Farnesylation of Pex19p Is Required for Its Structural Integrity and Function in Peroxisome Biogenesis*

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The conserved CaaX box peroxin Pex19p is known to be modified by farnesylation. The possible involvement of this lipid modification in peroxisome biogenesis, the degree to which Pex19p is farnesylated, and its molecular function are unknown or controversial. We resolve these issues by first showing that the complete pool of Pex19p is processed by farnesylation transferase in vivo and that this modification is independent of peroxisome induction or the Pex19p membrane anchor Pex3p. Furthermore, genomic mutations of PEX19 prove that farnesylation is essential for proper matrix protein import into peroxisomes, which is supposed to be caused indirectly by a defect in peroxisomal membrane protein (PMP) targeting or stability. This assumption is corroborated by the observation that mutants defective in Pex19p farnesylation are characterized by a significantly reduced steady-state concentration of prominent PMPs (Pex11p, Ant1p) but also of essential components of the peroxisomal import machinery, especially the RING peroxins, which were almost depleted from the importomer. In vivo and in vitro, PMP recognition is only efficient when Pex19p is farnesylated with affinities differing by a factor of 10 between the non-modified and wild-type forms of Pex19p. Farnesylation is likely to induce a conformational change in Pex19p. Thus, isoprenylation of Pex19p contributes to substrate membrane protein recognition for the topogenesis of PMPs, and our results highlight the importance of lipid modifications in protein–protein interactions.

A large number of eukaryotic intracellular proteins are post-translationally modified by the covalent attachment of either 15 or 20 carbon isoprenoids known as farnesyl or geranylgeranyl, respectively. This process (referred to as protein prenylation) affects lipases, kinases, inositol and protein-tyrosine phosphatases, lamins, and most of the small GTPases (1–3). Protein prenylation was shown to enable reversible association of modified proteins with lipid bilayers and to modulate protein–protein interactions (4–6).

The farnesyl group is attached to the cysteine of the C-terminal motif known as the CaaX box, where “a” indicates aliphatic amino acids and X is usually serine, methionine, glutamine, alanine, or threonine (3). Farnesyltransferase (FTase)3 consists of two subunits, the α-subunit and the β-subunit (Ram2p and Ram1p in yeast). The α-subunit is shared by the geranylgeranyl transferase (GGTase I), whereas the β-subunit is unique for FTase (7).

The peroxisome biogenesis protein (peroxin) Pex19p is one of a few farnesylated non-GTPases that are conserved between yeast and humans. Pex19p was initially identified as a prenylated protein (PxF) (8, 9) or housekeeping gene product (HK33) (10). A loss-of-function mutation in human PEX19 is associated with complementation group CG-1/CG-14 of Zellweger syndrome (11). In the absence of Pex19p, cells lack functional peroxisomes (11–13). Pex19p is mostly cytosolic and interacts with all peroxisomal membrane proteins (PMPs) analyzed (14–16).

Different and not all exclusive models have been proposed for Pex19p function. First, Pex19p might be an import receptor for PMPs that recognizes its substrates in the cytosol and delivers them to the peroxisomal membrane (15, 17, 18). This function would be analogous to that of the peroxisomal import receptors Pex5p and Pex7p, which recognize and deliver matrix proteins with PTS1 (peroxisomal targeting signal type 1) and PTS2 to peroxisomes (19). Second, Pex19p might act as a PMP chaperone that prevents newly synthesized PMPs from aggregation and degradation in the cytosol (17, 20). Third, Pex19p might act as a PMP membrane insertion factor (14, 16). Fourth, Pex19p might be required as an association/dissociation factor of membrane protein complexes (21) and has been reported to be required for the targeting of Pex3p from the ER to the peroxisomal membrane (22). Finally, Pex19p function is dependent on Pex3p, which serves as a docking factor at the peroxisomal membrane (12, 22–24). All models agree on the importance of PMP recognition for Pex19p function (25).

Pex19p shows only a moderate degree of sequence conservation, with less than 20% amino acid identity between yeast and

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3 The abbreviations used are: FTase, farnesyltransferase; PMP, peroxisomal membrane protein; ER, endoplasmic reticulum; GST, glutathione S-transferase; GFP, green fluorescent protein.
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human Pex19p. Its CaaX box, however, has been retained throughout evolution (see Fig. 1). Information on the status and the requirement of Pex19p farnesylation has so far been available only through often conflicting side observations. Mammalian PEX19 was described to be partially farnesylated in CHO-K1 cells (11), but other studies with human fibroblasts challenged the relevance of Pex19p farnesylation (15, 26). It was speculated that in Saccharomyces cerevisiae, farnesylation is required for an essential aspect of Pex19p function (12). This notion was recently contradicted (27). Work on other yeasts similarly suggested that farnesylation would be dispensable for Pex19p function (13, 28, 29).

In this study, we determined the in vivo farnesylation status of Pex19p and its dependence on peroxisome induction and on Pex3p. We discovered that Pex19p is fully modified by FTase and investigated whether Pex19p farnesylation is required for PMP recognition and stability. By peptide blots, two-hybrid analysis, and fluorescence polarization titration, we showed that farnesylation increases the affinity for PMPs by a factor of about 10. Last, we provide evidence that the interaction between farnesylated Pex19p and PMPs is achieved through a farnesylation-induced structural change in Pex19p rather than through direct farnesyl-PMP interaction. Our results exemplify the biological relevance of isoprenylation-dependent protein-protein interactions.

