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Clofibrate-inducible, 28-kDa peroxisomal integral membrane protein is encoded by *PEX11*

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Abstract We cloned a human *PEX11* cDNA by expressed sequence tag homology search using yeast *Candida boidinii PEX11*, followed by screening of human liver cDNA library. *PEX11* encoded a peroxisomal protein Pex11p comprising 247 amino acids, with two transmembrane segments and a dilysine motif at the C-terminus. Pex11p comigrated in SDS-PAGE with a 28-kDa peroxisomal integral membrane protein (PMP28) isolated from the liver of clofibrate-treated rats and was crossreactive to anti-PMP28 antibody, thereby indicating *PEX11* to encode PMP28. Pex11p exposes both N- and C-terminal parts to the cytosol. *PEX11* was not responsible for ten complementation groups of human peroxisome deficiency disorders.

Key words: Peroxisome biogenesis; Peroxin; Dilysine motif; Membrane protein; Clofibrate; Complementation group

1. Introduction

Peroxisomal proteins, including membrane proteins, are encoded by nuclear genes, translated on free polyribosomes in the cytosol, mostly at their final sizes [1]. Peroxisomes are formed by division of preexisting peroxisomes after posttranslational import of newly synthesized proteins [1]. Genetic analysis of peroxisome-deficient mutants of yeast and mammalian cells have led to identification of a number of protein factors essential for peroxisome biogenesis [2,3]. Genetical heterogeneities comprising 12 complementation groups (CGs) have been identified in mammals, including humans [4–12]. Thereby, more than 12 genes are likely to be involved in mammalian peroxisome biogenesis. We have to date cloned *PEX2* [13], *PEX6* encoding a member of the AAA ATPase family [14], *PEX12* [15,16], and *PEX1* coding for another AAA protein [17], by genetic phenotype complementation assay of CHO cell mutants. These *PEX* proteins, peroxins, were required for peroxisome assembly in mammalian cells. Several human orthologues of yeast peroxins have been isolated by means of expressed sequence tag (EST) search on human database using yeast *PEX* genes [18–21]. Six peroxin genes, *PEX2* [13,22], *PEX5* for peroxisome targeting signal type 1 (PTS1) receptor [23,24], *PEX6* [14,25,26], *PEX12* [15,16,27], *PEX1* [17,19,20], and *PEX10* [21] were shown to be responsible for human peroxisome biogenesis disorders.

However, the process of membrane vesicle formation and proliferation of peroxisomes is little understood, despite the development of genetic approaches described above. Treatment with peroxisome proliferators, including hypolipidemic drugs such as clofibrate, induces in rats a variety of peroxisomal enzymes such as β-oxidation enzymes and proliferate peroxisomes [28,29]. Peroxisomal membrane biogenesis has been investigated in mammals [1,30–34], including several reports by making use of such inducible characteristics [30,31,35].

We herein isolated human *PEX11* cDNA (*HsPEX11*) encoding a peroxin Pex11p, by EST homology search using *Candida boidinii* (*Cb*) *PEX11* [36]. Human Pex11p was recognized by antibody raised against clofibrate-inducible, 28-kDa peroxisomal membrane protein (PMP28).

2. Materials and methods

2.1. Isolation of PMP28 and preparation of antisera

Peroxisomal membranes were prepared by sodium carbonate treatment [37] of highly purified peroxisomes [38,39] from male Fisher F-344 rats that had been treated for 7 days with clofibrate [29]. PMP28 was isolated by preparative SDS-PAGE, as described [30]. Antisera to PMP28 were raised in a rabbit as described [30].

2.2. Cell line

Wild-type CHO-K1 cells were cultured as described [8].

