IP buffer was added. The mixture was centrifuged for 15 min at 6,000 × *g* to remove undissolved material. The resulting pellet was resuspended in 10 ml of Tween 20-IP buffer and, after thorough mixing, again centrifuged. Subsequently, both supernatants were combined, and the volume was brought up to 50 ml with Tween 20-IP buffer. The resulting extract contains fully denatured proteins and can if necessary be stored overnight at 4 °C.

Subsequently, the extract was loaded on a 0.25-ml column of IgG-Sepharose 6 Fast flow (Amersham Biosciences; pre-washed in wash buffer 1 (50 mM Tris-Cl, pH 7.6, 150 mM NaCl, 0.05% Tween 20)). After loading, the column was washed with 40 column volumes of wash buffer 1, followed by washing with 12 column volumes of wash buffer 2 (5 mM NH_4Ac, pH 5.0). Finally, the bound protA-containing proteins were eluted from the column using elution buffer (0.5 M acetic acid, pH 3.4). Eluate fractions of 0.5 ml were lyophilized and analyzed by SDS/PAGE and Western blotting.

Mass Spectroscopy—For mass spectroscopy, fractions with protA-containing proteins were separated by SDS-PAGE and stained with colloidal Coomassie Brilliant Blue G250, and the protein bands were excised from the gel. Excised bands were digested with trypsin at 37 °C overnight. The tryptic peptides were extracted and spotted on a target for MALDI mass fingerprint analysis. Measurements were done on a Bruker Reflex IV instrument. Fingerprint mass analysis was performed using the ProFound algorithm. For tandem mass spectrometry analysis the same instrument was employed acquiring the post-source delay spectrum from the desired parent ion.

RESULTS

Pex5p Is Modified in a Specific Set of S. cerevisiae pex Mutants—To investigate the steady-state levels of Pex5p in *S. cerevisiae*, we grew cells to the end of the logarithmic growth phase on glucose medium and determined Pex5p levels in wild type and *pex* mutant cells by quantification of immunoblots

TABLE I
S. cerevisiae strains used in this study

Strain	Characteristics	Reference
UTL7A	*MATα*, *ura3-52*, *trp1*, *leu2–3,112*	W. Duntze, Bochum, Germany
pex1	UTL7A *pex1*::*loxP*	This study
pex2	UTL7A *pex2*::*LEU2*	11
pex3	UTL7A *pex3*::KanMX4	11
pex4	UTL7A *pex4*::*LEU2*	29
pex5	UTL7A *pex5*::KanMX4	11
pex6	UTL7A *pex6*::KanMX4	11
pex7	UTL7A *pex7*::KanMX4	11
pex8	UTL7A *pex8*::*LEU2*	14
pex10	UTL7A *pex10*::*loxP*	This study
pex11	UTL7A *pex11*::*LEU2*	33
pex12	UTL7A *pex12*::*LEU2*	34
pex13	UTL7A *pex13*::KanMX4	35
pex14	UTL7A *pex14*::KanMX4	11
pex15	UTL7A *pex15*::*CreloxP*-KanMX4	This study
pex17	UTL7A *pex17*::*LEU2*	35
pex18	UTL7A *pex18*::KanMX4	36
pex19	UTL7A *pex19*::*CreloxP*-KanMX4	This study
pex21	UTL7A *pex21*::*CreloxP*-KanMX4	This study
pex22	UTL7A *pex22*::*CreloxP*-KanMX4	This study
pex1 pex2	UTL7A *pex2*::*LEU2 pex1*::*CreloxP*-KanMX4	This study
pex1 pex4	UTL7A *pex1*::*loxP pex4*::KanMX4	This study
pex1 pex5	UTL7A *pex1*::*loxP pex5*::*CreloxP*-KanMX4	This study
pex1 pex6	UTL7A *pex6*::*loxP pex1*::*CreloxP*-KanMX4	This study
pex1 pex7	UTL7A *pex7*::*LEU2 pex1*::*CreloxP*-KanMX4	This study
pex1 pex8	UTL7A *pex8*::*LEU2 pex1*::*CreloxP*-KanMX4	This study
pex1 pex13	UTL7A *pex13*::*URA3 pex1*::*CreloxP*-KanMX4	This study
pex1 pex15	UTL7A *pex15*::*loxP pex1*::*CreloxP*-KanMX4	This study
pex4 pex2	UTL7A *pex2*::*LEU2 pex4*::KanMX4	This study
pex4 pex3	UTL7A *pex3*::*LEU2 pex4*::KanMX4	This study
pex4 pex5	UTL7A *pex4*::*LEU2 pex5*::KanMX4	This study
pex4 pex6	UTL7A *pex6*::*loxP pex4*::KanMX4	This study
pex4 pex7	UTL7A *pex7*::*LEU2 pex4*::KanMX4	This study
pex4 pex8	UTL7A *pex8*::*LEU2 pex4*::KanMX4	This study
pex4 pex10	UTL7A *pex10*::*loxP pex4*::KanMX4	This study
pex4 pex11	UTL7A *pex11*::*LEU2 pex4*::KanMX4	This study
pex4 pex13	UTL7A *pex13*::*URA3 pex4*::KanMX4	This study
pex4 pex15	UTL7A *pex15*::*loxP pex4*::KanMX4	This study
pex4 pex22	UTL7A *pex22*::*loxP pex4*::KanMX4	This study
ubc1	UTL7A *ubc1*::*CreloxP*-KanMX4	This study
ubc2	UTL7A *ubc2*::*CreloxP*-KanMX4	This study
ubc4	UTL7A *ubc4*::*CreloxP*-KanMX4	This study
ubc5	UTL7A *ubc5*::*CreloxP*-KanMX4	This study
ubc6	UTL7A *ubc6*::*CreloxP*-KanMX4	This study
ubc7	UTL7A *ubc7*::*CreloxP*-KanMX4	This study
ubc8	UTL7A *ubc8*::*CreloxP*-KanMX4	This study
ubc11	UTL7A *ubc11*::*CreloxP*-KanMX4	This study
ubc12	UTL7A *ubc12*::*CreloxP*-KanMX4	This study
ubc13	UTL7A *ubc13*::*CreloxP*-KanMX4	This study
pex1 ubc1	UTL7A *pex1*::*loxP ubc1*::*CreloxP*-KanMX4	This study
pex1 ubc2	UTL7A *pex1*::*loxP ubc2*::*CreloxP*-KanMX4	This study
pex1 ubc4	UTL7A *pex1*::*loxP ubc4*::*CreloxP*-KanMX4	This study
pex1 ubc5	UTL7A *pex1*::*loxP ubc5*::*CreloxP*-KanMX4	This study
pex1 ubc6	UTL7A *pex1*::*loxP ubc6*::*CreloxP*-KanMX4	This study
pex1 ubc7	UTL7A *pex1*::*loxP ubc7*::*CreloxP*-KanMX4	This study
pex1 ubc8	UTL7A *pex1*::*loxP ubc8*::*CreloxP*-KanMX4	This study
pex1 ubc11	UTL7A *pex1*::*loxP ubc11*::*CreloxP*-KanMX4	This study
pex1 ubc12	UTL7A *pex1*::*loxP ubc12*::*CreloxP*-KanMX4	This study
pex1 ubc13	UTL7A *pex1*::*loxP ubc13*::*CreloxP*-KanMX4	This study
pex4 ubc1	UTL7A *pex4*::*LEU2 ubc1*::*CreloxP*-KanMX4	This study
pex4 ubc2	UTL7A *pex4*::*LEU2 ubc2*::*CreloxP*-KanMX4	This study
pex4 ubc4	UTL7A *pex4*::*LEU2 ubc4*::*CreloxP*-KanMX4	This study
pex4 ubc5	UTL7A *pex4*::*LEU2 ubc5*::*CreloxP*-KanMX4	This study
pex4 ubc6	UTL7A *pex4*::*LEU2 ubc6*::*CreloxP*-KanMX4	This study
pex4 ubc7	UTL7A *pex4*::*LEU2 ubc7*::*CreloxP*-KanMX4	This study
pex4 ubc8	UTL7A *pex4*::*LEU2 ubc8*::*CreloxP*-KanMX4	This study
pex4 ubc11	UTL7A *pex4*::*LEU2 ubc11*::*CreloxP*-KanMX4	This study
pex4 ubc12	UTL7A *pex4*::*LEU2 ubc12*::*CreloxP*-KanMX4	This study
pex4 ubc13	UTL7A *pex4*::*LEU2 ubc13*::*CreloxP*-KanMX4	This study
PEX5-protA	UTL7A ::*PEX5-ProtA*-KanMX4	This study
pex4 PEX5-protA	UTL7A *pex4*::*LEU2*::*PEX5-ProtA*-KanMX4	This study
cim3–1	*Mata cim3–1 ura3–52 leu2delta his3delta200*	30
cim3–1 pex4	*cim3–1 pex4*::KanMX4	This study
cim3–1 pex10	*cim3–1 pex10*::KanMX4	This study
cim3–1 ubc4	*cim3–1 ubc4*::*CreloxP*-KanMX4	This study