EXPERIMENTAL PROCEDURES

Oligonucleotides, Plasmids, and Strains—Oligonucleotides and plasmids are listed in supplemental Tables 1 and 2. Plasmids pRAM1, pPC86-PEX19, pPC97-ANT1, pPC97-PEX3, and pPex10-GFP were cloned by introduction of PCR products generated from genomic DNA into the respective vectors, as described in supplemental Table 2. pPC86-PEX19C347R was derived from pPC86-PEX19 using primers RE1425/1426 and the QuikChange II kit (Stratagene). The pseudo wild-type, pEX19p, was generated by integration of PCR amplificates containing 5\textsuperscript{th} base, however, has been retained necessary, contrast was linearly adjusted by the “best fit” function of the acquisition software, Axiovision version 4.2 (Zeiss).

Protein Expression—GST-Pex19p and GST-Ras1p were expressed in Escherichia coli and purified as described for GST-Pex19p (18). S. cerevisiae FTase was expressed and purified as described for mammalian FTase (36). For expression of GST-Pex19p in S. cerevisiae, strains were grown in SD-ura to mid-log phase and induced by 0.5 mm copper sulfate for 2 h. Cells were harvested and resuspended in phosphate-buffered saline containing 1 mm dithiothreitol, Roche Applied Science complete protease inhibitor mixture, and 1 mm phenylmethylsulfonyl fluoride. Cells were broken by glass beads, and the lysate was centrifuged at 15,000 rpm (SS-34) for 50 min. Glutathione-Sepharose 4B (GE Healthcare) was incubated with the supernatant and washed with phosphate-buffered saline, and the bound protein was eluted by phosphate-buffered saline containing 10 mm glutathione.

In Vitro Farnesylation—Pex19p was farnesylated on a column by 10 \( \mu \text{m} \) purified Ram1p/Ram2p in buffer F (50 mm HEPES, pH 7.4, 5 mm MgCl\(_2\), 50 mm NaCl, 5 mm dithiothreitol) with 20 \( \mu \text{m} \) farnesyl pyrophosphate (Sigma) for 30 min at room temperature, washed in buffer F, and eluted by 10 mm reduced glutathione. For limited proteolysis, fluorescence titration, and CD spectroscopy, Pex19p was washed on a column with buffer F and then with 10 mm potassium phosphate buffer (pH 7.4) and cleaved by thrombin (24 units/mg Pex19p) at 4 °C for 16 h.

Anion Exchange Chromatography and Gel Filtration—Anion exchange chromatography was carried out using a ResourceQ column (GE Healthcare) with buffer F as running buffer. Bound protein was eluted by a linear salt gradient to 500 mm. Gel filtration was carried out on a Superdex 200 16/60 pg column (GE Healthcare) using buffer F as running buffer.

Limited Proteolysis—100 \( \mu \text{g} \) of purified Pex19p were incubated with 20 ng of trypsin at 30 °C for 45 min. Samples were taken at different time points. Proteolysis was stopped by adding 5X SDS-sample buffer and incubation at 95 °C for 5 min. Samples were analyzed by SDS-PAGE and Coomassie Blue staining.

Peptide Blot Assays—PMP blots were synthesized in parallel by the SPOT technique (37). Pex19p in vitro binding assay with peptide arrays was carried out essentially as described (18). Purified GST-Pex19p or farnesylated GST-Pex19p were added to the peptide-containing membranes at 100 \( \mu \text{g/ml} \). Monoclonal anti-GST antibodies (Sigma) were used to detect bound Pex19p. Uniformity of spotting was verified by incubating the Pex19p-probed membrane with Pex19p\textsuperscript{FARN}, which yielded a comparable picture (not shown).

Fluorescence Polarization Titration—The Pex13p peptide GIFA1MKFLKIKLYR was synthesized and labeled with fluorescein isothiocyanate by Biosyntan (Berlin, Germany). Titrations of the fluorescein isothiocyanate-Pex13p peptide with Pex19p and Pex19p\textsuperscript{FARN} were performed in a Fluoromax SPEX II fluorometer equipped with a 1971 autopolarizer (L configuration; Horiba Jobin Yvon, Munich, Germany) at 20 °C. Increasing amounts of Pex19p were added to 107 \( \text{cm} \) peptide in 1.2 ml of 20 mm HEPES, pH 7.4, 150 mm NaCl, 5 mm MgCl\(_2\). The solution was carefully mixed, and the fluorescence polarization signal (excitation 488 nm, emission 517 nm) was recorded for at least
5 min after each addition. The polarization signal was determined as $p = ((VV/VH)/(HV/HH) - 1)/((VV/VH)/(HV/HH) + 1)$ from four combinations of the inlet and outlet polarizer (where $V$ represents vertical and $H$ represents horizontal position of the polarization plane; first character inlet, second outlet polarizer). Concentrations of free and bound Pex19p or Pex19p\textsuperscript{FARN} were calculated from the starting concentrations of Pex19p and peptide applied and the amplitude of the titration curve. For both titration experiments, data were fitted using GraFit version 3.0 software (Erithacus) according to an $A + B = AB$ binding model.

