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Human Adrenoleukodystrophy Protein and Related Peroxisomal ABC Transporters Interact with the Peroxisomal Assembly Protein PEX19p

Christian J. Gloeckner,* Peter U. Mayerhofer,† Pablo Landgraf,† Ania C. Muntau,† Andreas Holzinger,† Josef-Karl Gerber,* Stefan Kammerer,‡ Jerzy Adamski,*,[1] and Adelbert A. Roscher†,[1]

*GSF-National Research Center for Environment and Health, Institute of Mammalian Genetics, Ingolstaedter Landstrasse 1, 85764 Neuherberg, Germany; †Dr. v. Hauner Children's Hospital, Department of Clinical Chemistry and Metabolism, Ludwig-Maximilians-University, Lindwurmstrasse 4, 80337 Munich, Germany; and ‡SEQUENOM Inc., 11555 Sorrento Valley Road, San Diego, California 92121

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Four ABC half transporters (ALDP, ALDRP, PMP70, and PMP69) have been identified in the mammalian peroxisomal membrane but no function has been unambiguously assigned to any of them. To date X-linked adrenoleukodystrophy (X-ALD) is the only human disease known to result from a defect of one of these ABC transporters, ALDP. Using the yeast two-hybrid system and *in vitro* GST pull-down assays, we identified the peroxin PEX19p as a novel interactor of ALDP, ALDRP, and PMP70. The cytosolic farnesylated protein PEX19p was previously shown to be involved in an early step of the peroxisomal biogenesis. The PEX19p interaction occurs in an internal N-terminal region of ALDP which we verified to be important for proper peroxisomal targeting of this protein. Farnesylated wild-type PEX19p and a farnesylation-deficient mutant PEX19p did not differ in their ability to bind to ALDP. Our data provide evidence that PEX19p is a cytosolic acceptor protein for the peroxisomal ABC transporters ALDP, PMP70, and ALDRP and might be involved in the intracellular sorting and trafficking of these proteins to the peroxisomal membrane.

Key Words: **X-linked adrenoleukodystrophy; ABC transporter; PEX19; targeting; peroxisome; protein–protein interactions; yeast two-hybrid.**

Abbreviations used: ABC, transporter ATP binding cassette transporter; ALD (X-ALD), (X-linked) adrenoleukodystrophy; ALDP, adrenoleukodystrophy protein; ALDRP, adrenoleukodystrophy related protein; GFP, green fluorescent protein; NBF, nucleotide binding fold; PEX19p, human peroxisomal assembly protein 19; Pex19p, yeast peroxisomal assembly protein 19; PMP, peroxisomal membrane protein; PMP70, 70 kDa peroxisomal membrane protein; PTS, peroxisomal targeting sequence; TM, transmembrane domain; VLCS, very-long-chain acyl-coenzyme A synthetase; VLCFA, very-long-chain fatty acids; X-Gal, 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside.

[1] To whom correspondence may be addressed. For Jerzy Adamski: Fax: +49-89-5160-3343; E-mail: Adelbert.Roscher@kk-i.med.uni-muenchen.de. For Adelbert A. Roscher: Fax: +49-89-3187-3225; E-mail: adamski@gsf.de.

In higher organisms proper function and assembly of peroxisomes is of vital importance as evidenced by a variety of severe human diseases caused by malfunction of these organelles (1). These include defects in many genes required for peroxisome biogenesis, termed PEX genes (2, 3). Peroxins, the proteins encoded by PEX genes, are responsible for the evolutionarily highly conserved process of posttranslational trafficking of proteins to the peroxisomal membrane or into the peroxisomal matrix (3, 4). The most common peroxisomal disorder is X-linked adrenoleukodystrophy (X-ALD, OMIM No. 300100), causing severe neurodegenerative disease with a broad spectrum of clinical manifestations (5). Biochemical hallmarks of the disease are increased tissue and plasma concentrations of very-long-chain fatty acids (VLCFA; C > 22:0) (5, 6). It had been generally accepted that accumulation of VLCFA in X-ALD is due to an impaired activity of very-long-chain acyl-CoA synthetase (VLCS) required for the activation of VLCFAs to their CoA thioesters (7–9). Surprisingly, it turned out that not defects in VLCS, but mutations in the ALD gene, encoding an integral peroxisomal membrane protein (ALDP), are undoubtedly the primary genetic cause of this disease (10, 11). ALDP belongs to the superfamily of ATP binding cassette (ABC) transporters capable of transporting a wide variety of ligands, ranging from ions to proteins, across biological membranes. In eukaryotic organisms

