Dynamic and Functional Assembly of the AAA Peroxins, Pex1p and Pex6p, and Their Membrane Receptor Pex26p
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Two AAA peroxins, Pex1p and Pex6p, are encoded by PEX1 and PEX6, the causal genes for peroxisome biogenesis disorders of complementation group 1 (CG1) and CG4, respectively. PEX26 responsible for peroxisome biogenesis disorders of CG8 encodes Pex26p, the recruiter of Pex1p-Pex6p complexes to peroxisomes. We herein assigned the binding regions between human Pex1p and Pex6p and elucidated pivotal roles of the AAA cassettes, called D1 and D2 domains, in Pex1p-Pex6p interaction and peroxisome biogenesis. ATP binding in both AAA cassettes but not ATP hydrolysis in D2 of both Pex1p and Pex6p was prerequisite for Pex1p-Pex6p interaction and their peroxisomal localization. The AAA cassettes, D1 and D2, were essential for peroxisome-restoring activity of Pex1p and Pex6p. In HEK293 cells, endogenous Pex1p was partly localized likely as a homo-oligomer in the cytoplasm, whilst Pex6p and Pex26p were predominantly localized on peroxisomes. Interaction of Pex1p with Pex6p conferred a conformational change and dissociation of the Pex1p oligomer. These results suggested that Pex1p possesses two distinct oligomeric forms, a homo-oligomer in the cytosol and a hetero-oligomer on peroxisome membranes, possibly playing distinct functions in peroxisome biogenesis.

Peroxisomes are single-membrane organelles present in nearly all of eukaryotic cells. The functional importance of peroxisomes in human cells is highlighted by peroxisome biogenesis disorders (PBDs) including Zellweger syndrome, neonatal adrenoleukodystrophy, infantile Refsum disease, and rhizomelic chondrodysplasia punctata, of which the primary cause is the impaired biogenesis of peroxisomes (1). More than 30 proteins, termed as PEX gene products or peroxins, are required for peroxisome assembly (2-4). Only several peroxins have been delineated in their biological functions, such as Pex5p and Pex7p as soluble receptors for proteins with peroxisome-targeting signal type 1 (PTS1) and PTS2, respectively (5,6). The roles and molecular mechanisms of most of the peroxins are less understood.

Genetic heterogeneity consisting of 13 complementation groups (CGs) has been identified in PBDs (1,7,8). We isolated human PEX26 cDNA by functional complementation assay using Chinese hamster ovary (CHO) cell mutant ZP167 belonging to CG8, and demonstrated that Pex26p recruits Pex1p-Pex6p complexes to peroxisomes (7,9). Pex1p and Pex6p are members of the large AAA-protein family involved in a wide range of different cellular processes including vesicular transport, DNA repair, proteolysis, and mitochondrial functions (10-12). One possible common functional feature of the AAA proteins is a protein folding or unfolding in an ATP-dependent manner. AAA proteins share one or two AAA-cassettes that is characterized by a conserved sequence of 200-250 amino acids, termed D1 or D2 domain, including the Walker A and B motifs for ATP-binding and ATP-hydrolysis (13,14). Additional less conserved domains are often found at N-terminal and C-terminal regions (N and C domains) and are implicated to be involved in binding to the adaptor (15-17). It is noteworthy that yeast and human AAA peroxins were recently suggested to be involved in the export of Pex5p from peroxisomes (18,19). However, the molecular mechanisms of Pex5p shuttling between the cytoplasm and peroxisomes remain elusive. Pex1p interacts with Pex6p in vivo (20-22). N
terminal region of Pex6p was suggested to bind to yeast Pex15p (23) or human Pex26p (7).

As a first step to understanding the function of AAA family peroxins, Pex1p and Pex6p, in peroxisome biogenesis, we herein mapped the region involved in mutual binding and investigated intracellular localization of Pex1p and Pex6p. Such interaction likely represents a relationship between peroxisomal localization and functions of AAA peroxins. Moreover, we propose that peroxisome biogenesis requires two distinct oligomer forms of Pex1p.