Circular Dichroism and Secondary Structure Prediction—CD spectra were recorded using a Jasco J-710 spectropolarimeter (Jasco, Grossumstadt, Germany). Far UV spectra were recorded from 190 to 250 nm (10-fold oversampling) at 20 °C with proteins at a concentration of 0.2 mg/ml in 10 mM potassium phosphate buffer (pH 7.4) in cylindric quartz cuvettes (Hellma, Müllheim, Germany) with 0.1 cm path length. Secondary structure predictions were calculated using the algorithms CDDSTR, SELCON3, and CONTINLL as implemented in CDPro (38).

Miscellaneous—Preparation of yeast whole cell extracts and immunoblotting were performed according to standard procedures. Immunoreactive complexes were visualized using anti-rabbit or anti-mouse IgG-coupled horseradish peroxidase in combination with the ECL\textsuperscript{TM} system from Amersham BioSciences. The antibodies used have been obtained from commercial sources as the monoclonal anti-GST (Sigma) and anti-yeast 3-phosphoglycerate kinase, Pgk1p (Proteins, Inc., Eugene, OR) or described previously (namely Pex19p (12), Pex3p (39), Pex15p (40), Pex13p (41), Pex11p (42), Cta1p (43), Fox3p (44), and Pex14p (45)). The antibodies against Pxa1p and the anti-green fluorescent protein (GFP) were kind gifts from M. Schneider and W. H. Kunau, respectively (Bochum, Germany). The yeast two-hybrid analysis was performed essentially as described (46). β-Galactosidase activities were assayed according to the manufacturer’s instructions and expressed as μmol of chlorophenol red-β-D-galactopyranoside hydrolyzed/min/cell (Clontech, Palo Alto, CA).

The enzyme activity of catalase (EC 1.11.1.6) and cytochrome $c$ oxidase (EC 1.9.3.1) were measured according to Ref. 35.
RESULTS

All Pex19p Is Processed by Farnesyltransferase—To determine the level of Pex19p farnesylation in vivo, we analyzed cell lysates of S. cerevisiae wild-type cells by immunoblotting with antibodies directed against Pex19p. It is well known that farnesylation increases the electrophoretic mobility of Pex19p (11). In wild-type cells, Pex19p appeared as a double band, with the slower migrating form being less abundant (Fig. 1A). However, in a knock-out strain of the FTase β-subunit Ram1p, both bands shifted to a higher apparent molecular weight region (Fig. 1A). Complementation of the ram1 knock-out by reintroduction of Ram1p on a plasmid restored the wild-type mobility of the majority of Pex19p (11). In wild-type cells, Pex19p appeared as a double band, with the slower migrating form being less abundant (Fig. 1A). However, in a knock-out strain of the FTase β-subunit Ram1p, both bands shifted to a higher apparent molecular weight region (Fig. 1A). Complementation of the Δram1 knock-out by reintroduction of Ram1p on a plasmid restored the wild-type mobility of the majority of Pex19p (11). Importantly, as shown in Fig. 1A, the non-farnesylated form of Pex19p of a Δram1 mutant (arrowhead) cannot be detected in extracts from wild-type yeast (arrow). These results indicate that (i) Pex19p farnesylation is dependent on Ram1p and that (ii) the complete pool of Pex19p is modified by Ram1p in wild-type cells. Our results further show that the slower migrating form of Pex19p in wild-type cells does not represent non-farnesylated Pex19p but that the molecular weight shift most likely is due to another so far unknown modification. Phosphorylation can supposedly be excluded, since the double band persisted when extracts were treated with calf intestine phosphatase (not shown). Farnesylated and non-farnesylated Pex19p can be distinguished in wild-type cells, when the farnesylation machinery is challenged by overexpression of Pex19p (Fig. 1C). Farnesylation remained unaltered when peroxisome biogenesis was induced by oleate (Fig. 1D). In both glucose- and oleate-grown cells, about 3% of the Pex19p was associated with cellular organelles of a 20,000 × g sedimentation fraction (Fig. 1D), confirming Pex19p as a largely cytosolic protein that is temporarily or loosely associated with membranes. Also, the absence of Pep3p did not interfere with processing of Pex19p (Fig. 1D), indicating that Pep3p is not the recruitment factor for Pex19p farnesylation or subsequent farnesyl-dependent processing steps at the ER. Thus, farnesylation of Pex19p is complete and stable and has been maintained throughout evolution from yeast to humans, indicated by the nearly complete conservation of the farnesylation site (Fig. 1E).