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ABC transporters typically consist of two hydrophobic transmembrane domains and two hydrophilic domains each containing a nucleotide binding fold (12). ALDP, however, is a half transporter composed of only one transmembrane domain and one nucleotide binding fold and has to form homo- or heterodimers to be functional. In humans, three other closely related peroxisomal ABC half transporters, ALDRP (ALD-related protein), PMP70 (PXMP1) and PMP69 (PXMP1-L) have been identified (13–16). These may act as interaction or dimerization partners for ALDP (17, 18). Using the yeast two-hybrid system it has recently been demonstrated that homo- as well as heterodimerization can occur between the carboxy-terminal halves of ALDP, ALDRP, and PMP70 (19). It has also been suggested that a loss of ALDP dimerization plays a role in the pathogenesis of X-ALD. Nevertheless, it is unclear, how a malfunction of ALDP might be correlated to the accompanying deficient activity of VLCS being responsible for VLCFA accumulation (18, 20, 21). Moreover, the factors that mediate trafficking and targeting of ALDP have not been investigated yet. Imanaka and co-workers had shown, that the related peroxisomal ABC half transporter PMP70 is translated in the cytosol and transported to the peroxisome by cytosolic factors using *in vitro* targeting experiments (22). Very recent evidence suggests that the peroxin PEX19p might act as a cytosolic binding protein for PMP70 (23). The observation that approximately 70% of the X-ALD mutations result in loss of ALDP immunoreactivity (18, 24) is consistent with the hypothesis that ALDP, once translated in the cytosol, needs further protein interaction to be stabilized and properly targeted to the peroxisome.

To further elucidate the targeting and interaction network of peroxisomal ABC half transporters, we screened for interaction partners using the yeast two-hybrid system. For that purpose, defined portions of ALDRP were tested against a cDNA library derived from human brain tissue. We provide evidence that internal N-terminal domains of ALDP, ALDRP, and PMP70 strongly interact with human PEX19p, a 33-kDa farnesylated protein known to be involved in early steps of the peroxisomal biogenesis (25). These interactions were confirmed in *in vitro* pull-down assays. As the interaction of PEX19p occurs in a region which is important for the targeting of ALDP, our results are consistent with the idea that PEX19p is an acceptor protein for ALDP and related peroxisomal ABC transporters and might be involved in their trafficking to the peroxisomal membrane.

MATERIALS AND METHODS

Yeast two-hybrid screen and interaction assay. PCR using standard protocols was performed to obtain specifically selected cDNA fragments. PCR primers contained appropriate restriction sites to achieve in-frame ligation to the LexA gene. Additionally, a spacer of 3 glycine codons was inserted between LexA and the fragment of interest. The reverse primer contained a termination codon. Produced PCR fragments were restriction digested and cloned into the pEG202 vector. The correctness of the constructs was confirmed by DNA sequencing. Several constructs (ALDRP1-218, ALDRP181-297, ALDRP273-489, and ALDRP365-741) were then screened against a human brain tissue cDNA library in pJG4-5 vector (Clontech No. HL4500AK). Before being used in the library screen, baits were tested in repression assays for their ability to enter the nucleus (26). The library screen was performed in the yeast strain EGY48 (*Matα, trp1, his3, ura3,* (*lexAop*)6-*Leu2*). Yeast transformants were selected for leucine auxotrophy (Leu2-reporter) on galactose/raffinose drop-out medium lacking leucine and for activation of β-galactosidase (*lacZ*-reporter). Yeast clones containing interacting proteins were identified by growth on galactose/raffinose-medium without leucine and blue color development on galactose/raffinose-X-Gal-medium. pJG4-5 plasmids of positive clones were rescued, amplified in *E. coli* (strain XL1-Blue MRF′), and sequenced. Determined sequences were analyzed by performing a BLAST search (27).

The LexA yeast two-hybrid system (interaction trap) was kindly provided by R. Brent (Massachusetts General Hospital). Assay procedures and appropriate controls are described elsewhere (26).