**EXPERIMENTAL PROCEDURES**

**Yeast Two-hybrid Assay**--- Yeast two-hybrid plasmids were constructed by inserting a desired gene fragment downstream of the GAL4 DNA binding domain (BIND) in pDBLeu and the GAL4 activation domain (ACT) in pPC86 (24). pPC-PEX1 was constructed by cloning BamHI-NotI fragment of Flag-HsPEX1 in pCMVSPORT (21) into the BamHI-NotI site of pPC86. Plasmids coding for N-terminal deletion mutants of Pex1p, Dral-NotI fragment of HsPEXI was ligated into the Smal-NotI site in pPC86. Plasmids for C-terminal deletion mutants of Pex1p were generated by replacing SpeI-NotI region of full-length PEX1 in pPC86 with SpeI-NotI fragment of the PCR products amplified using a forward primer P1F9 (5'-TGGTATCCGGTCAGGCTCTGTCAGAG-3') with a reverse primer P1-1151stopR (5'-CGCCATGCCCCATACGAC-3') with a reverse primer P1-1151stopR (5'-CGCCATGCCCCATACGAC-3'). To clone the full-length HsPEXI in pDB, the HindIII (blunted)-NotI site of the Flag-HsPEXI in pCMVSPORT (25) was ligated into SalI (blunted) and NotI sites in pDBLeu. For cloning of plasmids for C-terminal deletion mutants, pDB-Pex6p(1-676) and pDB-Pex6p(1-882) were generated by replacing BssHII-NotI fragment of PCMVSPORT-HsPEX6(1-676) and -HsPEX6(1-882) that had been constructed by replacing XhoI-NotI fragment of the PCR products amplified using a primer P6F2 (5'-GGGCTCGGACCGCAGTC-3') with P6-677stopR (5'-CGCCGCGCGCCGCCTAAGCCAGGAGGAGGACACG-3'), respectively. To construct plasmids for N-terminal deletion mutants of Pex6p, two types of SalI-NotI fragments were amplified by PCR using primers P6SalI385F (5'-GTGGTATCCGGTCAGGCTCTGTCAGAG-3') and P6SalI593F (5'-TGAGGTCAGGGTCGCTGCTGTCGAG-3') with P6-883stopR. Each fragment was cloned into the SalI-NotI site in pDBLeu, with following insertion of NotI-NhelI fragment of HsPEX6 at the NotI-NhelI site. Yeast two-hybrid assays were performed using a ProQuest two-hybrid system (Invitrogen, Carlsbad, CA). *Saccharomyces cerevisiae* MaV203 (*Mata, leu2-3, 112, trp1-901, his3Δ200, ade2-101, gal4Δ, gal80Δ, SPAL10::URA3, GAL1::lacZ, HIS3*), MATA::HIS3 at mutation sites were K605E (A1), sense 5'-AAAACTTGCCCCATACGAC-3') with a reverse primer P1-1151stopR (5'-CGCCATGCCCCATACGAC-3'). To clone the full-length HsPEXI in pDB, the HindIII (blunted)-NotI site of the Flag-HsPEXI in pCMVSPORT (25) was ligated into SalI (blunted) and NotI sites in pDBLeu. For cloning of plasmids for C-terminal deletion mutants, pDB-Pex6p(1-676) and pDB-Pex6p(1-882) were generated by replacing BssHII-NotI fragment of PCMVSPORT-HsPEX6(1-676) and -HsPEX6(1-882) that had been constructed by replacing XhoI-NotI fragment of the PCR products amplified using a primer P6F2 (5'-GGGCTCGGACCGCAGTC-3') with P6-677stopR (5'-CGCCGCGCGCCGCCTAAGCCAGGAGGACACG-3'), respectively. To construct plasmids for N-terminal deletion mutants of Pex6p, two types of SalI-NotI fragments were amplified by PCR using primers P6SalI385F (5'-GTGGTATCCGGTCAGGCTCTGTCAGAG-3') and P6SalI593F (5'-TGAGGTCAGGGTCGCTGCTGTCGAG-3') with P6-883stopR. Each fragment was cloned into the SalI-NotI site in pDBLeu, with following insertion of NotI-NhelI fragment of HsPEX6 at the NotI-NhelI site. Yeast two-hybrid assays were performed using a ProQuest two-hybrid system (Invitrogen, Carlsbad, CA). *Saccharomyces cerevisiae* MaV203 (*Mata, leu2-3, 112, trp1-901, his3Δ200, ade2-101, gal4Δ, gal80Δ, SPAL10::URA3, GAL1::lacZ, HIS3*) was used as a host strain (24). Two-hybrid manipulations and assays were conducted as recommended by the manufacturer, as described (24).