2.3. Cloning of human PEX11

Human EST AA476689 and R99800 showed high homology, 20% identical at a deduced amino acid sequence level, in the region encoding the N-terminal part comprising 100 amino acids of *C. boidinii PEX11* protein (formerly *PMP30*) [36]. Twenty subpools of human liver cDNA library constructed in a vector pCMVSPORTI (Gibco BRL) [17] were screened by PCR using nucleotide oligomers, sense HsPEX11.F (5′-GGTTCAGACTAGGCAATGTGG-3′) and antisense HsPEX11.R (5′-CTCGTTTCATCTGCAGGGAG-3′), corresponding to nucleotide residues at positions 235–255 and 297–316, of the EST AA476689 and R99800, respectively. One positive subpool No. 19 was screened by colony hybridization using the resulting PCR products as probe. Four positive colonies hybridized to the probe were identified. A single clone, F6-19-7, was isolated.

2.4. Morphological analysis

Peroxisomes in CHO cells were visualized by indirect immunofluorescence light microscopy, as described [4]. We used rabbit antibodies to PTS1 peptide [11]. Antigen-antibody complex was detected by FITC-labelled sheep anti-rabbit immunoglobulin G antibody (Cappel), under a Carl Zeiss Axioskop FL microscope.

2.5. Expression of epitope-tagged Pex11p

An epitope, flag-tagging to the N-terminus of Pex11p was done by a PCR-based technique. *Flag-HsPEX11* was amplified using a forward primer HsPEX11.FLAG.F (5′-GCGCGGATCCAGGACGCCTTCACCCGC-3′) and a reverse primer HsPEX11.FLAG.R (5′-GCGCGTCGACACATACAACTCTTCATGCT-3′). The PCR products were blunt-ended, digested with *Bam*HI, then ligated into a *Bam*-

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Abbreviations: CG, complementation group; EST, expressed sequence tag; *PEX11*, cDNA encoding the peroxin Pex11p; PMP28, 28-kDa peroxisomal membrane protein; PTS1, peroxisome targeting signal type 1

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HI-*Apa*I (blunted) vector fragment containing flag-tag sequence, originated from pUcD2Hyg-*flag-RnPEX12* [16]. C-terminal myc-tagging to flag-Pex11p was likewise done using a forward primer HsPEX11.BglF (5′-GCGCAGATCTTGGACGCCTTCACCCGC-3′) and a reverse primer HsPEX11.myc (5′-GCGCGGGCCCTCACAAGTCTTCTTCAGAAATAAGCTTTTGTTCGTCGACACGGGTCTTCAGCTTCATC-3′). A plasmid pUcD2Hyg-*flag-HsPEX11-myc* was constructed by inserting *Apa*I-*Bgl*II fragment of the PCR product into a *Bam*HI-*Apa*I vector fragment containing flag-tag sequence derived from pUcD2Hyg-*flag-HsPEX12*. Flag-tagged Pex11p and flag-Pex11p-myc were detected using mouse monoclonal antibodies to flag (M2) (Scientific Imazing Systems) and human c-myc (9E10) (Santa Cruz Biotech), and Texas Red-labelled sheep anti-mouse IgG second antibody (Amersham) in cells that had been permeabilized with 25 μg/ml of digitonin [15,40] or 0.1% Triton X-100.

2.6. In vitro transcription and translation of PEX11

*Sal*I-*Xba*I fragment of pCMVSPORT-*HsPEX11* and *Not*I (blunted)-*Kpn*I fragment of pUcD2Hyg-*flag-HsPEX11* were separately inserted into *Xba*I-*Sal*I site and *Hin*cII-*Kpn*I site, respectively, of pTZ19R vector (Amersham). In vitro translation of transcription product of *HsPEX11* or *flag-HsPEX11* in pTZ19R was done in a rabbit reticulocyte lysate cell-free protein synthesizing system, using [^{35}S]methionine and [^{35}S]cysteine as label, as described [41]. To reduce nonspecific binding, translation products were incubated, prior to immunoprecipitation, with formaldehyde-fixed and heat-inactivated *Staphylococcus aureus* cells (Calbiochem), in 1% NP-40/10 mM Tris-HCl, pH 7.4/150 mM NaCl/2 mM EDTA/1 mM phenylmethylsulfonyl fluoride/50 U/ml of aprotinin/2 mg/ml of leupeptin/2 mg/ml of antipain, and centrifuged. Supernatants were subjected to immunoprecipitation using antibodies to PMP28 and flag, as described [8].