Using a yeast two-hybrid interaction assay, human peroxisomal ABC transporters were then tested for interaction with PEX19p. For this purpose constructs of the N-terminal region of ALDP, ALDRP and PMP70 were cloned into the pEG202 vector and checked for interaction with PEX19p (clone pJG4-5-PEX19pΔ6) as described above.

Expression and purification of GST-fusion proteins. For *in vitro* binding assays the PEX19p and a CaaX-Box-mutated PEX19p (C296S-PEX19p) were cloned into the pGEXΔ*Bam*HI expression plasmid for the production of glutathione-*S*-transferase (GST) fusion-proteins (28). Full-length human PEX19p cDNA (GenBank No. NM_002857) was recently cloned in our lab and the mutations in the CaaX motif were introduced by PCR (29). Expression in the *E. coli* strain (BL21-DE3) and affinity purification of GST-PEX19p and GST-C296S-PEX19p fusion-proteins were performed as described previously (29, 30).

In vitro farnesylation of PEX19p at the C-terminal CaaX-box. 6 μg of purified GST-PEX19p or GST-C296S-PEX19p were incubated with 25 μl TNT rabbit reticulocyte lysate supplying the essential CaaX farnesyltransferase, 2 μl reaction buffer (TNT Coupled Reticulocyte Lysate System, Promega) and 2 μl farnesyl pyrophosphate (10 mM, Sigma) in a total volume of 50 μl for 2 h at 30°C (29). Both the farnesylated wild type protein and the farnesyl-deficient C296S mutant protein were bound to GSH-Sepharose beads and used in a GST pull-down assay as described below.

In vitro translation and ^{35}S-methionine labeling of peroxisomal ABC transporter proteins. For *in vitro* translation and labeling, full length ALDP, ALDRP, PMP70, and several N-terminally truncated constructs of ALDP (Δ66, Δ186, and Δ281) were cloned into pcDNA3.1 plasmid (Invitrogen). The proteins were transcribed *in vitro* from the T7-promotor of pcDNA3.1, translated and labeled with ^{35}S-methionine (Amersham) using the TNT Coupled Reticulocyte Lysate System (Promega) following standard protocols.

In vitro interaction assay (GST pull-down). The purified GST fusion proteins were bound to glutathione-Sepharose beads (Pharmacia) in PBS for 1 h at 4°C and washed three times in PBS. For the *in vitro* interaction assay the GST-fusion proteins (0.5–1.5 μg protein bound to 1 μl bead suspension) were incubated with 5 μl of *in vitro* translated protein in 200 μl binding buffer (100 mM NaCl, 50 mM potassium phosphate pH 7.4, 1 mM $MgCl_2$, 10% glycerol, 0.1% Tween 20, 1.5% BSA) for 2 h at 4°C. Samples were pelleted and washed four times in 1 ml binding buffer without BSA. Pellets were resuspended in SDS-sample buffer and boiled for 5 min before being analyzed by SDS–PAGE according to standard protocols (31). To verify stability of the

GST-fusion proteins and equal loading, gels were stained with Coomassie blue. The gels were dried and exposed to X-ray film for 24 h.

Transfection and analysis of ALDP–GFP fusion proteins. For *in vivo* targeting studies deletion constructs of ALDP were fused to the N-terminus of the green fluorescent protein (GFP). For this purpose ALDP1-281 and Δ281 ALDP were cloned into pEGFPN1 vector (Clontech). Plasmids were transfected into Cos7 cells using Lipofectamine (Life Technologies) following protocols provided by the manufacturer. After 48 h transfected cells were fixed with 3% formalin/PBS for 30 min and permeabilized with 1% Triton X-100 for 5 min. Catalase was used as marker for peroxisomes. Cells were incubated with a 1:100 dilution of a polyclonal rabbit antibody against catalase (Biodesign) for 1 h. The cells were then washed thoroughly with PBS and incubated with a 1:50 dilution of the secondary tetrarhodamine isothiocyanate (TRITC)-labeled anti-mouse IgG antibody (Jackson Immunoresearch Laboratory Inc.) for 1 h. The cells were embedded in an anti-fading reagent (Vectashield, Vector Laboratories Inc.). Fluorescence microscopy was performed using an Axiovert 135 inverted fluorescence microscope (Zeiss).