FIGURE 2. Pex19p farnesylation is required for peroxisome function. A, genomic Pex19p mutants. In all cases, the kanMX4 marker was used to select for integration into the genome. CKQQ, the terminal four amino acids of wild-type S. cerevisiae Pex19p (CaaX box). For generation of the pseudo-wild type (ψ wild-type), the kanMX4 marker was introduced after the STOP codon of wild-type PEX19. In the pex19Δψ mutant, the cysteine of the farnesylation site was genomically replaced by arginine. In the pex19Δψ mutant, the farnesylation site CKQQ was removed by inserting a STOP codon followed by kanMX4 after PEX19 base pair 1038, corresponding to amino acid 346. B, appearance of Pex19p modification in genomic pex19 farnesylation mutants. The indicated strains were grown on glucose and oleate medium and analyzed by immunoblot with the antibodies indicated. PGK1p was used as loading control. C, growth assay on oleate liquid medium. Strains were precultured in synthetic medium (SD) with 0.3% glucose, washed, and inoculated at 0.05 A 600 units/ml in 0.1% oleate and 2% ethanol medium. At the indicated time points, 1-ml samples were taken, sedimented by centrifugation, and washed, and A 600 was determined.
Efficient Peroxisome Biogenesis Requires Pex19p Farnesylation

— To investigate the requirement for Pex19p farnesylation in vivo and to overcome potential overexpression artifacts associated with complementing plasmids, we constructed a series of genomic mutants using the kanMX4 selection marker (31). We introduced a single point mutation in the Pex19p farnesylation site (C347R), removed the whole CaaX box by deleting the last four amino acids of Pex19p (ΔH9004 C4), or integrated the selection marker right after the PEX19 gene to obtain a pseudo-wild type (ΔH9274 wild-type) as a control for possible effects of marker integration on the stability of the transcripts (Fig. 2A). We confirmed the apparent higher molecular weight of Pex19p in the genomic mutants, indicating that Pex19p was not farnesylated in these strains (Fig. 2B). Notably, all mutants were expressed at wild-type levels, thereby excluding PEX19 overexpression effects that might circumvent the requirement for the farnesyl moiety (12). The pseudo-wild type behaved like the wild-type strain in all of our assays.

When grown on oleate as the only carbon source, peroxisomes become essential for growth of yeast, because they are the only site of fatty acid β-oxidation (47). To test whether Δram1 or strains expressing the Pex19p mutant versions are capable of utilizing oleic acid as the sole carbon source, we performed growth tests in liquid culture. As shown in Fig. 2C, the growth of the two genomic pex19 farnesylation mutations, C347R or ΔC4, as well as the Δram1 mutant was significantly reduced compared with the wild-type growth rate, which indicates that these mutants failed to metabolize oleate. On ethanol, all mutants behaved like wild-type (Fig. 2C), which excludes pleiotropic effects and more likely indicates a clear peroxisomal defect due to the lack of Pex19p farnesylation.

Defects in Peroxisomal Matrix Protein Import
— To investigate the reason for the growth defect on oleate, we analyzed the distribution of GFP-SKL, as a peroxisomal matrix marker protein, by direct fluorescence microscopy (Fig. 3A). As expected, in wild-type cells, GFP-SKL exhibited a punctate staining pattern, which is typical for a peroxisomal localization and indicates a functional import of PTS1-proteins. In contrast, the GFP-SKL was mislocalized to the cytosol in ΔH9004 pex19 cells (Fig. 3A), where no peroxisomal structures are detectable (48). In farnesylation mutants as well as in the ΔH9004 ram1 mutant, different species of cells were observed. Some mutant cells showed a wild-type-like punctate pattern (Fig. 3A, top), whereas in others, GFP-SKL was totally misdirected to the cytosol (Fig. 3A, bottom). A third species of farnesylation mutant cells exhibited an intermediate phenotype of punctate peroxisomal structures with a cytosolic background staining, indicative of a partial import defect (Fig. 3A, middle).

For further investigation, a sedimentation analysis was performed, and the distribution of catalase (Cta1p) and 3-oxo-acyl-CoA thiolase (Fox3p) were subjected to immunoblot analysis with antibodies raised against peroxisomal catalase (Cta1p) and 3-oxo-acyl-CoA thiolase (Fox3p) or by catalase activity measurements (C). The total activity of Δpex19 was set as 100%.
this mutant strain (Fig. 3B). Interestingly, the farnesylation mutant pex19<sup>C347R</sup> exhibited a significant cytosolic mislocalization of both marker enzymes, which was more pronounced for Cta1p. Thus, the mutant strain is characterized by a partial mislocalization for peroxisomal matrix proteins. The Δram1 mutant did exhibit a similar phenotype concerning the distribution of the marker enzymes with the difference that the overall concentration of the proteins was decreased. We assume that the decreased concentrations of marker enzymes arise from secondary effects. In the Δram1 mutant strain, the general farnesylation defect affects not only Pex19p but also the function of other usually farnesylated proteins. This could affect regulatory processes, which may lead to the reduced protein concentrations. The partial mislocalization of peroxisomal marker proteins in the Δram1 strain was corroborated by enzyme activity measurements of Cta1p (Fig. 3C).

Taken together, these results show that the import of PTS1 and PTS2 proteins is partially disturbed in the Δram1 and the Pex19p mutant strains, which is in agreement with a requirement of Pex19p farnesylation for proper peroxisome biogenesis.