2.7. Other methods

Nucleotide sequence was determined by the dideoxy-chain termination method using a Dye-terminator DNA sequence kit (Applied Biosystems). Alignment was done using GENETYX-Mac program (SDC, Tokyo). For Northern blotting, liver RNA was isolated from a rat treated for 7 days with clofibrate, and from an untreated rat. The blot was hybridized with *Bam*HI fragment (nucleotide residues at 115–598) of *HsPEX11* labelled with α-[^{32}P]dCTP (Amersham). Western blot analysis on polyvinylidene difluoride membrane (Bio-Rad) was done using a horseradish peroxidase-conjugated second antibody, donkey anti-rabbit IgG antibody, and ECL Western blotting detection reagent (Amersham).

3. Results and discussion

3.1. Anti-PMP28 antibody

By treatment of rats with clofibrate, PMP28 was highly induced, while 22-kDa PMP [30] was at a normal level (Fig. 1, lanes 1 and 2). We raised antiserum against PMP28 by immunizing rabbits with PMP28 isolated from liver peroxisomes of clofibrate-treated rats. The antibody specifically recognized PMP28 among numerous peroxisomal proteins in immunoblots of purified peroxisomes and homogenates from the liver of clofibrate-treated rats (Fig. 1, lanes 4 and 6). PMP28 was resistant to the sodium carbonate extraction [30,37], thereby suggesting PMP28 to be an integral membrane protein (lane 5). PMP28 was evidently induced by clofibrate, barely detectable in peroxisomes as well as homogenates from the liver of untreated rats (lanes 3 and 7).

3.2. Cloning of PMP28 cDNA

As a step to investigate biogenesis of PMP28, we attempted to clone a cDNA for mammalian PMP28. We used the EST homology search method as a cDNA cloning strategy. We first selected yeast PMP Pex11p as a potential candidate, judged from its molecular mass and inducible property [36,42]. BLAST search, using a TBLASTN program [43] for mammalian orthologue of *C. boidinii PEX11* (*CbPEX11*) [36], identified a cDNA clone, human EST: AA476689. We did further BLAST search using a human EST clone AA476689 and identified human EST: R99800. We isolated four positive clones by screening a human liver cDNA library with AA476689- and R99800-derived probes; one plasmid F6-19-7 contained 1205-bp cDNA with an ORF encoding a 247-amino acid protein of 28 353 Da (Fig. 2). Homology analysis suggested that this ORF was most likely to encode human orthologue of the *CbPEX11* and *PEX11* from *Saccharomyces cerevisiae* (*ScPEX11*) [42,44]. Thereby, we termed this cDNA *HsPEX11*. The *HsPEX11* protein, *Hs*Pex11p, was shorter by nine amino acids than *Cb*Pex11p in the primary sequence, but longer by 11 amino acids than *Sc*Pex11p. The average amino acid identity to *Cb*Pex11p and *Sc*Pex11p was 20% and 25%, respectively. Two putative membrane-spanning, hydrophobic segments were identified in human Pex11p (Fig. 2, overlines). Human Pex11p also contained a dilysine motif, ER retention motif [45], at the C-terminus and a potential *N*-glycosylation site, Asn at residue 9, in the N-terminal part, both of which were found neither in *Cb*Pex11p nor *Sc*Pex11p (Fig. 2).

The size of PMP28 was indistinguishable from that of Pex11p synthesized in vitro by coupled transcription/translation which was specifically immunoprecipitated by anti-PMP28 antibody (Fig. 3A, lanes 1–4), thereby indicating that a cloned *PEX11* cDNA encodes bona fide PMP28. This result implies the synthesis of PMP28, i.e. Pex11p, at its final size, consistent with a general rule for peroxisomal proteins [1]. Moreover, in vitro synthesized flag-Pex11p (Fig. 3A, lanes 5–7) as well as flag-Pex11p expressed in CHO-K1 cells (data