TABLE II
Plasmids used in this study

Plasmid	Characteristics	Reference
pEMBLyex4	*E. coli*/*S. cerevisiae* shuttle plasmid, contains *GAL* promoter; Amp^R, *URA3*	37
pEMBLyex4-PAS2	pEMBLyex4 expressing the wild type *PEX4* gene	29
pEMBLyex4-$PAS2^{Ser}$	pEMBLyex4 expressing the inactive *PEX4-C115S* gene	29
pRS416	*E. coli*/*S. cerevisiae* shuttle vector, CEN, *URA3*, Amp^R	38
pRSpas1-Glu-744	pRS416 expressing *PEX1* K744E	28
YEp352	*E. coli*/*S. cerevisiae* shuttle vector 2-μm; *URA3*, Amp^R	39
YEp105	YEp46 derivative; contains expression Cassette P*cup1*·MYC-UB·T*cyc1*; 2-μm, Amp^R, *TRP1*	T. Sommer, MDC,[a] Berlin, Germany
pBKK8	YEp352 with expression cassette P*cup1*·MYC-UB·T*cyc1*	This study
pUB203	YEp46 derivative; contains expression Cassette P*cup1*·UB.K48R·T*cyc1*; 2-μm, Amp^R, *TRP1*	T. Sommer, MDC,[a] Berlin, Germany
pBKK9	YEp352 with expression cassette P*cup1*·UB.K48R·T*cyc1*	This study

[a] *MDC,* Max-Delbrück Center.

(Fig. 1*A*). The results indicate that in mutant cells affected in either the formation of the peroxisomal membrane (*pex3* and *pex19*), or in peroxisomal matrix protein import (*pex2*, *pex8*, *pex10*, *pex12*, *pex13*, and *pex14*), Pex5p levels were much increased compared with wild type control cells. In contrast, in mutant cells that display no PTS1 import defect (*e.g. pex11*, *pex18*, and *pex21*), Pex5p levels remained rather similar to those observed in wild type cells. Also in *pex* mutants proposed to be disturbed in receptor recycling (*pex1*, *pex4*, *pex6*, *pex15*, and *pex22*), Pex5p levels were rather close to wild type levels. Therefore, the strong reductions in Pex5p levels observed in certain *P. pastoris*, human, and plants *pex* mutants (18, 20, 21) are apparently not observed in *S. cerevisiae pex* mutants. Essentially identical results were observed when the cells were grown on oleate (data not shown).

Notably, in five *pex* mutants (*pex1*, *pex4*, *pex6*, *pex15*, and *pex22*), we observed, in addition to the normal Pex5p protein band, α-Pex5p immunoreactive bands of higher M_r (Fig. 1*B*). These bands were most prominent in *pex4* and *pex22* cells and less abundant in *pex1*, *pex6*, and *pex15* cells, but were never seen in *pex5* cells, strongly suggesting that they indeed represent modified Pex5p molecules. Similar modification bands were also seen with oleate-induced cells (data not shown). Notably, the modification pattern of these α-Pex5p immunoreactive bands appeared to fall into two classes (Fig. 1*B*), which correlated well with the two interaction groups to which the corresponding peroxins belong (Pex4p/Pex22p and Pex1p/Pex6p/Pex15p (16, 18)). Currently, we can not explain this difference in modification pattern.

Pex5p Is Ubiquitinated in pex1 and pex4 Mutants—We set out to identify the nature of the modifications of Pex5p observed in these specific *pex* mutants. First, we analyzed the possibility that the additional bands represent phosphorylated forms of Pex5p. However, dephosphorylation of immunoprecipitated Pex5p did not affect the presence of the bands (data not shown). Also, a drastic change in conformation of the PTS1 receptor Pex5p may influence its behavior on SDS/polyacrylamide gels. However, iodoacetamide treatment of samples, which should result in a more efficient unfolding of proteins, had no effect on the presence of the modifications (data not shown). To investigate whether Pex5p was modified by ubiquitination, we transformed cells of selected *pex* mutants with a plasmid that enables synthesis of an N-terminally Myc epitope-tagged form of ubiquitin. When a Myc-tagged ubiquitin moiety (calculated molecular mass of 10 kDa) instead of wild type ubiquitin (8.5 kDa) becomes conjugated to a target protein, a shift toward a higher molecular weight is observed. Analysis of the transformants indicated that this indeed occurred with the modified Pex5p bands (shown for *pex1* and *pex4* in Fig. 2, compare *lanes 1* and *4* with *lanes 2* and *5*). Synthesis of Myc-tagged ubiquitin did not result in any detectable modification