**Mutagenesis in the Walker Motifs of Pex1p and Pex6p**--- Oligonucleotide-directed site-specific mutagenesis was performed by overlap extension by PCR (26), using pCMVSPORT/HsPEXI as a template. Two sets of reactions were carried out: the first using primers of P1F5 (5'-ATGCATGCCTAGTCAGG-3'), corresponding to nucleotide residues 1276-1293 of HsPEXI cDNA, and antisense mutagenic oligonucleotide; the second using P1R3 (5'-CATCCCTGAGTCACCTG-3'), corresponding to residues 3194-3211 of HsPEXI cDNA, and sense mutagenic oligonucleotide. The mutagenic oligonucleotides with base-changes underlined at mutation sites were K605E(A1), sense 5'-AAGTGGAGAACATCAAATTTAGCCA-3' and antisense 5'-GTTTGACTCCACTCCCTCCTTCCC-3'; D662N(B2), 5'-AGGTGAATTTGAATTTG-3' and 5'-AAAGGTTTCCCTCTGTGGAGGAC-3'; D940N(A1), 5'-CATCCCTGAGTCACCTG-3' and 5'-AAGTGGAGAACATCAAATTTAGCCA-3'; and K887E(A2), 5'-AACAGGAGAACACGAGAACACG-3' and 5'-CATCCCTGAGTCACCTG-3'. The amplified products were combined and used as a template for the second PCR, using P1F5 and P1R3 as primers. Second PCR produced a 1936-base pair, amplified fragment containing the mutation and harboring *EcoRI* and SpeI sites.
Following the digestion with EcoRI and SpeI, the fragment was cloned into the EcoRI-SpeI sites each of pCMVSPORT/HsPEX1 and pCMVSPORT/HsPEX1-HA. Next, following sets of oligonucleotides were used for mutagenesis of HsPEX6. First, P6F3 (5'-TCTCCTGATAGCTGCTGCGAC-3') corresponding to residues 752–769 of HsPEX6 and antisense-5' AGTTCATT and sense-5' GTGGTCTC and antisense-5' AGGGTCTC digested with PCR products with 2192-base pairs were second using P6F3 and P6R1 primers. The second set of oligonucleotides used were K476E(A1), sense-5' CTAGCAGGCAGCAAACTTGCGCTGGATG and antisense-5' CACCGGCG, K750E(A2), 5'-AGAAGGTTGGGCCGGG-3' and 5'-GGGCCGGG-3' primer. The mutagenic oligonucleotides used were K476E(A1), sense-5' CTAGCAGGCAGCAAACTTGCGCTGGATG and antisense-5' CACCGGCG, K750E(A2), 5'-AGAAGGTTGGGCCGGG-3' and 5'-GGGCCGGG-3', respectively. All constructs were confirmed by sequencing.