RESULTS

Identification of PEX19p as an Interactor of ALD-Related Protein

To identify proteins which interact with peroxisomal ABC transporters, we performed a cDNA library screen using the LexA yeast two-hybrid system with parts of ALD-related protein (ALDRP), recently cloned in our lab, as a bait (32).

Prior to the library screen we verified if baits were able to enter the nucleus. Generally for large proteins and especially for complex membrane proteins a separation into several smaller baits (not bigger than 60 kDa) is necessary, probably because of impairment of nuclear import by molecular mass or by hydrophobic domains. ALDRP constructs corresponding to amino acids 1–218, 181–297, 273–489, and 365–741 (ALDRP1-218, ALDRP181-297, ALDRP273-489, and ALDRP365-741, respectively) were imported into the nucleus (data not shown).

A cDNA library from human brain tissue (Clontech) was screened with all constructs. With the N-terminal construct containing the first 218 amino acids of ALDRP (ALDRP1-218) we obtained a strong interaction with human PEX19p (data not shown). The cDNA of the isolated clone (PEX19pΔ6) encompassed nearly the complete coding sequence (missing only the first 6 amino acids out of 299) of PEX19p. The interaction between ALDRP and PEX19p was confirmed *in vitro* using the GST pull-down assay as described later on.

PEX19p Interacts with the Human Peroxisomal ABC Transporters ALDP, ALDRP, and PMP70 in a Yeast Two-Hybrid Assay

To test if the closely related ABC transporters ALDP and PMP70 also interact with PEX19p as initially established for the N-terminal domain of ALDRP, a yeast two-hybrid interaction assay was performed. Therefore, the regions of ALDP and PMP70, that are homologous to the N-terminal part of ALDRP (ALDRP1-218), were cloned into the pEG202 vector (ALDP1-203 and PMP70 1-199) and used as a bait against the clone PEX19pΔ6 in the pJG4-5 vector. The ALDP and PMP70 constructs comprise, similar to ALDRP, the N-terminal region including the first two transmembrane helices but not the third and further C-terminal domains (Fig. 1, top). Like ALDRP, both, ALDP and PMP70 interacted strongly with PEX19p. These interactions activated the LEU2 gene allowing a growth on media lacking leucine (Fig. 1, panel 1). The activation of LEU2 is due to a PEX19p interaction with ALDP, ALDRP or PMP70, because suppression of PEX19p expression by glucose hampers yeast growth (Fig. 1, panel 2). The strength of the interaction is illustrated by blue color development within 24 h due to the activation of *LacZ* gene when grown on X-Gal media (Fig. 1, panel 3).

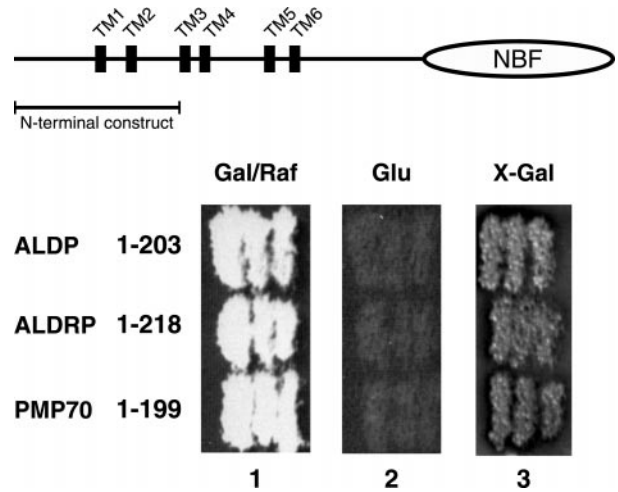

FIG. 1. Interaction of peroxisomal ABC transporters with PEX19p in the yeast two-hybrid assay. A scheme of peroxisomal ABC half-transporter structure with the six transmembrane helices (TM) and the nuclear binding fold (NBF) is represented on the top. The N-terminal regions marked by the bar in the scheme were fused to LexA and tested against PEX19p (clone PEX19pΔ6) for interaction in a yeast two-hybrid assay (bottom). Panel 1 presents the Leu2-reporter activity (growth on galactose/raffinose media without leucine); panel 2 presents the negative control on glucose media without leucine, where the Leu2-reporter is inhibited. Panel 3 presents the color development caused by LacZ-reporter activity for yeasts plated on galactose/raffinose-X-Gal-medium.