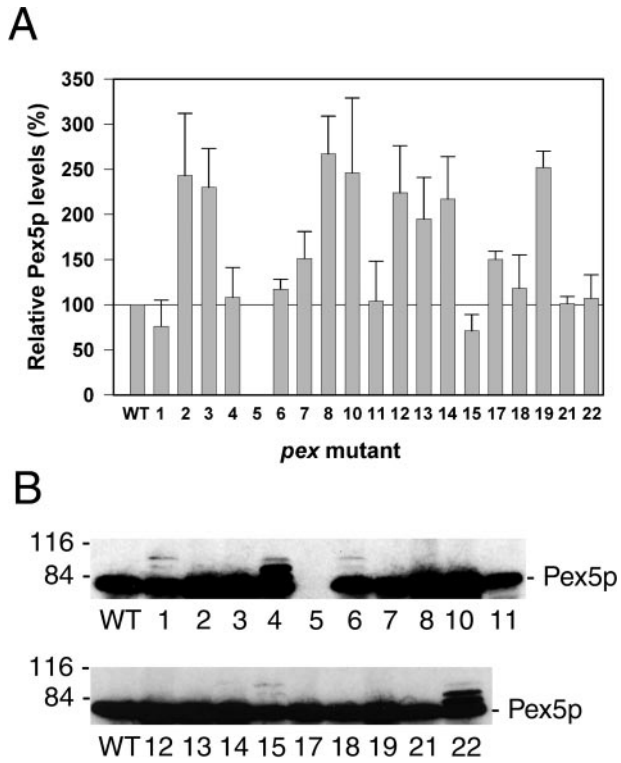

FIG. 1. **Steady-state levels of Pex5p.** *A*, the steady-state levels of Pex5p are enhanced in *pex* mutant cells affected in PTS1 import. Trichloroacetic acid extracts were prepared from glucose-grown cells of *S. cerevisiae* wild type and *pex* mutant cells. Equal amounts of protein were separated by SDS-PAGE and immunoblotted using antibodies specific for *S. cerevisiae* Pex5p. Subsequently, Pex5p levels were quantified by laser densitometric scanning of Western blots. Three independent samples were analyzed by Western blotting and averaged. Relative Pex5p levels are expressed as percentages of the values obtained for wild type cells, which was set to 100%. *B*, Pex5p is modified in *pex1*, *pex4*, *pex6*, *pex15*, and *pex22* mutant cells. Trichloroacetic acid extracts were prepared from glucose-grown cells of *S. cerevisiae* wild type and *pex* mutant cells. Equal amounts of protein were separated by SDS-PAGE, and Western blots were prepared and developed using *S. cerevisiae* Pex5p antibodies as described in *panel A*. To enable visualization of α-Pex5p-specific bands of higher molecular weight than Pex5p, films were overexposed. The values to the *left* of the blots indicate marker proteins (sizes in kilodaltons). In both panels: *WT*, wild type; the *numbers* refer to the respective *S. cerevisiae pex* mutants listed in Table I.

of Pex5p in wild type cells. Furthermore, overproduction of untagged ubiquitin did not affect Pex5p levels nor its modification. These data are indicative of Pex5p ubiquitination in this specific set of *pex* mutants.

Additional proof for this hypothesis was obtained by immunoprecipitation of Pex5p from the relevant *pex* mutants

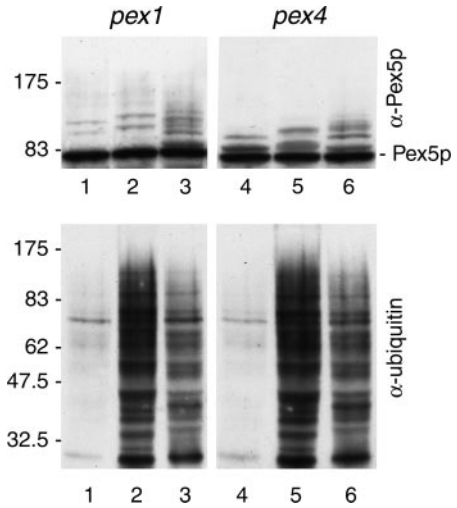

FIG. 2. **Overproduction of modified forms of ubiquitin demonstrates ubiquitination of Pex5p in *S. cerevisiae pex1* and *pex4* mutants.** *S. cerevisiae pex1* and *pex4* cells were transformed with plasmids pBKK8, expressing *MYC-UB*, and pBKK9, expressing *UB.K48R*, using vector YEp352 as a control. Transformants were grown to the end of the exponential growth phase in selective glucose minimal medium in the presence of 0.1 mM $CuSO_4$ to induce expression of the ubiquitin genes by the *CUP1* promoter. Subsequently, Western blots were prepared from trichloroacetic acid extracts, with equal amounts of protein loaded per lane. The blots were decorated with specific antibodies against *S. cerevisiae* Pex5p (*top panels*) or monoclonal antibodies against bovine ubiquitin to demonstrate ubiquitin overproduction (*lower panels*). *Lane 1*, *pex1* (YEp352); *lane 2*, *pex1* (pBKK8); *lane 3*, *pex1* (pBKK9); *lane 4*, *pex4* (YEp352); *lane 5*, *pex4* (pBKK8); and *lane 6*, *pex4* (pBKK9). The values to the *left* of the blots indicate sizes of marker proteins (in kilodaltons).

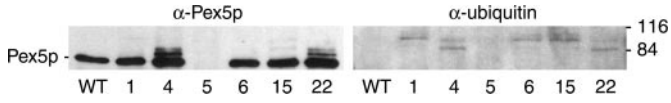

FIG. 3. **Immunoprecipitation of Pex5p from cells of specific *S. cerevisiae pex* mutants demonstrates ubiquitination of the PTS1 receptor.** *S. cerevisiae* wild type cells and cells of the indicated *pex* mutants were grown on glucose minimal medium to the late exponential growth phase. Cells were harvested, and Pex5p was immunoprecipitated as described under "Materials and Methods." Equal volumes of immunoprecipitates were loaded on SDS/polyacrylamide gels and Western blots were prepared. Blots were decorated with specific antibodies against *S. cerevisiae* Pex5p (*left panel*) or monoclonal antibodies against bovine ubiquitin (*right panel*). The *left panel* shows that Pex5p can indeed be immunoprecipitated. It must be noted that some of the α-Pex5p specific bands of higher molecular weight than Pex5p can only be visualized upon extreme overexposure (not shown). Nevertheless, in *pex1*, *pex4*, *pex6*, *pex15*, and *pex22* mutants the ubiquitin antibodies recognize specific protein bands in the α-Pex5p immunoprecipitates. *WT*, wild type; the *numbers under the blots* refer to the *S. cerevisiae pex1*, *pex4*, *pex5*, *pex6*, *pex15*, and *pex22* mutants, respectively.

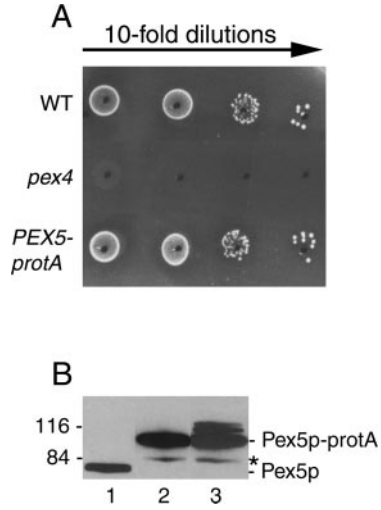

FIG. 4. **Analysis of Pex5p-protA species.** *A*, addition of the protein A tag to Pex5p does not significantly affect peroxisome biogenesis in *S. cerevisiae*. Cells of the indicated strains were grown overnight on glucose minimal media. Subsequently, dilutions were prepared, and 2 μl of each dilution was spotted onto oleate plates. The plates were then incubated for 3–5 days at 30 °C and scored for the appearance of colonies. *B*, addition of the protein A tag to Pex5p has no effect on the formation of modified Pex5p molecules. Cells of wild type (*lane 1*), *PEX5-protA* (*lane 2*), and *pex4 PEX5-protA* (*lane 3*) were grown on glucose mineral medium to the late exponential growth phase and harvested. Trichloroacetic acid extracts were prepared and immunoblotted for Pex5p, using equal amounts of protein per lane. The values to the *left* of the blots indicate sizes of marker proteins (in kilodaltons). The *asterisk* indicates a minor degradation product of Pex5p-protA that is not observed in extracts of wild type cells.

followed by analysis of the precipitates by Western blotting using monoclonal antibodies raised against bovine ubiquitin. α-Pex5p-specific antibodies were used as control. The results (Fig. 3, *right panel*) show that in α-Pex5p precipitates from *pex1*, *pex6*, and *pex15* cells an identical pattern of proteins was detected with the ubiquitin antibodies, indicating the presence of ubiquitinated Pex5p species in these mutants. Similarly, in α-Pex5p precipitates from *pex4* and *pex22* cells a set of ubiquitinated proteins was observed, albeit with a different protein pattern than observed for *pex1*, *pex6*, and *pex15* cells. These patterns were reminiscent of the Pex5p modification patterns seen in the immunoblotting experiments (*cf.* Fig. 1*B*). However, the most abundant modified Pex5p species in *pex4* and *pex22* cells observed in those experiments (see Fig. 1*B*) was hardly detectable in the α-Pex5p precipitates.