Mammalian Two-hybrid Analysis---We used plasmids of the CheckMate system (Promega, Madison, WI). The reporter pG5lac expresses the firefly luciferase under the control of a minimal TATA box and five GAL4-binding sites. The coding sequences of PEX1 and PEX6 were cloned as BamHI-NotI fragments of pCMVSPORT–Flag–PEX1 and BamHI-NotI fragment of pCMVSPORT–Flag–PEX6, respectively, into the pBIND vector in frame with the yeast GAL4 DNA-binding domain, termed pBIND–PEX1 and pBIND–PEX6. The coding sequences of PEX1 and PEX6, namely the BamHI-NotI fragments of pCMVSPORT–Flag–PEX1 and pCMVSPORT–Flag–PEX6, were separately cloned into the pACT vector in frame with the herpes simplex virus VP16 activation domain, named pACT–PEX1 and pACT–PEX6. To generate Walker motif mutants of pACT–PEX1, pCMVSPORT–PEX1 harboring Walker motif mutations were digested with BamHI and SalI, and the 3.0-kb BamHI–SalI fragments were then ligated into the pACT–PEX1 vector. To construct in pBIND vector Walker motif mutants of PEX6 and deletion mutants including a CG4 patient-derived PEX6del207-292 (25), corresponding mutant forms of pCMVSPORT–PEX6 were digested with BssHI and BglII. Resulting BssHI-BglII fragments were separately inserted into the pBIND–PEX6 vector. DNA transfection into PEX1-defective, pex1 ZP107 and PEX6-deficient, pex6 ZP164 cells was achieved by the lipofection method using LipofectAMINE (Invitrogen) (27). Luciferase activity was
determined using $10^5$ cells with a luciferase assay system, PicaGene (Toyo Ink, Tokyo, Japan), and was represented as a fold-activation by taking as 1 the value with mock vectors, pACT and pBIND, termed relative light unit (RLU).

**Morphological Analysis**--- DNA transfection into CHO-K1, pex1 ZP107, and pex6 ZP164 cells was performed by the lipofection. Peroxisomes were visualized by indirect immunofluorescence light microscopy using rabbit antibodies against human catalase (25), PTS1 peptide (28), and influenza virus hemagglutinin (HA) (29). Antigen–antibody complexes were detected with fluorescein isothiocyanate-labeled goat antibodies against rabbit immunoglobulin G (MP Biomedicals-Cappel, Irvine, CA), under a Carl Zeiss Axioskop FL microscope. Peroxisome-restoring activity of the Walker motif mutants of PEX1 and PEX6 were verified by expressing in pex1 ZP107 and pex6 ZP164, respectively. The number of peroxisome-positive cells was counted in three separate areas.

**Subcellular Fractionation**--- HEK293 cells were homogenized on ice by ten strokes of an Elvehjem–Potter homogenizer in a homogenizing buffer: 0.25 M sucrose, 5 mM Hepes-KOH, pH 7.4, 25 μg/ml leupeptin, 25 μg/ml antipain. The homogenates were centrifuged at 750 x g for 5 min at 4°C. Post-nuclear supernatant (PNS) fraction was centrifuged at 100,000 x g for 1 h to separate organelles (heavy and light mitochondrial, microsomal fractions) and the cytosol.

**Blue Native-PAGE Analysis**--- The compositions of blue native gels and gel buffers were as described (30). The resolving gel contained an acrylamide gradient from 4 to 16%. Sample (15 μl) containing 50 μg of protein was mixed with 15 μl of 10 x Blue Native-PAGE sample buffer containing 0.3 M aminocaproic acid, 2 mM EDTA, 0.2 M Bis-Tris, pH 7.0, 5% Coomassie Brilliant Blue G-25 (Nacalai Tesque, Kyoto, Japan), and was incubated for 20 min on ice. The protein complexes were separated at 4°C for 4 h at 100 V. For Western blot analysis of the first dimension, the protein complexes were transferred to polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). For the second, denaturing gel dimension, a lane of the blue native gel containing the sample was cut into a strip of gel slice. The gel slices were equilibrated for 30 min at 60°C with SDS-PAGE sample buffer containing 2% SDS and 10 mM dithiothreitol. They were transferred into the slots of a 7.5% SDS-PAGE gel and electrophoresed at 30 mA.

**Other Methods**--- CHO cells were cultured in Ham's F-12 medium supplemented with 10% fetal bovine serum under 5% CO₂, 95% air (31). Western blot analysis on polyvinylidene difluoride membrane was done with primary rabbit antibodies against Pex1p (27), Pex6p (21), Pex14p (32), and Pex26p (7), and a second antibody, donkey anti-rabbit immunoglobulin G antibody conjugated with horseradish peroxidase, with ECL (enhanced chemiluminescence) Western blotting detection reagent (Amersham Biosciences, Tokyo, Japan).