Mapping of the PEX19p Interaction Domain of ALDP

To validate the observation that segments within the N-terminal domain of the peroxisomal ABC transporters ALDP, ALDRP, and PMP70 are sufficient for interaction with PEX19p, we performed an *in vitro* interaction assay (GST pull-down) using ALDP as our model system. Full-length PEX19p was expressed as GST fusion protein and bound to glutathione-Sepharose beads. Several deletion constructs of ALDP lacking the first 66, 186, and 281 amino acids (Δ66, Δ186, and

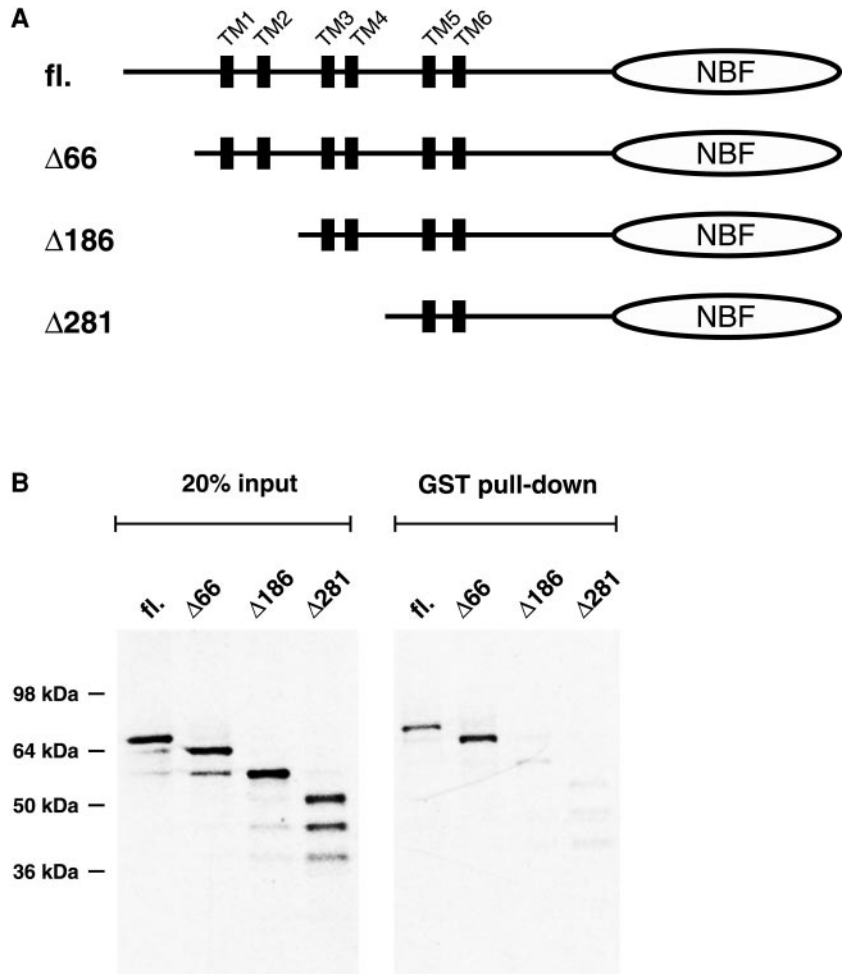

FIG. 2. Mapping of the PEX19p interaction domain on ALDP using a GST pull-down. (A) Scheme of ALDP-constructs: A full-length (fl.) construct and the deletion constructs Δ66, Δ186 and Δ286 were generated. They were [^{35}S]methionine labeled by *in vitro* translation using a rabbit reticulocyte lysate and tested for interaction with GST-PEX19p in a pull-down assay. The GST-PEX19p copurified proteins were separated on 12% SDS polyacrylamide gel electrophoresis. (B) Autoradiography of the gel: On the left, 20% of the total amount of the ^{35}S-labeled proteins (input) used in the GST pull-down was loaded and illustrates the molecular masses of these proteins (left part). The labeled proteins that were copurified with GST-PEX19p are depicted on the right. Lower molecular mass bands might correspond to alternative internal start codons or proteolytic cleavage of the *in vitro* translated proteins. No signal was obtained for a GST negative control (see Fig. 4).

Δ281) were ^{35}S labeled by *in vitro* translation (Fig. 2A) and subjected to a GST-PEX19p pull-down assay.