To confirm that all modification bands in the *pex4* mutant indeed represented ubiquitinated species of Pex5p, we further analyzed these bands by mass spectroscopy. In baker's yeast, Pex5p is a low abundance protein that is highly unstable *in vitro*. To enable its purification and subsequent analysis, we constructed *S. cerevisiae* strains producing Pex5p carboxyl-terminally tagged with *Staphylococcus aureus* protein A. We first investigated whether addition of the tag had an effect on the function of Pex5p. Growth analysis indicated that a wild type strain producing Pex5p-protA instead of wild type Pex5p was equally capable of growing on oleate plates as wild type controls, indicating that peroxisome biogenesis was still functional (Fig. 4*A*). Additionally, Western blot analysis demonstrated that in *pex4* cells in which *PEX5* had been replaced by *PEX5-protA* the additional Pex5p-immunoreactive protein bands were still being synthesized (Fig. 4*B*). Subsequently, we purified denatured protein A-containing proteins from the *pex4 PEX5-protA* strain using IgG-Sepharose affinity chromatography (see "Materials and Methods"). The isolated proteins were separated by SDS-PAGE, and gels were stained with colloidal Coomassie. This revealed the expected pattern of three protein bands, with the lowest of the three being the most prominent (*cf.* Fig. 4*B*, *lane 3*), which were analyzed by mass spectroscopy (see "Materials and Methods"). The resulting mass fingerprint list identified Pex5p in all three bands. Moreover, in both minor bands of higher molecular weight, five additional parent masses (1523.8, 1345.9, 1267.7, 1067.2, and 1039.5 Da) were observed belonging to tryptic peptides from ubiquitin. To prove the identity of this post-translational modification the parent mass ($M+H^+$) at 1523.8 Da was further characterized by MALDI-post-source delay analysis, and the resulting fragment spectrum was analyzed by the SEQUEST algorithm. This procedure identified the amino acid sequence 30–42 (IQDKEGIPPDQQR) of yeast ubiquitin. Thus, we conclude that in *pex4* mutant cells, Pex5p becomes ubiquitinated. Most likely, in *pex4* (and probably *pex22*) cells the most prominent ubiquitinated

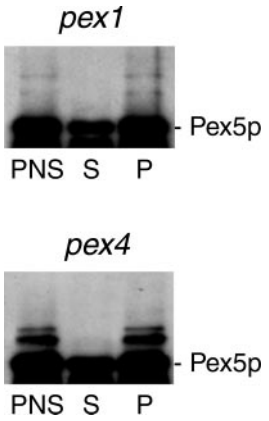

FIG. 5. **Ubiquitinated forms of Pex5p co-localize with organelles.** Subcellular distribution of ubiquitinated forms of Pex5p in *S. cerevisiae pex1* and *pex4* mutant cells. After subcellular fractionation equivalent volumes of the post-nuclear supernatant (*PNS*), 100,000 × *g* supernatant (*S*), and 100,000 × *g* pellet (*P*) fractions were analyzed by immunoblotting using specific antibodies against *S. cerevisiae* Pex5p.

form of Pex5p represents a mono-ubiquitinated species. It has been observed before that mono-ubiquitinated proteins are poorly recognized by monoclonal antibodies against bovine ubiquitin (*cf.* Ref. 26), which may explain why this prominent ubiquitinated species was hardly detectable in the immunoprecipitation experiment (see Fig. 3).

To analyze the Pex5p ubiquitination in somewhat more detail, in addition to transforming *pex1* and *pex4* cells with a plasmid that results in the synthesis of Myc-tagged ubiquitin (Fig. 2, *lanes 2* and *5*), we also transformed the same cells with a plasmid that enables synthesis of a mutant ubiquitin (Ub.Lys48Arg; Fig. 2, *lanes 3* and *6*). In cells that also produce wild type ubiquitin, synthesis of this form of ubiquitin will reduce the conjugation of multiple ubiquitin molecules to target proteins via the lysine 48 residue thereby interfering with their turnover by the proteasome (see Ref. 27). As a result, lowly ubiquitinated target proteins are expected to accumulate. In the *pex1* mutant the ubiquitination pattern of Pex5p changed upon expression of Ub.Lys48Arg, with a clear increase in the amount of ubiquitinated Pex5p molecules (Fig. 2, compare *lanes 1* and *3*). Also in cells of the *pex4* mutant, overproduction of Ub.Lys48Arg resulted in an increase of ubiquitinated Pex5p, especially of forms containing an estimated two to four ubiquitin moieties (Fig. 2, compare *lanes 4* and *6*).

Ubiquitinated Pex5p Is Pelletable—Pex5p is a cycling receptor that has a dual location in the wild type cell, *i.e.* it is present in the cytosol and in a peroxisome-bound form. In *pex1* and *pex4* mutants, peroxisomal matrix proteins are mislocalized to the cytosol, whereas the peroxisomal membrane proteins remain present in so-called peroxisomal membrane remnants. To obtain information as to the function of the observed ubiquitination of Pex5p in peroxisome biogenesis, we analyzed the subcellular location of the ubiquitinated species. Thus, post-nuclear supernatants of gently lysed protoplasts were prepared from *pex1* and *pex4* cells and separated by centrifugation at 100,000 × *g* into an organellar pellet fraction, which also contains the peroxisomal membrane remnants, and a cytosolic fraction. Western blot analysis demonstrated that in *pex1* and *pex4* cells unmodified Pex5p was located in both organellar and cytosolic fractions (Fig. 5). It must be noted, however, that *in vitro* Pex5p is a highly unstable protein and especially the cytosolic pool of Pex5p is subject to proteolysis. Therefore, the relative amount of unmodified Pex5p in the various fractions does not represent the physiological state. Nevertheless, the lowly abundant ubiquitinated forms of Pex5p, that apparently represent a rather stable fraction of the protein, co-fractionated

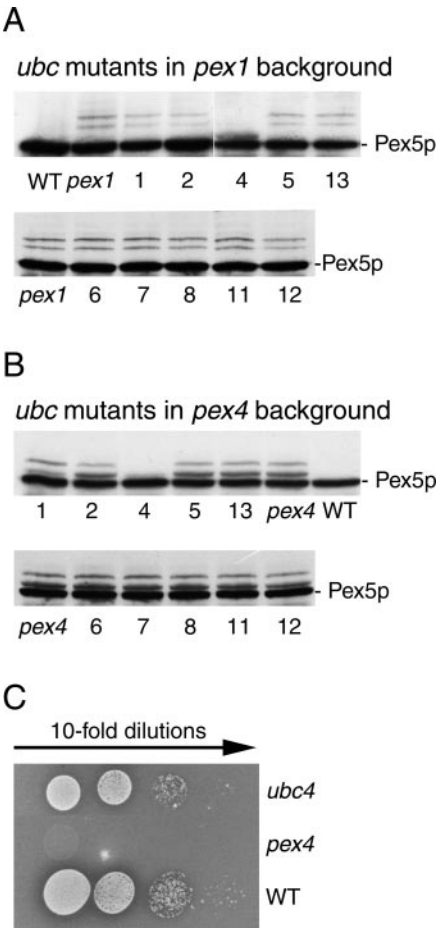

FIG. 6. **Pex5p ubiquitination in *pex1* and *pex4* mutants depends on *UBC4*.** Deletion mutants of *UBC* genes were constructed in the *pex1* background (*A*) or the *pex4* background (*B*). Subsequently, trichloroacetic acid extracts were prepared from glucose-grown cells of the double mutants using identically grown wild type, *pex1*, and *pex4* cells as controls. Equal amounts of protein were separated by SDS-PAGE and blotted with antibodies specific for *S. cerevisiae* Pex5p. The *numbers* refer to the respective *pex1 ubc* and *pex4 ubc* double mutants (see also Table I). *C*, deletion of *UBC4* in wild type *S. cerevisiae* does not significantly affect growth on oleate. Cells of the indicated strains were grown overnight on glucose minimal media. Subsequently, 10-fold dilutions were prepared, and 2 μl of each dilution was spotted onto oleate plates. The plates were then incubated for 3–5 days at 30 °C and scored for the appearance of colonies.

exclusively with the organellar fraction, suggesting that these forms of Pex5p are localized at peroxisomal remnants rather than present in the cytosol.