**RESULTS**

**Anatomy of Pex1p and Pex6p**

We first attempted to define the regions of the two human AAA peroxins involved in the Pex1p-Pex6p interaction as well as those required for restoring the impaired protein import in respective pex1 and pex6 cell mutants. We constructed various truncated Pex1p and Pex6p variants and verified them for interaction by yeast two-hybrid assays. Patient-derived mutants, Pex1pΔ634-690 and Pex1pL664P, were abrogated in the interaction with Pex6p (Fig. 1A). According to the predicted domain structure (33,34), the sequence of Pex1p and Pex6p were divided into three parts: the N-terminal region, the AAA-cassette (D1 and D2), and the C-terminal region. The deletion of N-terminal region, such as 551-amino-acid truncation in Pex1p(552-1283), did not influence the Pex1p-Pex6p binding. C-terminal side deletion mutants, Pex1p(1-1216) and Pex1p(1-1150), showed a reduced and barely detectable level of the interaction with Pex6p. Thereby, it is more likely that the AAA-cassette and C-terminal part of Pex1p(552-1283) are sufficient for interacting with Pex6p (Fig. 1A). Moreover, based on the results using the patient PBDE-04-derived Pex1p mutants, D1 domain is critical for the binding to Pex6p and the C-terminal domain is also involved in the interaction. These results are consistent with the observation that Pex1p-Pex6p heteromic
complexes are assembled in an ATP-dependent manner (20).

Pex1p-binding site of Pex6p was similarly investigated with truncation mutants of Pex6p (Fig. 1B). In the yeast two-hybrid assays, Pex6p variants truncated in the N- and C-terminal region, Pex6p(385-980) and Pex6p(1-882), interacted with Pex1p. Regarding to the AAA-cassette of Pex6p, deletion of D1 (Pex6p(593-980)) or D2 (Pex6p(1-676)) significantly eliminated the interaction with Pex1p. Both N- and C-terminal regions of Pex6p might have a role for stabilizing interaction with Pex1p.

Pex1p(552-1283) and Pex6p(385-980) were sufficient to maintain the mutual interaction (Fig. 1C). Collectively, these findings demonstrate the importance of the AAA-cassette (D1 and D2) together with C-terminal region of both Pex1p and Pex6p for the stable interaction.

Various truncated forms of Pex1p and Pex6p were separately subcloned into mammalian expression vector pCMVSPORT. Pex1p variants were verified for complementing activity by expressing in PEX1-defective, pex1ZP107 cells. In ZP107 cells transfected with wild-type PEX1, numerous PTS1- and catalase-positive peroxisomes were detected in a punctate staining pattern, indicating the restoration of PTS1 protein and catalase import (Fig. 1D, a and b). In contrast, in ZP107 cells expressing Pex1p552-1283 PTS1- and catalase-positive particles were not discernible (Fig. 1D, c and d). We earlier reported that L664P and del634-690 mutants of Pex1p were incompetent to restore peroxisome biogenesis (27). Other truncation mutants shown in Fig. 1A also failed to restore import of PTS1 proteins and catalase in ZP107 cells. Similarly, PTS1- and catalase-positive particles were not discernible in PEX6-deficient, pex6 ZP164 cells that had been transfected with full-length PEX6 but not with all of the truncation mutants examined, including Pex6p(385-980) (Fig. 1D, e-h). Collectively, the N-terminal region and the AAA-cassette as well as the C-terminal part of Pex1p and Pex6p were required for the complementing activity.