Full-length and Δ66 ALDP constructs showed interaction with PEX19p. Deletion constructs Δ186 and Δ281, however, did not interact with PEX19p since at the same level of input only faint signals were observed (Fig. 2B). These results show that the binding site of PEX19p lies in a region of about 120 amino acid residues (between amino acid 67 and 186) within the N-terminal domain.

In Vivo Targeting Studies of ALDP

To test the hypothesis that the region of ALDP interacting with PEX19p might be involved in the intracellular trafficking of ALDP deletion constructs were tested for their targeting to peroxisomes. For that purpose an ALDP-deletion construct (Δ281 ALDP) comprising a region which did not interact with PEX19p in the pull-down assay (Fig. 2) was compared to a construct containing amino acids 1–281 of ALDP in its cellular localization. The proteins were labeled by N-terminal fusion to the green fluorescent protein and expressed in Cos7 cell culture. The peroxisomal localization of the constructs was determined by the co-localization of catalase, detected by immunofluorescence staining. ALDP1-281 shows a proper peroxisomal localization while Δ281 ALDP exhibits a non-peroxisomal pattern (Fig. 3).

Farnesylation Is Not Necessary for the in Vitro Interaction of PEX19p with Peroxisomal ABC Transporters

Farnesylation of a C-terminal CaaX-motif of PEX19p (CLIM) was shown to be important for its biological

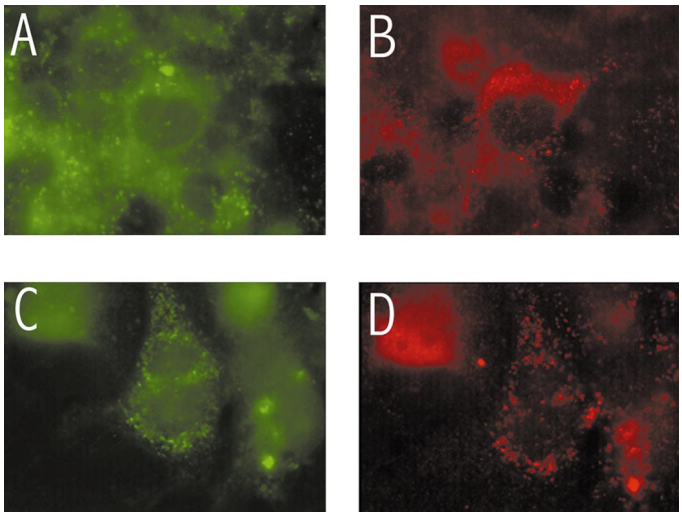

FIG. 3. Targeting of ALDP-GFP fusion protein constructs. The cellular distribution of Δ281 ALDP-GFP, missing the N-terminal amino acids 1–281 (A), and ALDP1-281-GFP, coding for the N-terminal amino acids 1–281 only (C), is compared with the localization of catalase detected by immunofluorescence staining (B, D) in Cos7 cells. ALDP1-281-GFP is co-localizing with catalase. Δ281 ALDP-GFP shows a diffuse cytosolic pattern with some spots, that do not co-localize with catalase.

function (25, 33). To test the influence of the farnesylation of PEX19p on its interaction with peroxisomal ABC transporters, a CaaX-box mutant of PEX19p (C296S-PEX19p) was compared with wild-type PEX19p in a GST pull-down after *in vitro* farnesylation. As shown previously, C296S-PEX19p can not be farnesylated *in vitro* (29). To conduct the assay, equal amounts of GST-PEX19p and GST-C296S-PEX19p were incubated in rabbit reticulocyte lysate under conditions that ensure attachment of the farnesyl group at the cysteine residue of the CLIM motif of PEX19p (Fig. 4A). Both, wild-type farnesylated and mutated farnesyl-deficient protein were then tested for interaction with *in vitro* translated and ^{35}S-labeled full-length ALDP, ALDRP, and PMP70. The mutant PEX19p (C296S-PEX19p) shows no significantly different binding capacity to the peroxisomal ABC-transporters *in vitro* compared to wild-type PEX19p (Fig. 4B).