Ubiquitination of Pex5p in pex1 and pex4 Cells Is Dependent on Ubc4p—The ubiquitination of Pex5p observed in *pex4* cells is obviously not the result of the action of Pex4p, so far the only known ubiquitin-conjugating enzyme (also known as Ubc10p) to be involved in peroxisome biogenesis. To understand which Ubc protein is responsible for the ubiquitination of Pex5p in *pex1* and *pex4* cells, we constructed null mutants for *UBC1*, *UBC2*, *UBC4*, *UBC5*, *UBC6*, *UBC7*, *UBC8*, *UBC11*, *UBC12*, and *UBC13* in wild type, *pex1*, and *pex4* backgrounds and analyzed their effect on the formation of ubiquitinated Pex5p. The resulting data (Fig. 6, *A* and *B*) indicate that, for both *pex1* and *pex4* cells, introduction of the *ubc4* mutation affected Pex5p ubiquitination severely. In contrast, none of the other *ubc* mutations had any significant effect on the Pex5p ubiquitination patterns. Notably, when the indicated *ubc* mutations were introduced in the wild type background no significant effect was observed on steady-state levels of Pex5p, and modified Pex5p bands were not observed (data not shown).

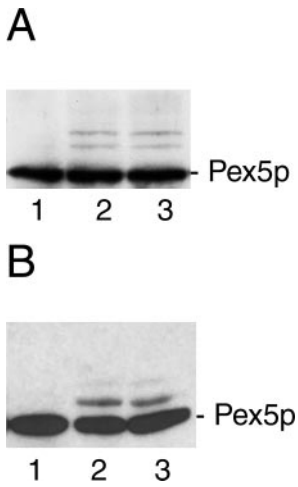

FIG. 7. **Ubiquitination of Pex5p also occurs when Pex1p and Pex4p are inactivated.** *A*, *S. cerevisiae pex1* cells were transformed with plasmid pRSpas1-Glu744 (*lane 2*) expressing the inactive *PEX1-K744E* gene, and the presence of ubiquitinated forms of Pex5p was determined by immunoblotting of trichloroacetic acid extracts of glucose-grown cells. Wild type (*lane 1*) and *pex1* cells (*lane 3*) transformed with vector pRS416 were used as controls. *B*, *S. cerevisiae pex4* cells were transformed with plasmids pEMBLyex4-PAS2 expressing the wild type *PEX4* gene (*lane 1*), vector pRS416 (*lane 2*), and pEMBLyex4-PAS2^{Ser} (*lane 3*) expressing the inactive *PEX4-C115S* gene. Cells were cultured on selective galactose medium to induce the expression of the plasmid-encoded mutant *PEX4* genes. Subsequently, the presence of ubiquitinated forms of Pex5p in trichloroacetic acid extracts was determined by immunoblotting using α-Pex5p-specific antibodies.

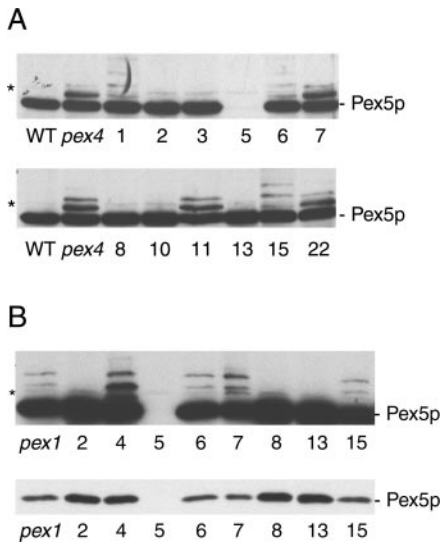

FIG. 8. **Ubiquitination of Pex5p in *pex1* and *pex4* mutants depends on the presence of peroxins involved in PTS1 import.** *A*, selected *PEX* genes were deleted in the *S. cerevisiae pex4* background. Subsequently, cells of the obtained double *pex* mutants were cultured on glucose mineral medium, and the presence of ubiquitinated forms of Pex5p in trichloroacetic acid extracts was determined by immunoblotting using α-Pex5p-specific antibodies. Equal amounts of protein were loaded per lane. *B*, a similar analysis for the *S. cerevisiae pex1* mutant. The *lower part of panel B* shows a short exposure of the same blot to demonstrate that in certain double mutants (*i.e. pex1 pex2*, *pex1 pex8*, and *pex1 pex13*) Pex5p levels have increased as compared with the parental strain. The indicated numbers refer to the *PEX* gene deleted in the *pex4* (*A*) or *pex1* (*B*) backgrounds. The *asterisks* indicate the presence of a faint α-Pex5p immunoreactive protein band that is occasionally observed on Western blots.

Subsequently, we analyzed whether *ubc4* mutants were significantly affected in their ability to grow on oleate, a measure of the functionality of peroxisomal matrix protein import. No significant difference was observed between wild type and *ubc4* cells, indicating that Ubc4p has no direct effect on peroxisome biogenesis (Fig. 6*C*).

Formation of Ubiquitinated Pex5p Molecules Occurs Also When Inactive Forms of Pex1p and Pex4p Are Present—To ascertain that the ubiquitination of Pex5p observed in *pex1* and *pex4* mutants results from the absence of the activity of the peroxins Pex1p and Pex4p, rather than being a direct consequence of the absence of the proteins, we introduced in *pex1* and *pex4* cells versions of the corresponding genes mutated in the active sites of their gene products (Fig. 7). Thus, *pex1* cells were transformed with a plasmid expressing the inactive gene *PEX1-K744E*, mutated in the second ATP binding site of the two AAA modules of Pex1p, which blocks its function in peroxisome biogenesis (28). Similarly, a plasmid carrying an inactive variant of the *PEX4* gene (*PEX4-C115S* (29)) was introduced in the *pex4* mutant. Notably, these mutant proteins have the same subcellular location as the wild type proteins (28, 29). In both cases, in the presence of the inactive forms of Pex1p and Pex4p, formation of ubiquitinated Pex5p molecules was similar to that observed in the *pex1* and *pex4* controls, respectively. Thus, we conclude that formation of ubiquitinated forms of Pex5p in the *pex1* and *pex4* mutants is directly connected to absence of the activity of Pex1p or Pex4p in peroxisome biogenesis.