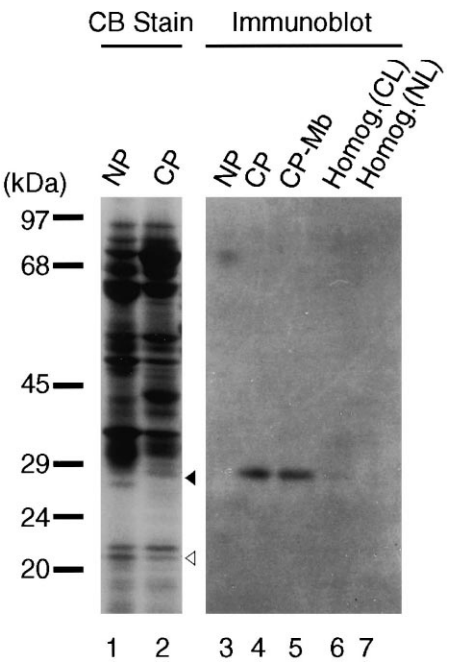

Fig. 1. Western blot analysis with anti-PMP28 antibody. Lanes 1 and 2, Coomassie blue-stained SDS-PAGE gel; lanes 3–7, immunoblot with rabbit anti-PMP28 antibody. Lanes: 1, normal rat liver peroxisomes (NP, 50 μg); 2, liver peroxisomes (CP, 30 μg) from clofibrate-treated rats; 3, normal peroxisomes (15 μg); 4–6, peroxisomes (15 μg), membranes from peroxisomes (15 μg), and liver homogenates (100 μg), respectively, from clofibrate-treated rats; 7, normal rat liver homogenates (100 μg). Molecular mass markers are on the left. The solid and open arrowheads indicate PMP28 and 22-kDa PMP [30], respectively.

not shown) were recognized by both antibodies to flag and PMP28, confirming the *PEX11* to encode PMP28.

Peroxisomes and peroxisomal proteins are induced in rat liver by administration of hypolipidemic compounds, such as clofibrate [28,29]. On Northern blotting, *PEX11* mRNA was detected as a single band of about 1.5 kb in liver RNA from clofibrate-treated rat (Fig. 3B, upper panel). *PEX11* mRNA was highly induced in rats by clofibrate, consistent with the result in immunoblot (see Fig. 1), under which acyl-CoA oxidase mRNA was elevated (middle panel), by about 50-fold [16,29]. It is interesting to note that PMP with an estimated mass of 26 kDa was inducible in rats by treatment with di(2-ethylhexyl)phthalate, a plasticizer, or thyroid hormones [31,35]. Accordingly, 26-kDa PMP is likely to be the same protein as PMP28 cloned in the present work.

3.3. Intracellular localization and topology of Pex11p

Subcellular localization of Pex11p was determined by immunofluorescent microscopy of ectopically expressed Pex11p that had been tagged with an epitope at its N- or C-terminus. When N-terminally *flag*-tagged *HsPEX11* was expressed in CHO-K1 cells, Pex11p was detected in a punctate staining pattern (Fig. 4A, a). The pattern was superimposable on that obtained using anti-PTS1 antibody (Fig. 4A, b), thereby suggesting that flag-Pex11p was localized to peroxisomes. Similar results were obtained when C-terminally epitope-tagged Pex11p-myc was expressed in CHO-K1 (Fig. 4B, a and b).

Membrane topology of Pex11p was determined by a differential permeabilization procedure. CHO-K1 cells expressing flag-Pex11p were permeabilized with 25 μg/ml of digitonin, under which plasma membranes are selectively permeabilized and intraperoxisomal proteins are inaccessible to exogenous antibodies [15,16,40]. Flag-Pex11p was observed in a punctate staining pattern, whereas there was nearly no staining of cells with anti-PTS1 antibody (Fig. 4A, c and d). Similar results,

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Hs Pex11p :M--DAF------TR---FTNQTQGRDRLFRATQYTCMLLRYLLEP 34
Cb Pex11p :MVYGELIYHPVVTKLLKFLDSSASREKLLRLLQYLCRFLTFYTFK 45
Sc Pex11p :MVCDTLVYHPSVTRFVKFLDGSAGREKVLRLLQY---LARFLAVQ 42