DISCUSSION

In humans, four ABC half transporters have been localized to the peroxisomal membrane but no precise function has been unequivocally assigned to any of them. X-ALD is the only human disease known so far that results from mutations in the *ALD* gene. It is, however, unclear how a defective transport function of ALDP might be related to the abnormality in fatty acid metabolism observed in X-ALD as a biochemical hallmark (20, 21). One approach to unravel protein functions is to elucidate the cellular network by identifying protein interaction partners. The yeast two-hybrid system has previously been used to show that peroxisomal ABC transporters can form either homodimers or heterodimers with one of the related ABC half transporters (19, 34). In this study the aim was to identify ALDP interaction partners beyond those required for dimerization.

Using a yeast two-hybrid screen we initially identified a strong interaction of ALDRP with a small protein first cloned and described as the human peroxisomal farnesylated protein PxF (29). PxF is, typical for housekeeping genes, ubiquitously expressed (30). Since PxF has subsequently been shown to be crucial for peroxisomal biogenesis (25) it is now designated PEX19p. By performing yeast two-hybrid and *in vitro* interaction

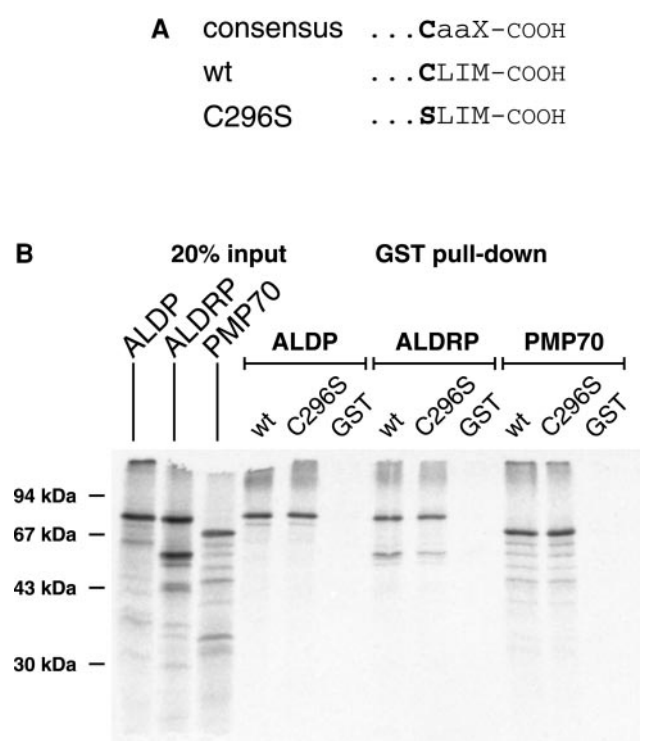

FIG. 4. Effect of *in vitro* farnesylation of PEX19p on its interaction with the peroxisomal ABC transporters. (A) Scheme of C-terminal farnesylation motif (CaaX-box) of PEX19p. Single letter notation of the consensus sequence: C, cysteine; a, aliphatic amino acid; X, any amino acid. A PEX19p wild-type (wt) and a CaaX-box mutated (C296S) PEX19p were expressed as GST-fusion proteins and subjected to an *in vitro* farnesylation assay as described under Materials and Methods. Farnesylated wt PEX19p and farnesyl-deficient C296S mutated PEX19p were tested for interaction with *in vitro* translated and ^{35}S-labeled full-length ALDP, ALDRP and PMP70 in a GST pull-down. Copurified proteins were separated on 12% SDS–polyacrylamide gel electrophoresis. (B) Autoradiography: 20% of the total amount of the ^{35}S-labeled ABC transporter proteins (input) used in the GST pull-down is shown on the left. Retention of labeled protein with GST-PEX19p and GST-C296S-PEX19p, respectively, is depicted on the right. GST only was used as a negative control. Lower molecular mass bands might correspond to alternative internal start codons or proteolytic cleavage of the *in vitro* translated proteins.