Ternary-complex Formation of Pex1p, Pex6p, and Pex26p

Next, the interaction between Pex1p and Pex6p was likewise assessed by means of two-hybrid system in mammalian cells. Wild-type Pex1p was fused to ACT in pACT vector, and wild-type Pex6p was fused to BIND in pBIND vector. As a mock control, expression of only ACT-Pex1p or BIND-Pex6p in pex6 ZP164 cells did not show transcriptional activation of the reporter luciferase gene (Fig. 2B). Co-expression of ACT-Pex1p and BIND-Pex6p gave rise to a significant level of luciferase activity, indicative of positive interaction. We also performed the two-hybrid assay in PEX26-defective, pex26 ZP167 cells. Luciferase activity comparable to that seen in ZP164 was detected, hence indicating that the Pex1p-Pex6p interaction is less likely modulated by Pex26p (data not shown). Next, two plasmid constructs, one encoding wild-type Pex26p fused to transcriptional activation domain of GAL4 in pACT vector and the other containing wild-type PEX1 ligated into pBIND vector, were co-transfected into ZP164 cells with or without PEX6 in pCMVSPORT. Co-expression of ACT-Pex26p and BIND-Pex1p showed high luciferase activity in the presence of Pex6p, whilst the luciferase activity was very low in the absence of Pex6p similar to that by ACT-Pex26p alone (Fig. 2B). These results strongly suggested that Pex1p and Pex26p binding is dependent to Pex6p, consistent with our earlier report on the co-immunoprecipitation assays (7). Moreover, interaction between Pex6p and Pex26p was observed at a level slightly lower than that obtained in the presence of Pex1p (Fig. 2B).

Mutation of Walker Motif Affects the Function of Pex1p and Pex6p

The AAA-cassette D1 and D2 domains and C-terminal region are involved in the interaction of Pex1p and Pex6p (see Fig. 1A). Mutation of the lysine residue in the P-loop of the Walker A eliminates the ATP-binding, while that of acidic residues in the Walker B blocks the ATP hydrolysis (14). To determine whether these cassettes are essential for the Pex1p-Pex6p interaction, we introduced site-directed mutations into the Walker motifs A and B of Pex1p and Pex6p (Fig. 2A). We constructed Pex1p variants, termed A1(K605E) and A2(K887E), where lysine residues at positions 605 and 887 in the Walker motifs A1 and A2 were substituted by glutamic acid, and B1(D662N) and B2(D940N) with replacement of aspartic acid residue at 662 and 940 with asparagines in the Walker motifs B1 and B2. We likewise constructed Pex6p mutants, A1(K476E) and A2(K750E) in the Walker A and B2(D803N) in the Walker B. With respect
to B1 in Pex6p, the conserved aspartic acid residue in the Walker B motif was not present, rather replaced to threonine. Therefore, we tentatively introduced D532N mutation at three-amino-acid downstream, termed Pex6p(B1).

We verified these Walker motif mutants for interaction in the mammalian two-hybrid system. Co-expression of ACT-Pex1pB2(D940N) with BIND-Pex6p gave rise to about 80% luciferase activity as compared to the wild-type Pex1p, indicating a similar level of binding of B2 mutant to Pex6p (Fig. 2C). In contrast, the mutants, A1, B1, and A2, showed a clearly reduced level, lower than 30% of the wild-type Pex1p, of binding to Pex6p. Co-expression of BIND-Pex6p(B2) mutant with ACT-Pex1p showed the binding-activity similar to the Pex1p B2 mutant, whilst A1 and A2 mutants were abrogated in the interaction with ACT-Pex1p (Fig. 2C). Mutant BIND-Pex6p(B1) was not affected in binding to ACT-Pex1p. These results strongly suggested that the ATP binding to D1 and D2 domains of both Pex1p and Pex6p are essential for the interaction of these AAA peroxins. It is also possible that ATP hydrolysis by Pex1p B1 site is indispensable for the binding to Pex6p.