Hs Pex11p :KAGKEKVVMKLKKLESSVSTGRKWFRLGNVVHAIQAT--EQSIH- 76
Cb Pex11p :RNFNIETIQLIKKIQSSIGIRRTPLRFSKNLPHLKNLNKIYSNE- 89
Sc Pex11p :NSS-----LLARQLQAQFTTVRKFLRFLKPLNHLQAAAKFYDNKL 82

Hs Pex11p :ATDLVPRLCLTLANLNRVIYFICDTILWVRSVGLTSGINKE---- 117
Cb Pex11p :LLDSTLKIGDLIKNFGYALYFQFDTLQWLKLLGLLTSKNSGSLYF 134
Sc Pex11p :ASDNVVRVCNVLKNIFFAAYLSLDQVNLLRILKVIPVTVLTGK-- 125

Hs Pex11p :KWRTRAAHHYYYSLLLSLVRDLYEISLQMKRVTCDRAKKEKSASQ 162
Cb Pex11p :KIDKLAANFWLIGLTGSIITDLRNLKISYD---SNKALLNEINSQ 176
Sc Pex11p :KIPRWSNWCWLFGLLSGLAMDLRKIQTSHAQIAAFVKAK--SQSQ 168

Hs Pex11p :DPLWFSVAEEETEWLQSFLLLLFRSLKQHPPLLLDTVKNLCDILN 207
Cb Pex11p :NNNSNNDTLDEKLIEQNNDLILKNNEKIN-LNKRDLFKNILDSLI 220
Sc Pex11p :--------GDE---HEDHKKVLGKAYQDRYTALRRLFWDAADSFI 202

Hs Pex11p :PLDQLGIYKSNPGIIGLGGLVSSIAGMITVAYPQMKLKTR      247
Cb Pex11p :ALKGSQLIDLNDGVLGFAGIITSIIGIEDIWNAT-KA        256
Sc Pex11p :VLNNLGYLSSNEEYVALSGVVTSILGMQDMW----KA-T      236
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Fig. 2. Amino acid sequence alignment of human *PEX11* protein and Pex11p from *C. boidinii* and *S. cerevisiae*. Deduced amino acid sequence of human (*Hs*) *PEX11* was compared with those of Pex11p from yeast *C. boidinii* (*Cb*) and *S. cerevisiae* (*Sc*). (-) is a space. Identical amino acids between human and other species are shaded. Putative membrane-spanning segments are overlined. Conserved lysine residues in the dilysine motif are designated by dots; open arrowhead indicates the position of a potential *N*-glycosylation site. The DDBJ/EMBL/GenBank database accession number for the human *PEX11* cDNA is AB015594.