Defects in PMP Stability—The proposed function of Pex19p is that of a soluble import receptor and/or a chaperone for newly synthesized PMPs (15, 17, 18, 20). Therefore, Pex19p is only indirectly involved in matrix protein import. To investigate the reason for the observed partial matrix protein import defect in the Δram1 and the Pex19p mutant strains, we analyzed the cellular distribution of PMPs by density gradient centrifugation. Immunoblot analysis revealed that in farnesylation mutants, peroxisomal structures are detectable at a density similar to that of wild-type peroxisomes (data not shown), indicating that the targeting of PMPs to peroxisomes and thus peroxisome biogenesis is not generally disturbed in the farnesylation mutants. Next, we analyzed the stability of PMPs, which is known to be reduced in a Δpex19 deletion strain (48) and asked whether Pex19p farnesylation would affect the steady-state concentration of PMPs (Fig. 4A). As expected, the Δpex19 strain exhibited a significantly reduced steady-state level of all PMPs tested (Fig. 4A, lane 2). The Δram1 and the Pex19p farnesylation mutant strains showed no significant difference from the wild type concerning the presence of the components of the docking complex, Pex13p and Pex14p, as well as Pex15p and Pex3p, the class II PMP. However, the amounts of the most abundant peroxisomal membrane protein and proliferation factor, Pex11p, of the peroxisomal ABC transporter Pxa1p, and importantly also of the RING finger peroxins Pex2p and Pex10p were as reduced in the farnesylation mutants as in the Δpex19 deletion strain. The detection of Pex2p and Pex10p was achieved by genomic integration of Protein A at the C terminus of both proteins. To corroborate the presumed instability of the proteins, we monitored the amount of a GFP fusion of Pex10p, which was expressed under the control of the MET25 promoter in the mutant cells (Fig. 4B). The cellular concentration of the fusion protein was reduced in farnesylation mutants, and the addition of methionine to culture media repressed the expression of the fusion protein, showing the control of the MET25 promoter. These results indicate that the observed altered steady-state concentration of the PMPs in farnesylation mutants is not caused by a differential transcriptional regulation but is supposed to be due to a reduced stability or increased turnover.
The RING finger peroxins Pex2p and Pex10p are components of a larger assembly, the importomer of the peroxisomal protein import machinery (32). Since the steady-state concentration of the RING finger peroxins was drastically reduced in the farnesylation mutants, we investigated whether this is also reflected by the protein composition of the importomer. The importomer was isolated by affinity chromatography of Pex2p, genomically tagged with ProtA (Fig. 4C). The bait protein, Pex2p-Protein A, was isolated in reduced amounts in farnesylation mutants as well as in the /H9004 pex19 strain because of the lower steady-state concentration (Fig. 4, A and C). The amounts of the two other RING finger peroxins, Pex10p and Pex12p, were drastically reduced in the eluates of the farnesylation mutant strains compared with wild type, whereas the amounts of the docking components Pex13p and Pex14p were less affected (Fig. 4C). Since the RING peroxins are required for peroxisomal matrix protein import, their virtual absence in the importomer can explain the observed mislocalization of matrix proteins to the cytosol and the defect in growth on oleic acid medium of the farnesylation mutants.

Expression and in Vitro Farnesylation of Yeast Pex19p—To study the function of the farnesyl group of Pex19p in vitro, we expressed S. cerevisiae Pex19p in E. coli and purified the recombinant protein (Fig. 5A, lane 6). The apparent molecular mass of the tag-free non-farnesylated form of Pex19p is 39,937 Da (including linker amino acids Gly-Ser-His). The molecular mass of the farnesyl group is 204 Da, so the expected molecular weight of Pex19pFARN corresponds to 40,141 Da. C, size exclusion and anion exchange chromatography. GST-Pex19p was prepared as in A, cleaved by thrombin, and analyzed by gel filtration (Superdex 200 pg; GE Healthcare) or anion-exchange chromatography (ResourceQ; GE Healthcare). Solid line, Pex19p; dashed line, Pex19pFARN. AU, absorbance units. D, Pex19p was prepared as in C and treated with trypsin at 30 °C. Samples were taken at the indicated time points and analyzed by SDS-PAGE and Coomassie staining. The arrows indicate salient differences in the protein pattern. E, CD spectra of Pex19p and Pex19pFARN. F, increase in α-helical domains upon farnesylation. A conformational change was deduced from independent structural predictions of Pex19p and Pex19pFARN.
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TABLE 1
Secondary structure predictions from CD and primary structure

| Pex19p       | Pex19p<sub>FARN</sub> | Change |
|--------------|------------------------|--------|
| H            | S          | T          | U     | H            | S          | T          | U     | Change |
| CONTIN/LL    | 84.8       | 0.5        | 4.7    | 9.9  | 95.1        | 0.0        | 2.3        | 2.6   | +10.3  |
| av CONTIN/LL  | 57.5       | 33.7       | 6.4    | 2.5  | 93.5        | 0.2        | 5.7        | 0.7   | +36.1  |
| av SMPS6     | 61.8       | 8.0        | 12.7   | 18.7 | 67.6        | 6.9        | 11.3       | 15.5  | +5.7   |
| av total     | 52.2       | 19.4       | 13.5   | 16.1 | 67.0        | 6.7        | 12.6       | 14.9  | +14.8  |
| PHD<sup>a</sup> | 42.6       | 0.0        | 0.0    | 57.4 |             |            |            |       |        |
| SOPM<sup>b</sup> | 54.3       | 4.9        | 6.7    | 34.3 |             |            |            |       |        |
|               | Averaged from three algorithms with all six basis data sets. |        |
|               | Averaged results for basis data sets SP29, SP37, SP43, SDP48, SMP50, and SMP57. |        |
|               | Averaged for the algorithms CDSSTR, SELCON3, and CONTIN/LL. |        |
|               | Averaged results for basis data sets SP29, SP37, SP43, SDP48, SMP50, and SMP57. |        |