Ubiquitination of Pex5p in pex1 and pex4 Cells Depends on the Presence of Other Peroxins—Recently, Collins *et al.* (10) studied steady-state Pex5p levels in *pex* mutants of *P. pastoris* and observed that the strongly decreased Pex5p levels observed in certain mutants (*e.g. pex4* and *pex22*) could be restored to wild type levels when these were combined with mutations in those *PEX* genes that were directly required for peroxisomal protein import. To understand whether the ubiquitination of Pex5p observed in *S. cerevisiae pex4* mutants is also dependent on other peroxins, we created double mutants by separately deleting selected *PEX* genes in the *pex4* background. Subsequently, we studied Pex5p ubiquitination in the constructed strains. The results (Fig. 8*A*) indicate that ubiquitination of Pex5p can no longer be observed when the *pex4* null mutation was combined with deletions in *PEX* genes that are required for the formation of the peroxisomal membrane (*PEX3*) or for peroxisomal matrix protein import (*PEX2*, *PEX8*, *PEX10*, and *PEX13*). No effect was observed on Pex5p ubiquitination when the *pex4* null allele was combined with deletions in genes not involved in PTS1 import (*PEX7* and *PEX11*). Also when two *pex* mutations in genes proposed to be involved in recycling of Pex5p were combined (*pex4 pex1*, *pex4 pex6*, *pex4 pex15*, and *pex4 pex22*), the ubiquitination remained present, although in specific cases the ubiquitination pattern changed to a certain extent (*pex4 pex1*, *pex4 pex6*, and *pex4 pex15*).

We have performed a similar analysis for the *pex1* mutant (Fig. 8*B*) and obtained essentially the same results. Notably, in the double mutants that no longer have ubiquitinated forms of Pex5p, the steady-state levels of this protein increased as compared with the parental strain (shown for *pex1* double mutants in Fig. 8*B*, *lower blot*), confirming that the *PEX* genes deleted in these mutants act prior to *PEX1* and *PEX4* (*cf.* Ref. 10). Thus, we conclude that ubiquitination of Pex5p in the *pex1* and *pex4* mutants depends on the presence/function of those peroxins that are required for functional PTS1 import.

Inhibition of Proteasome Function Results in the Accumulation of Ubiquitinated Pex5p—Our data suggested that the lower levels of Pex5p in wild type cells, as compared with *pex* mutant cells affected in PTS1 import, may actually result from proteasomal degradation of a portion of the PTS1 receptor during its recycling to the cytosol. Therefore, we analyzed Pex5p in a mutant disturbed in the function of the proteasome, *i.e. cim3-1*, which carries a mutant allele of the gene encoding the proteasomal ATPase Rpt6p (30). If a significant amount of

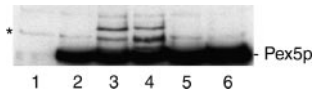

FIG. 9. **Ubiquitination of Pex5p also occurs in a mutant disturbed in proteasome function and depends on Ubc4p and Pex10p.** Trichloroacetic acid extracts were prepared from glucose-grown cells of *S. cerevisiae pex5* (*lane 1*), UTL7A (wild type, *lane 2*), *cim3-1* (*lane 3*), *cim3-1 pex4* (*lane 4*), *cim3-1 ubc4* (*lane 5*), and *cim3-1 pex10* (*lane 6*). Equal amounts of protein were separated by SDS-PAGE, and Western blots were prepared and developed using *S. cerevisiae* Pex5p antibodies. Essentially identical results were obtained when the cells were grown at either 20 or 37 °C, the restrictive temperature of the *cim3-1* mutant, suggesting that this mutant is already significantly affected in proteasome function at the lower temperature. The *asterisk* indicates the presence of a faint α-Pex5p immunoreactive protein band that is occasionally observed.

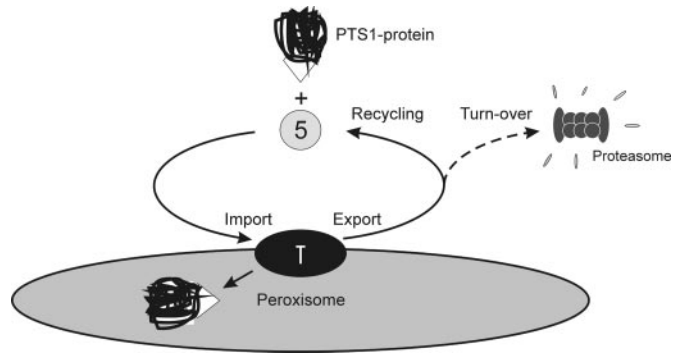

FIG. 10. **Schematic model of quality control of Pex5p at the peroxisomal membrane during receptor export.** The PTS1 receptor (5) is thought to bind its cargo, a PTS1 protein, in the cytosol and bring it to the docking/translocation machinery (*T*) at the peroxisomal membrane. After import of the PTS1 protein into the lumen of the peroxisome, the receptor is exported to the cytosol. At this moment, it is decided either to recycle the receptor to assist in a new round of PTS1 import, or to degrade the receptor by the proteasome. In mutants blocked in docking/translocation, this decision is not required, and Pex5p remains stable in the cytosol. However, in mutants blocked in receptor export, turnover of Pex5p may be stimulated.

Pex5p indeed becomes degraded by the proteasome, inhibition of proteasome function should result in the accumulation of ubiquitinated Pex5p molecules. Fig. 9 indicates that in the *cim3-1* background α-Pex5p-specific proteins of higher molecular weight than Pex5p are indeed present. Notably, when the *cim3-1* mutation was combined with a deletion of either *UBC4* or *PEX10*, these modified forms of Pex5p were absent. In contrast, a *cim3-1 pex4* mutant still contained (possibly even somewhat enhanced levels of) these modified Pex5p molecules. Taken together, these results strongly suggest that the modified forms of Pex5p observed here represent ubiquitinated species of the PTS1 receptor

DISCUSSION

We describe the identification of ubiquitinated forms of Pex5p in mutants of *PEX* genes that encode two groups of physically interacting proteins (Pex4p/Pex22p and Pex1p/Pex6p/Pex15p). The identification of ubiquitinated Pex5p in a specific set of *S. cerevisiae pex* mutants and its dependence on other *PEX* genes is consistent with the role ascribed to certain peroxins in peroxisome biogenesis (2). Our data indicate that these ubiquitinated forms of Pex5p are exclusively synthesized in mutants lacking the peroxins Pex1p, Pex4p, Pex6p, Pex15p, and Pex22p, all of which have been implicated in Pex5p recycling. Modification of Pex5p was never observed in those *pex* mutants that lack one of the peroxins thought to be involved in the formation of the peroxisomal membrane (Pex3p and Pex19p) or in docking/translocation of PTS1 proteins (Pex2p, Pex8p, Pex10p, Pex12p, Pex13p, Pex14p, and Pex17p). Rather, the formation of ubiquitinated Pex5p molecules in *pex1* and *pex4* cells appeared to depend on the presence of these peroxins, which all act in the steps prior to receptor recycling. Such dependence implies that these Pex5p molecules have actually followed most of the translocation route at the peroxisomal membrane and have become blocked at a stage where Pex5p is normally recycled to the cytosol. Presumably, at this membrane-bound stage of the receptor cycle ubiquitination of Pex5p has occurred. Indeed, we demonstrated that the ubiquitinated forms of Pex5p were exclusively present in the organellar pellet upon differential centrifugation of lysed *pex1* and *pex4* spheroplasts, suggesting that these molecules are located at the peroxisomal membrane. Thus, our data seem to be consistent with previous experiments that suggested a limited import of PTS1 matrix proteins in *P. pastoris pex4* and *pex22* (10), *Hansenula polymorpha pex4* (19), and *Arabidopsis thaliana pex6* (21) cells.