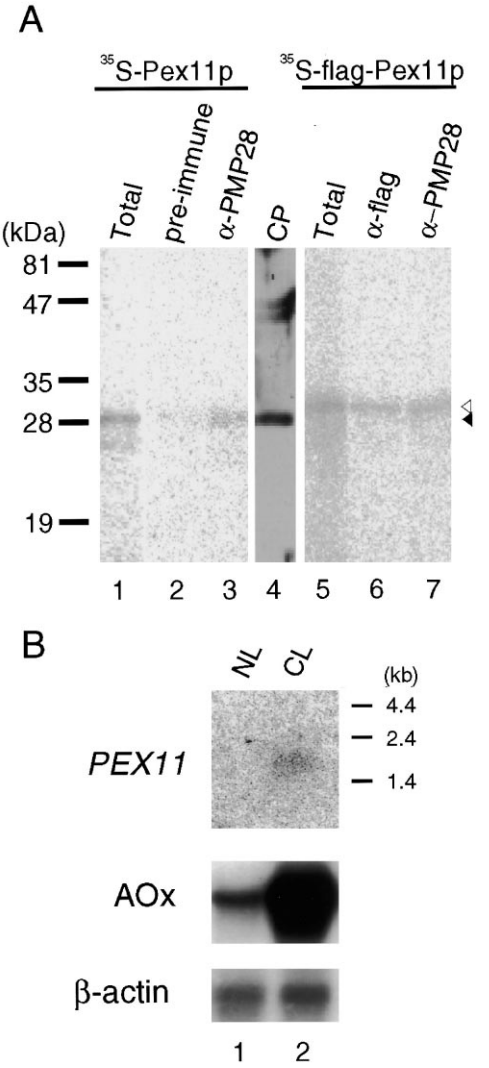

Fig. 3. Identification of Pex11p. A: Size comparison of in vitro transcription/translation product of human *PEX11* cDNA and rat liver PMP28. ^{35}S-labelled, in vitro transcription/translation product of *HsPEX11* and rat liver peroxisomes was subjected to SDS-PAGE and transferred to polyvinylidene difluoride membrane. ^{35}S protein bands in lanes 1–3 and 5–7 were detected by a FujiX BAS1000 Bio-Imaging Analyzer at an exposure for 16 h; immunodetection was done for lane 4 with anti-PMP28 antibody. Lanes: 1, in vitro transcription/translation product (1 μl) of *HsPEX11*; 2 and 3, immunoprecipitation of ^{35}S-Pex11p (1 μl) was done with preimmune and anti-PMP28 immune sera, respectively; 4, liver peroxisomes (CP, 20 μg) from clofibrate-treated rats; 5, in vitro transcription/translation product (1 μl) of *flag-HsPEX11*; 6 and 7, immunoprecipitation of ^{35}S-flag-Pex11p (1 μl) was done with antibodies to flag and PMP28, respectively. Solid and open arrowheads indicate ^{35}S-Pex11p and ^{35}S-flag-Pex11p, respectively. B: Northern blot analysis of *PEX11* mRNA. Total RNA (20 μg) from the livers of a normal (NL) and a clofibrate-treated (CL) rat was separated, transferred to Zeta-Probe GT membrane (Bio-Rad), and hybridized with ^{32}P-labelled cDNA probes for human *PEX11* (upper panel) and rat acyl-CoA oxidase (AOx, middle panel), respectively. Human β-actin (lower panel) cDNA was used as a control probe to check the amount of RNA loaded. Washing was done twice with 0.15 M NaCl/10 mM sodium phosphate, pH 7.4/1 mM EDTA/0.5% SDS at 60°C. *PEX11* mRNA band was detected using a FujiX BAS1000 Bio-Imaging Analyzer (Fuji Photo Film) at an exposure for 16 h; AOx and β-actin bands, by autoradiography at exposures for 28 and 24 h, respectively.