—In addition, we directly compared Pex19p and wild-type PEX19 with a CaaX box mutation (Pex19p<sup>C347R</sup>) together with Ant1p, Pex11p, or the central domain of Pex13p. The CONTIN/LL algorithm (38, 51) indicated an increase in helical content by 10% and a decrease in unstructured domains by 7.3% upon introduction of the farnesyl moiety (Fig. 5E). All changes were qualitatively robust against change of algorithm as well as against change of data sets (Table 1).

Secondary structure predictions calculated from the primary sequence by PHD (52) or SOPM (53) predicted an α-helical content of 43 or 54%, close to our averaged experimentally predicted value of 52% (Table 1). From secondary structure prediction, we concluded that Pex19p has an α-helical content in the range of 50% and that this content increases upon farnesylation, possibly due to structuring of the otherwise disordered N terminus (20).

Farnesylation of Pex19p Strongly Enhances Binding to Peroxisomal Targeting Motifs in PMPs—Prenylation of proteins is known to affect membrane association as well as protein-protein interaction (5). We tested whether farnesylation would affect the binding of Pex19p to its binding sites in PMPs, which have been demonstrated to be part of the signal sequence responsible for peroxisomal membrane targeting (18). To that end, farnesylated and non-farnesylated GST-Pex19p were added to identical cellulose membranes, which contained an array of synthetic 15-mer peptides scanning the Pex19p binding regions with the core binding sequence at the central positions (18). Sites were chosen according to the prediction of our algorithm and included a selection of both novel and already characterized regions. Immunological detection of bound Pex19p revealed that lipid-modified Pex19p bound to the same peptides as did Pex19p but apparently with higher affinity (Fig. 6A). The previously identified sites in Pex13p, Pex11p, and Pex25p (18) bound significantly more Pex19p<sup>FARN</sup>. A second predicted site in Pex11p (amino acids 49–74) was efficiently recognized only by the modified form of Pex19p. Several other binding sites predicted for the PMPs Pex12p, Pex17p, Pex27p, and Ant1p were also verified by the <i>in vitro</i> Pex19p binding assay. Importantly, all identified sites bound Pex19p<sup>FARN</sup> more avidly than the unmodified protein. As a control, the N-terminal 31 amino acids of Pex13p failed to interact with either version of Pex19p, demonstrating that the farnesyl moiety did not cause nonspecific binding of Pex19p to random peptides.

These results were complemented by two-hybrid analysis. We tested interactions of two-hybrid constructs expressing wild-type <i>PEX19</i> or a CaaX box mutation (Pex19p<sup>C347R</sup>) together with Ant1p, Pex11p, or the central domain of Pex13p...
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**FIGURE 6.** Pex19p farnesylation is required for efficient association with PMPs. A, Pex19p farnesylation enhances binding to its binding site in several PMPs. Overlapping pentadecameric peptides sampling the Pex19p binding site of several PMPs with two-amino acid shifts between neighboring peptides were spotted onto nitrocellulose membranes in duplicate and tested for interaction with Pex19p. Membranes were probed with equal amounts of purified GST-Pex19p or GST-Pex19p<sub>FARN</sub>. Bound protein was detected by monoclonal anti-GST antibodies. The numbers denote the amino acid positions that delimit the analyzed binding site. Farnesylation of Pex19p increased the affinity to its binding site for all PMPs tested. Pex13p-(1–31) served as a negative control. B and C, two-hybrid analysis. AD/BD, GAL4 activation/binding domain; wt, PEX19 wild-type; mut, pex19<sup>C347R</sup>. B, Pex19p binding to PMPs was tested with the Pex13p loop domain (PEX13L, amino acids 173–258) as well as full-length PEX11 and ANT1. Inset, immunoblot of two-hybrid whole cell lysates decorated with anti-Pex19p antibodies. C, efficient two-hybrid interaction of Pex3p requires Pex19p farnesylation. D, quantification of Pex19p-PMP peptide binding by fluorescence polarization titration analysis. A fluorescence polarization titration of a fluorescein isothiocyanate-Pex13p peptide with Pex19p and Pex19p<sub>FARN</sub>. Calculation of binding constants revealed a <i>K<sub>D</sub></i> of 64 nM for non-farnesylated Pex19p. Farnesylation reduced the <i>K<sub>D</sub></i> to 7.6 nM (Fig. 6D). In summary, peptide blots, two-hybrid assays, and fluorescence polarization titration indicate an about 10-fold reduced PMP affinity of non-farnesylated Pex19p.