Apparently, the inactivation of one of the peroxins in the Pex4p-Pex22p or Pex1p-Pex6p-Pex15p complexes, results in Pex5p ubiquitination, a process that we show to be dependent on the ubiquitin-conjugating enzyme Ubc4p. Clearly, this ubiquitination is not related to the alleged function of Pex4p (Ubc10p), which in a hitherto unknown fashion is involved in Pex5p recycling. Moreover, *ubc4* mutants grow normally on oleate plates, precluding a direct role for the observed Pex5p ubiquitination in peroxisome biogenesis. But if the ubiquitination of Pex5p is not directly involved in recycling of the receptor to the cytosol, what then is the function of this protein modification? We propose that this function is related to quality control of Pex5p at the peroxisomal membrane (Fig. 10). In such a scheme, when peroxisomal import occurs normally, a receptor cycle, including Pex5p binding, import, and recycling, results in a highly efficient import of PTS1 proteins into the peroxisome matrix. However, occasionally the recycling of Pex5p may not function optimally. Under such conditions, the obstructing receptor molecules should be removed from the import/export site, which could be achieved by ubiquitination of Pex5p followed by its degradation via the proteasome. Such a process would be highly enhanced when recycling of Pex5p becomes blocked in specific *pex* mutants. This scenario is consistent with the reduced Pex5p levels in *P. pastoris pex1*, *pex4*, *pex6*, and *pex22* cells, in human *pex1* and *pex6* cell lines and in *A. thaliana pex6* cells (18, 20, 21, 31).

At first sight, such a scheme does not seem to fit completely with the phenotypes observed here for *S. cerevisiae*. In this yeast species, *pex1*, *pex4*, *pex6*, *pex15*, and *pex22* mutants show steady-state levels of Pex5p rather similar to those observed in wild type cells. Furthermore, although ubiquitinated forms of Pex5p are visible in these mutants, and are thought to be located at peroxisomal remnants, these are apparently not removed with high efficiency by the proteasome. There is, however, one major difference between our data and those described for *P. pastoris*. Although in *P. pastoris pex* mutants affected in docking/translocation of PTS1 proteins the steady-state levels of Pex5p do not differ significantly from those in wild type cells (10), we have observed that *S. cerevisiae* mutants lacking these peroxins have significantly higher Pex5p levels than wild type cells. We interpret this phenomenon as an indication that already in *S. cerevisiae* wild type cells a significant number of Pex5p molecules may actually be degraded. This view is confirmed by the observation that in a mutant blocked in proteasome functioning (*cim3-1*) ubiquitinated forms of Pex5p accumulate, in a Ubc4p-dependent manner. Notably, also deletion of the peroxisomal membrane protein Pex10p prevented formation of modified Pex5p species in the *cim3-1* mutant. This implies that also here the observed modification indeed takes place at the peroxisomal membrane and does not represent ubiquitination of wrongly folded Pex5p mol-

ecules in the cytosol. Additionally, the modified forms of Pex5p we observed seem to constitute mainly modified forms of Pex5p containing 1, 2, or 3 ubiquitin moieties on the basis of their apparent molecular weight on Western blots. Unfortunately, our data do not allow us to unequivocally conclude that these represent either mono-, di-, and tri-ubiquitinated Pex5p molecules, respectively, that have been conjugated to a single lysine residue in the receptor, or are conjugates of single ubiquitin moieties at different lysine residues in the target protein. Nevertheless, normally only multiubiquitinated proteins with chains carrying four or more Lys-48-linked ubiquitin moieties are degraded with high efficiency by the proteasome (32, for review see Ref. 27). Apparently, in *pex1*, *pex4*, *pex6*, *pex15*, and *pex22* mutants, a portion of Pex5p has obtained ubiquitin chains that are (still) too small to enable degradation by the proteasome, which results in its accumulation at peroxisomal remnants. Alternatively, in these mutants a number of Pex5p molecules at the peroxisomal membrane may be poorly accessible to the proposed quality control machinery, or may even be (partly) present inside the peroxisomal remnants, resulting in only a limited ubiquitination. Nevertheless, like in wild type cells, in these mutants the steady-state Pex5p levels are much lower than in mutants blocked in PTS1 import. This suggests that also in these mutants a significant portion of Pex5p has already been degraded via the proteasome, and only those ubiquitinated receptor molecules remain that either contain ubiquitin chains that are too small to be degraded by the proteasome, or are inaccessible. This notion is stressed by the observation that production of the Lys48Arg form of ubiquitin, which should reduce the size of multiubiquitin chains and thereby prevent proteasomal degradation, resulted in an increase in the level of lowly ubiquitinated Pex5p molecules in *pex1* and *pex4* cells.

Thus, a consistent model can be distilled from a comparison of the steady-state levels of Pex5p in *S. cerevisiae*, *P. pastoris*, human, and *A. thaliana* cells (Fig. 10). In this model, after cargo release into the lumen of the peroxisome, Pex5p has to be transported back to the cytosol. At this point apparently the cell has to decide either to recycle the receptor to assist in a new round of import, or to degrade it via the proteasome. Apparently, in certain organisms (*e.g. P. pastoris*) Pex5p is mainly recycled to the cytosol, and only a direct block in receptor recycling initiates a speedy turnover of the receptor by the proteasome. Presumably, under these conditions mainly multiubiquitinated forms of Pex5p are formed that are quickly degraded and therefore not easily visualizable. In other organisms, like *S. cerevisiae*, a different balance between these two processes exists, and already at optimal conditions a significant number of receptor molecules becomes turned over. Also here ubiquitination of the receptor is normally not detected. However, under certain circumstances (*e.g.* in a *pex1* or *pex4* mutant), the quality control mechanism apparently also produces lowly ubiquitinated forms of Pex5p, which are not degraded quickly enough by the proteasome, thereby allowing their visualization.

It must be noted that the scenario described above for the PTS1 receptor Pex5p is partly reminiscent of the ubiquitination of another *S. cerevisiae* peroxin, Pex18p, which together with the receptor Pex7p is specifically required for PTS2 import. Purdue and Lazarow (26) have demonstrated that, in wild type *S. cerevisiae* cells, Pex18p becomes ubiquitinated in a Ubc4p/Ubc5p-dependent manner and is continuously being degraded by the proteasome. Possibly, Pex18p is also part of a receptor cycle, and a quality control mechanism will decide between recycling and turnover of the protein. Clearly, much is still to be learned from an analysis of the processes that take place at the peroxisomal membrane during receptor recycling.

While this manuscript was under review, the group of Erdmann (40) reported that Pex5p is ubiquitinated in a Ubc4p-dependent manner in *S. cerevisiae pex* mutants disturbed in receptor recycling. In many respects the data in both manuscripts are in line with each other and complementary. However, a significant difference between both reports concerns the steady-state level of Pex5p in *pex* mutants, which is an essential component of the model shown in Fig. 10. In the present communication, we show the results of a careful analysis of Pex5p levels in multiple independent samples that demonstrates unequivocally that Pex5p levels in a specific set of *pex* mutants is 2- to 3-fold higher than in wild type cells (Fig. 1*A*). Platta and co-workers do not show any data regarding Pex5p steady-state levels in their report. Nevertheless, they indicate that all *pex* mutants have identical Pex5p levels. To obtain reproducible data on Pex5p levels, we have cultivated the relevant *pex* mutant strains on medium containing either glucose or oleate as carbon source and utilized multiple exposures of Western blots to enable reliable quantification of Pex5p levels. In contrast, in their report Platta and co-workers have only analyzed oleate grown cells, which contain extremely high levels of Pex5p. Furthermore, their report concentrates on the detection of the very minor amounts of ubiquitinated Pex5p present in a specific set of *pex* mutant cells, which in our hands required significant overexposure of blots (*cf.* Fig. 1*B*). We feel that, because of continuous overexposure of their blots, Platta and co-workers may have missed the 2- to 3-fold difference in steady-state Pex5p levels that we have consistently observed.