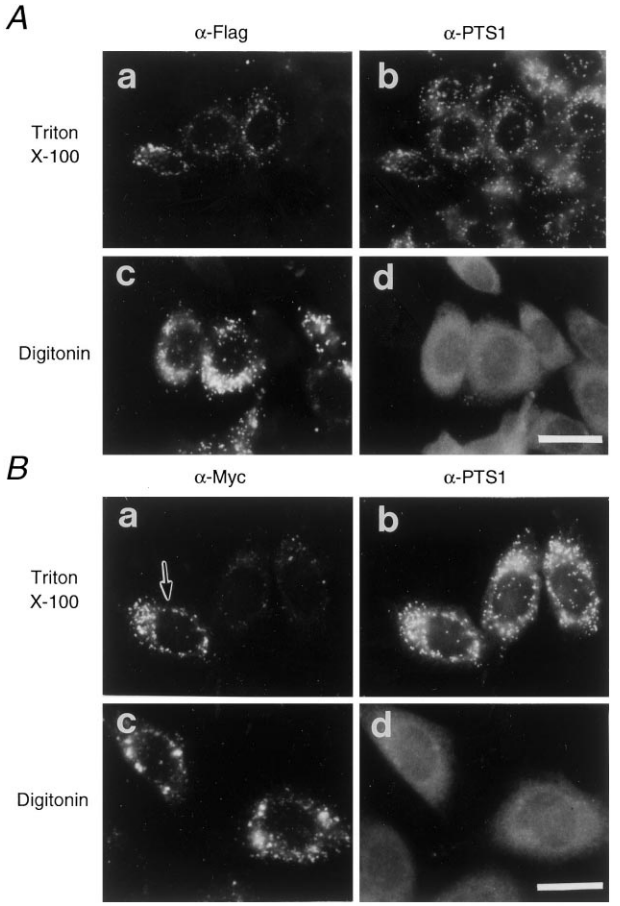

Fig. 4. Intracellular localization and topology of Pex11p. A: Human Pex11p tagged with flag at the N-terminus was expressed in CHO-K1 cells. Cells were treated with 0.1% Triton X-100 (a and b), or with 25 μg/ml of digitonin, under which the plasma membrane was permeabilized [15,16,40] (c and d). Cells were stained with mouse anti-flag antibody (a and c) and rabbit anti-PTS1 peptide antibody (b and d). Note that punctate structures, peroxisomes, are superimposable in a and b. Magnification, ×630; bar, 20 μm. B: CHO-K1 cells transfected with C-terminally *myc*-tagged human *PEX11* were treated with 0.1% Triton X-100 (a and b) or with 25 μg/ml of digitonin (c and d). Cells were stained with antibodies to myc (a and c) and PTS1 (b and d), respectively. Arrow in a indicates a cell expressing Pex11p-myc; two other cells apparently show a lower level of Pex11p-myc expression. Magnification, ×630; bar, 20 μm. Note that flag-Pex11p and Pex11p-myc were detected after both types of treatments.

including detection of C-terminally tagged myc on post-permeabilization with 25 μg/ml of digitonin, were obtained when *HsPEX11-myc* was expressed in CHO-K1 cells (Fig. 4B, c and d). Taken together, these results strongly suggest that both N- and C-terminal parts of Pex11p are exposed to the cytosol, presumably anchored by two membrane-spanning segments (see Fig. 2). It is noteworthy that *Sc*Pex11p was shown to be a peroxisomal integral membrane protein [42].

Furthermore, to investigate whether two conserved lysine residues in the dilysine motif are essential for peroxisomal targeting of Pex11p, we mutated and expressed *flag-HsPEX11* in CHO-K1 cells. Pex11p with mutation of residues ^{243}K^{245}K to ^{243}S^{245}S in the motif was detected in a punctate staining pattern, colocalized with PTS1-positive vesicles, peroxisomes, thereby indicating the mutation of the dilysine motif to show no apparent effect on localization of Pex11p (data not shown).

It is presently unknown if ER plays a role in peroxisome biogenesis in mammals. Flag-Pex11p mutated at the potential *N*-glycosylation site, ^{9}N to ^{9}D, was likewise detected in punctate structures colocalized with PTS1 proteins, implying no discernible change in Pex11p localization (data not shown). Moreover, this N9D mutation did not change the mobility of Pex11p in SDS-PAGE, suggesting no glycosylation of human Pex11p expressed in CHO cells (data not shown). We should await further study to delineate the potential role of ER in biogenesis of Pex11p.

Collectively, the data in the present work demonstrate that *PEX11* encodes peroxisome proliferator-inducible PMP28. *PEX11* was not responsible for peroxisome deficiency of 12 different CGs so far identified [3], including ten human CGs and two CHO CGs distinct from human CGs, as assessed by transfection of *HsPEX11* (data not shown). Pex11p was suggested to be involved in proliferation of peroxisomes in the yeast *S. cerevisiae* [42,44] and *C. boidinii* [36]. No apparent proliferation of peroxisomes was noted, however, when *HsPEX11* was expressed in CHO-K1 cells (see Fig. 4). Pex11p may function as a peroxisome biogenesis regulator, possibly by interacting with other *PEX* proteins [2,3,18], including RING peroxins, Pex2p [13,22,46] and Pex12p [15,16,27], AAA family peroxins Pex1p [17,19,20] and Pex6p [14,25,26], a tetratricopeptide repeat (TPR) protein Pex5p (PTS1 receptor) [23,24,47], a WD motif protein Pex7p (PTS2 receptor) [48–50], and Pex13p (PTS1 receptor docking protein) presumed in mammals [51]. Functional role of Pex11p in peroxisome biogenesis in mammals remains to be investigated.

Note: Passreiter et al. [52] very recently cloned rat *PEX11*, showing interaction of Pex11p with ADP-ribosylation factor and coatomer.

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