assays we confirmed that internal N-terminal parts of ALDP, ALDRP and PMP70 directly interact with PEX19p. These findings also suggest a functional relationship between the processes of peroxisome assembly driven by peroxins such as PEX19p and those of peroxisomal membrane transport accomplished by peroxisomal ABC transporters. The cellular site of interaction seems to be outside of the peroxisome since PEX19p appears to be mainly located in the cytosol (23), although immunocytochemical evidence suggests that it can also associate with the peroxisomal membrane (29, 35). Very recently Pex19p has been shown to interact with a broad range of integral peroxisomal membrane proteins, but not matrix proteins (23, 36). Such data have implicated that PEX19p may act as a broad specificity cellular chaperone for newly synthesized PMPs. Recently, an inactivating mutation in *PEX19* has been described that results in a complete lack of even residual peroxisomal membrane vesicles ("ghosts") in fibroblasts of a patient with Zellweger syndrome (33). Since ghosts are usually identified by anti-PMP70 antibodies, these findings indirectly demonstrate that a lack of the PEX19p acceptor site prevents PMP70 to reach its designated location within peroxisomal vesicular structures. Lack of interaction may thereby result in mislocalization and degradation of newly synthesized PMP70 and other PMPs (23).

We show here that PEX19p is interacting with ALDP and ALDRP which might contribute to the pathogenesis of X-ALD, the most common peroxisomal disorder. By using N-terminal constructs of ALDP we were able to delimit the interaction domain to an N-terminal stretch of ALDP comprising amino acid residues 67-186. The precise location of the specific interaction epitope was beyond the scope of this study since this would require experimental strategies delivering much higher resolution. Nonetheless, our *in vivo* targeting studies with ALDP deletion constructs suggest that the PEX19p interaction domain coincides with the region of ALDP required for targeting to the peroxisome. The data are consistent with the hypothesis that PEX19p may bind to a targeting element of ALDP thereby facilitating the intracellular trafficking of the transporter protein to the peroxisomal membrane.

In this context it is noteworthy that PEX19p is the only peroxin that can be farnesylated at a carboxyl-terminal CaaX motif (29). The covalent attachment of prenyl lipids, i.e., farnesyl groups or geranylgeranyl groups, by specific transferases has been shown to be indispensable for the intracellular sorting and membrane association of some proteins (37–40). Thus, we investigated whether *in vitro* farnesylation of PEX19p might influence its interaction with peroxisomal ABC transporters. Our findings were that a CaaX-box mutated and therefore farnesyl-deficient PEX19p (C296S-PEX19p) can bind peroxisomal ABC transporters as well as wild-type PEX19p. Though PEX19p farnesylation may not be a prerequisite for its *in vitro* interaction with peroxisomal ABC transporters it has been suggested to be essential for its overall biological action *in vivo,* e.g., in rescuing impaired peroxisomal assembly of PEX19p deficient fibroblasts (33). Moreover, in yeast the interaction of Pex19p with the cytosolic domain of Pex3p appears to be influenced in a farnesyl-dependent manner (25, 41). The posttranslational modification of PEX19p by farnesylation might thereby provide an additional regulatory mechanism to ensure proper sorting and trafficking of PMPs (42).

Our results might have implications in several ways. They may add to the understanding of the pathogenesis and phenotypic heterogeneity of X-ALD by demonstrating a mechanism by which newly synthesized ALDP may either be stabilized and/or translocated to the peroxisomal membrane. So far it is not clear why a surprisingly high percentage of missense mutations in the *ALD* gene is resulting in a loss of ALDP immunoreactivity (18, 24). Recent data suggest that ALDP instability may be a direct consequence of deficiency of dimerization that resides in the C-terminus of ALDP (19). The fact that interaction with PEX19p occurs within an internal N-terminal domain of ALDP implies that a much wider range of *ALD* mutations could potentially result in ALDP degradation as a consequence of "interaction deficiencies." Additional interaction domains of ALDP need to be postulated in order to explain the influence of ALDP mutations on peroxisomal β-oxidation at the level of the VLCS protein (7, 20). To date we and others also have not yet obtained conclusive data whether PMP69 is sharing interaction properties similar to the three other members of the peroxisomal ABC transporter family. These questions clearly need to be addressed for further understanding of the complex picture of X-ALD pathogenesis.

The demonstration of *in vitro* interaction of peroxisomal ABC transporters with PEX19p also supports the idea of direct functional interaction between peroxins and other peroxisomal membrane components during peroxisome maturation. Another indirect hint for this kind of interaction is provided by the observation that a PEX2p deficiency can be restored by overexpression of peroxisomal ABC transporters (43, 44). The identification of PEX19p as an acceptor molecule for ALDP suggests that PEX19p may modulate the function of some tissues afflicted by *ALD* mutations and could thereby influence clinical disease manifestation.

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