Acknowledgments—We thank Dr. Albert Sickmann and Uta Ricken for expert assistance during various parts of this work. We thank Dr. Carl Mann, Gif-Sur-Yvette Cedex, France for the *cim3-1* strain and Dr. Thomas Sommer for the gift of plasmids YEp105 and pUB203 and the monoclonal antibodies against bovine ubiquitin.

REFERENCES

1. Weller, S., Gould, S. J., and Valle, D. (2003) *Annu. Rev. Genomics Hum. Genet.* **4,** 165–211
2. Purdue, P. E., and Lazarow, P. B. (2001) *Annu. Rev. Cell Dev. Biol.* **17,** 701–752
3. Gould, S. J., Keller, G. A., Hosken, N., Wilkinson, J., and Subramani, S. (1989) *J. Cell Biol.* **108,** 1657–1664
4. Swinkels, B. W., Gould, S. J., Bodnar, A. G., Rachubinski, R. A., and Subramani, S. (1991) *EMBO J.* **10,** 3255–3262
5. Yang, X., Purdue, P. E., and Lazarow, P. B. (2001) *Eur. J. Cell Biol.* **80,** 126–138
6. Titorenko, V. I., Nicaud, J. M., Wang, H., Chan, H., and Rachubinski, R. A. (2002) *J. Cell Biol.* **156,** 481–494
7. Dammai, V., and Subramani, S. (2001) *Cell* **105,** 187–196
8. Oliveira, M. E., Gouveia, A. M., Pinto, R. A., Sa-Miranda, C., and Azevedo, J. E. (2003) *J. Biol. Chem.* **278,** 39483–39488
9. Distel, B., Erdmann, R., Gould, S. J., Blobel, G., Crane, D. I., Cregg, J. M., Dodt, G., Fujiki, Y., Goodman, J. M., Just, W. W., Kiel, J. A. K. W., Kunau, W. H., Lazarow, P. B., Mannaerts, G. P., Moser, H. W., Osumi, T., Rachubinski, R. A., Roscher, A., Subramani, S., Tabak, H. F., Tsukamoto, T., Valle, D., van der Klei, I., van Veldhoven, P. P., and Veenhuis, M. (1996) *J. Cell Biol.* **135,** 1–3
10. Collins, C. S., Kalish, J. E., Morrell, J. C., McCaffery, J. M., and Gould, S. J. (2000) *Mol. Cell. Biol.* **20,** 7516–7526
11. Hettema, E. H., Girzalsky, W., van den Berg, M., Erdmann, R., and Distel, B. (2000) *EMBO J.* **19,** 223–233
12. Hazra, P. P., Suriapranata, I., Snyder, W. B., and Subramani, S. (2002) *Traffic* **3,** 560–574
13. Agne, B., Meindl, N. M., Niederhoff, K., Einwachter, H., Rehling, P., Sickmann, A., Meyer, H. E., Girzalsky, W., and Kunau, W. H. (2003) *Mol. Cell.* **11,** 635–646
14. Rehling, P., Skaletz-Rorowski, A., Girzalsky, W., Voorn-Brouwer, T., Franse, M. M., Distel, B., Veenhuis, M., Kunau, W. H., and Erdmann, R. (2000) *J. Biol. Chem.* **275,** 3593–3602
15. Wang, D., Visser, N. V., Veenhuis, M., and van der Klei, I. J. (2003) *J. Biol. Chem.* **278,** 43340–43345
16. Birschmann, I., Stroobants, A. K., van den Berg, M., Schäfer, A., Rosenkranz, K., Kunau, W. H., and Tabak, H. F. (2003) *Mol. Biol. Cell.* **14,** 2226–2236
17. Matsumoto, N., Tamura, S., and Fujiki, Y. (2003) *Nat. Cell Biol.* **5,** 454–460
18. Koller, A., Snyder, W. B., Faber, K. N., Wenzel, T. J., Rangell, L., Keller, G. A., and Subramani, S. (1999) *J. Cell Biol.* **146,** 99–112
19. van der Klei, I. J., Hilbrands, R. E., Kiel, J. A. K. W., Rasmussen, S. W., Cregg, J. M., and Veenhuis, M. (1998) *EMBO J.* **17,** 3608–3618
20. Dodt, G., and Gould, S. J. (1996) *J. Cell Biol.* **135,** 1763–1774
21. Zolman, B. K., and Bartel, B. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 1786–1791
22. Erdmann, R., Wiebel, F. F., Flessau, A., Rytka, J., Beyer, A., Frohlich, K. U.,

and Kunau, W. H. (1991) *Cell* **64,** 499–510
23. Guldener, U., Heck, S., Fielder, T., Beinhauer, J., and Hegemann, J. H. (1996) *Nucleic Acids Res.* **24,** 2519–2524
24. Knop, M., Siegers, K., Pereira, G., Zachariae, W., Winsor, B., Nasmyth, K., and Schiebel, E. (1999) *Yeast* **15,** 963–972
25. Erdmann, R., Veenhuis, M., Mertens, D., and Kunau, W. H. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 5419–5423
26. Purdue, P. E., and Lazarow, P. B. (2001) *J. Biol. Chem.* **276,** 47684–47689
27. Hershko, A., and Ciechanover, A. (1998) *Annu. Rev. Biochem.* **67,** 425–479
28. Krause, T., Kunau, W. H., and Erdmann, R. (1994) *Yeast* **10,** 1613–1620
29. Wiebel, F. F., and Kunau, W. H. (1992) *Nature* **359,** 73–76
30. Ghislain, M., Udvardy, A., and Mann, C. (1993) *Nature* **366,** 358–362
31. Yahraus, T., Braverman, N., Dodt, G., Kalish, J. E., Morrell, J. C., Moser, H. W., Valle, D., and Gould, S. J. (1996) *EMBO J.* **15,** 2914–2923
32. Deveraux, Q., Ustrell, V., Pickart, C., and Rechsteiner, M. (1994) *J. Biol. Chem.* **269,** 7059–7061
33. Erdmann, R., and Blobel, G. (1995) *J. Cell Biol.* **128,** 509–523
34. Albertini, M., Girzalsky, W., Veenhuis, M., and Kunau, W. H. (2001) *Eur. J. Cell Biol.* **80,** 257–270
35. Huhse, B., Rehling, P., Albertini, M., Blank, L., Meller, K., and Kunau, W. H. (1998) *J. Cell Biol.* **140,** 49–60
36. Stein, K., Schell-Steven, A., Erdmann, R., and Rottensteiner, H. (2002) *Mol. Cell. Biol.* **22,** 6056–6069
37. Cesareni, G., and Murray, J. A. H. (1987) in *Genetic Engineering, Principles and Methods* (Setlow, J. K., ed) Vol. 9, pp. 135–149, Plenum Press, New York
38. Sikorski, R. S., and Hieter, P. (1989) *Genetics* **122,** 19–27
39. Hill, J. E., Myers, A. M., Koerner, T. J., and Tzagoloff, A. (1986) *Yeast* **2,** 163–167
40. Platta, H. W., Girzalsky, W., and Erdmann, R. (2004) *Biochem. J.* **384,** 37–45