**DISCUSSION**

An Evolutionarily Conserved Farnesylation Site—We show here that in *S. cerevisiae*, all cellular Pex19p is processed by FTase, irrespective of peroxisome induction. The Pex19p farnesylation site is conserved in all species (Fig. 1E), with PEX19 from trypanosomes being the only exception so far (56). This deviation is in concert with a strong conservation of the PEX19 farnesylation site in eukaryotes, since trypanosomes mark one of the earliest branching points in the eukaryotic lineage (57). Considering the low overall sequence conservation of Pex19p, the preservation of the farnesylation sites throughout kingdoms (Fig. 1E) indicates that the lipid modification is an essential component of the Pex19p protein. Farnesylation sites have originally been described as “CaaX” (Ca<sup>a1</sup>a<sup>2</sup>X) boxes with “aa” being small aliphatic amino acids and X being any amino acid (3). Pex19p from *S. cerevisiae* has an unusual farnesylation motif, with lysine and glutamine in the a<sup>1</sup>a<sup>2</sup> positions not being “small aliphatic amino acids.” Crystallographic inspection of mammalian FTase has revealed that there are virtually no restrictions on the a<sup>1</sup> site, whereas Gln in the a<sup>2</sup> site is “forbidden” (58). Although the human Pex19p CaaX motif (CLIM) is a “classical” one, this apparent conflict cannot be resolved by reference to differences between human and yeast FTase, because there are at least two human CKQQ proteins, one of which is described as farnesylated (59 – 61). The conservation of the CKQQ motif in the Pex19p proteins of other yeasts (Fig. 1E) makes us confident that the unusual CKQQ motif is also modified in these yeasts. However, Pex19p from *Y. lipolytica* (28) and *Pichia pastoris* (13) were reported not to be farnesylated. This notion was based on point mutations in the CaaX motif that did not cause a mobility shift of Pex19p. It is possible that farnesylation was not detectable in these experiments.

(amino acids 173–258). In all cases, Pex19p-PMP interaction was strongly reduced if Pex19p could not be farnesylated (Fig. 6B). Different affinities cannot be attributed to varying expression levels of wild-type and mutated Pex19p, as shown in the inset of Fig. 6B. Also for Pex3p, which is a class II PMP (17, 54) and interacts with a distinct domain of Pex19p (23, 55), Pex19p farnesylation was required for efficient interaction (Fig. 6C).

To quantify the farnesylation dependence of Pex19p-cargo interaction, we analyzed the interaction of recombinant Pex19p and Pex19p<sub>FARN</sub> with a fluorescently labeled Pex13p peptide by fluorescence polarization titration (Fig. 6D). The dissociation constants for Pex19p to its binding peptide in Pex13p were fitted by an A + B = AB model and resulted in a <i>K<sub>D</sub></i> of 64 nM for non-farnesylated Pex19p. Farnesylation reduced the <i>K<sub>D</sub></i> to 7.6 nM (Fig. 6D). In summary, peptide blots, two-hybrid assays, and fluorescence polarization titration indicate an about 10-fold reduced PMP affinity of non-farnesylated Pex19p.
The Pex19p double band observed by immunoblot analysis (Fig. 1A) was anticipated to represent farnesylated and non-farnesylated forms of Pex19p; the concurrent mobility shift of both bands in a Δram1 mutant now excludes this possibility. The second band could point to a second posttranslational modification.

Farnesylation before Membrane Recruitment—A bimodal localization is suggested for Pex19p with a few percent of the Pex19p protein at the peroxisome and the bulk amount in the cytosol (12, 24). Recent data suggest that Pex19p is first recruited to the ER (22). FTase is probably cytosolic (62), but methyltransferase, which catalyzes methylation of the farnesylated cysteine, has been localized to the ER in yeast and mammals (63). Yeast CaaX proteases that cut off the last three amino acids of the CaaX box sequence are also localized to the ER (64). We could not find differences in farnesylation levels under conditions that affect peroxisome biogenesis: growth on oleate or the absence of Pex3p (Fig. 1). Thus, we have to place Pex19p farnesylation epistatically upstream of recruitment to Pex3p. It will be interesting to learn whether Pex19p recruitment (22) is associated with subsequent processing steps at the ER.

A Role for Pex19p Farnesylation in Peroxisome Biogenesis—Having shown that Pex19p is fully farnesylated in vivo, we demonstrate that the endogenous concentration of Pex19p did not suffice to sustain normal peroxisome biogenesis when farnesylation was prevented. The Δram1 mutant as well as the Pex19p-CaaX box mutants were unable to grow on oleic acid medium (Fig. 2C), which is typical for mutants affected in peroxisome function (65). This was not due to a general growth defect, since the mutants grew normally on other non-fermentable carbon sources like ethanol (Fig. 2C). In contrast to pex19Δ cells, the mutants still contained peroxisomes, indicating that the non-farnesylated Pex19p still maintained part of its function in peroxisome biogenesis. The PEX19-defective human cell line PBD399 represents complementation group 14 (complementation group J in Japan) and expresses a truncated version of Pex19p with a 44-amino acid deletion at the C terminus (11, 15, 26). This underscores the importance of the farnesylation motif, although it is unclear whether the truncated Pex19p is stably expressed in this patient cell line. On the other hand, introduction of plasmid-borne Pex19p with a disabled CaaX box largely complements the biogenesis defect of a Δpex19 strain (12, 13, 15, 26–29). This discrepancy might be explained by overexpression effects in plasmid-based expression, which increases the local concentration at its place of action and thereby compensates for the 10-fold lower affinity to its substrate. However, the yeast farnesylation mutants exhibited a partial import defect for peroxisomal matrix proteins (Fig. 3), which might impair peroxisome function and thus explained the growth defect on oleic acid medium. The import defect is assumed to be caused indirectly by a defect in PMP targeting or stability. This assumption is corroborated by the observation that mutants defective in Pex19p farnesylation are characterized by a significantly reduced steady-state concentration of prominent PMPs (Pex11p, Ant1p) but also of essential components of the peroxisomal import machinery, especially the RING peroxins (Fig. 4), which were almost depleted from the importome. A selective instability of the RING peroxin Pex2p has also been observed in Yarrowia lipolytica pex19 mutant cells (28).

Several other peroxins, such as Pex13p and Pex14p, were rather stable in the Pex19p farnesylation mutant cells (Fig. 4). These proteins might either be intrinsically more stable prior to insertion or be targeted to the peroxisomal membrane more efficiently (i.e. even when the affinity to Pex19p is reduced), thereby circumventing the absolute requirement for farnesylated Pex19p. Similarly, the selective absence of the RING peroxin Pex2p in a Y. lipolytica pex19Δ strain might indicate that targeting of this PMP is most critical, whereas others can target to peroxisomes even without Pex19p, although very inefficiently in this organism (28). Clearly, more work is required to substantiate such a hypothesis.

A Role for Pex19p Farnesylation in Substrate Recognition—We provide three lines of evidence that Pex19p farnesylation is crucial for efficient binding of PMPs. First, we detected in peptide blots sampling PMP binding sites that recombiant and completely farnesylated Pex19p (Pex19pFARN) bound significantly stronger than the non-farnesylated protein to its binding sites (Fig. 6A). Second, in two-hybrid assays, abrogation of Pex19p farnesylation strongly reduced PMP interaction (Fig. 6, B and C) (12). Third, quantification of the interaction of Pex19p and Pex19pFARN with a Pex13p peptide by fluorescence polarization titration yielded K_{DP} values that differed by a factor of about 10 (Fig. 6D). Peptide scans and two-hybrid analysis are entirely different ways for the analysis of protein-protein interactions. In peptide scans, PMP peptides are synthesized on a solid surface and probed with recombiant in vitro farnesylated Pex19p, which allows only a limited degree of PMP secondary structure. On the other hand, in the two-hybrid assay, both proteins are expressed in the same cell and are expected to assume their native conformation. Finally, fluorescence polarization accurately measures the protein-peptide interaction in solution. The K_{DP} of 7.6 nM for Pex19pFARN suggests that PMPs are tightly bound by Pex19p.

In vivo evidence exists that human Pex19p is similarly dependent on farnesylation for efficient PMP recognition. Deletion of the CaaX box drastically reduced the affinity for several PMPs, including Pex13p, in yeast two-hybrid assays (16). Consistently, the farnesylation-dependent Pex19p-PMP interactions could not be detected in a bacterial two-hybrid system, where Pex19p cannot be farnesylated (66). However, the interaction of human Pex19p with Pex3p was not affected in a farnesylation mutant (16, 66), which might indicate that the interaction of Pex19p with Pex3p, which is a type II PMP and functions as a docking factor for the peroxisomal targeting of Pex19p (23), is somewhat different from the typical PMP recognition. This assumption is also corroborated by the domain structure of Pex19p with an N-terminal domain interacting with Pex3p and a C-terminal region recognizing type I PMPs (17, 55).

Evidence is also provided for the assumption that the farnesyl moiety does not directly contribute to the Pex19p binding to its target proteins. First, farnesylated Pex19p bound to the same sites in PMPs as did non-farnesylated Pex19p (Fig. 6A); if the lipid tail directly interacted with PMP binding sites, we would expect only Pex19pFARN to recognize these sites. Second,
Pex19p showed increased affinity to all PMPs tested, making an interaction based on an exclusive farnesyl-PMP recognition unlikely. Furthermore, Pex19p farnesylation also enhanced the interaction with Pex3p (Fig. 6C), which is likely to bind Pex19p by a different mode (17, 55). Pex3p is described to represent proteins whose targeting is not dependent on Pex19p (class II PMPs) (17, 54). Finally, our data point to a conformational change upon farnesylation (Fig. 5, C and D). Secondary structure predictions indicated an increase in α-helical content (Fig. 5E). We assume that this structural change allows Pex19p to recognize PMPs more efficiently.

In conclusion, we have shown in this work that farnesylation of the endogenous, fully farnesylated Pex19p is required for efficient PMP interaction, which in turn is essential for proper PMP topogenesis. Our results can be reconciled with all currently proposed roles for Pex19p function (import receptor, chaperone, insertion factor, association/dissociation factor; see Introduction), since these models agree on the physiological relevance of the Pex19p-PMP interaction (25). Our data do not preclude involvement of the farnesyl moiety of Pex19p also in membrane association, which, however, still needs to be analyzed.

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