Interaction between ACT-Pex26p and BIND-Pex1p was assayed in the presence of Pex6p variants and represented as the activity relative to the luciferase activity on co-expression with wild-type Pex6p (Fig. 2D). We attempted to delineate the regions of Pex6p involved in the interaction of Pex26p and Pex1p, by co-expressing the Walker motif as well as truncation mutants of Pex6p with ACT-Pex26p and BIND-Pex1p. Pex6pB1(D532N) and Pex6pB2(D803N) showed a normal or a little higher level of Pex26p-Pex1p interaction as the wild type, whilst the Pex6pA1(K476E) and Pex6pA2(K750E) severely eliminated the interaction (Fig. 2D). These results coincided with a binding profile of the Pex6p Walker mutants to Pex1p (Fig. 2C). Pex6p(1-882) deleted by ~100 amino acids from the C-terminus and Pex6p(1-676) defective in binding to Pex1p showed ~80% and ~30% levels of the interaction of normal Pex6p with Pex1p. N-terminal deletion variants, Pex6p(154-980) and Pex6p(188-980), did not affect the Pex1p interaction with Pex26p (data not shown), whilst Pex6p(385-980) competent to bind to Pex1p and a CG4 PBD patient-derived Pex6pΔ207-292 apparently abolished the Pex1p interaction with Pex26p. These results suggest that a putative Pex6p domain involved in binding to Pex26p resides in the amino-acid residues 207-292, presumably forming a complex with Pex26p and Pex1p.

Pex6p variants were also verified for binding to Pex26p by mammalian two-hybrid assays (Fig. 2E). Pex6pA1(K476E), Pex6pA2(K750E), and Pex6p(1-676) showed weaker interaction with Pex26p at 30-60% of the wild-type Pex6p, while the putative (B1, D532N) apparently bound to Pex26p as the wild type (Fig. 2E). Pex6pΔ207-292 was inactive in interacting with Pex26p. Pex6pB2(D803N) and Pex6p(1-882) showed nearly two-fold enhanced level of the interaction, suggesting that the inhibition of ATP hydrolysis at D2 domain prevented the Pex6p release from Pex26p, consistent with the finding by Birschmann et al. (23). Furthermore, it is more likely that for the binding of Pex6p to Pex26p its D1 and D2 domains are required to be in an ATP-bound form.

Next, we assessed the complementing activity of the Walker motif mutants by expressing in the peroxisome-deficient pex1 and pex6 cell mutants. PTS1-positive particles were discernible in ZP107 cells that had been transfected with PEX1B2(D940N) but not with PEX1A2(K887E), suggesting that PTS1 protein import required ATP binding to Pex1p D2 domain, not ATP hydrolysis (Fig. 3A, upper panel). In contrast, catalase-positive particles were barely detectable in both types of ZP107 cells transfected with PEX1A2(K887E) and PEX1B2(D940N), suggesting that catalase import requires both ATP binding and hydrolysis on Pex1p D2 domain. On the other hand, PEX1A1(K605E) and PEX1B1(D662N) mutations decreased to approx. 30-50% activity in the import of PTS1 proteins and catalase (Figs. 3A, upper panel and 3B, left panel). The impaired import of PTS1 proteins and catalase in pex6 ZP164 cells were very weakly restored by a Pex6p variant, Pex6pA1(K476E), but not with Pex6pA2(K750E) and Pex6pB2(D803N) (Figs. 3A, lower panel and 3B, right panel). These results suggested that ATP binding to Pex6p D1 and D2 domains and hydrolysis by D2 domain were indispensable in PTS1 proteins and catalase import. It is of interest to note that the D532N mutation in Pex6p(B1) does not affect such Pex6p activities (Figs. 3A, lower panel and 3B, right panel).

Intracellular Localization of Pex1p and Pex6p
We investigated subcellular localization of Pex1p and Pex6p. In CHO-K1 and pex6 ZP164 cells, Pex6p-HA was expressed and detected by staining with anti-HA antibody. In both types of cells, Pex6p-HA was discernible in a punctate-staining pattern (Fig. 4A, g and i) and in a manner superimposable on Pex1p-positive particles, peroxisomes (g and h). Pex1p-HA was likewise detected in punctate HA-staining pattern when expressed in CHO-K1 and pex1 ZP107 cells (Fig. 4A, a and d). HA-tagging to Pex1p and Pex6p did not affect their peroxisome-restoring activity in ZP107 and pex6 ZP164 cells, respectively (data not shown). A low level of cytosolic staining of Pex6p-HA was also seen. Thus, these results suggested that Pex1p-HA and Pex6p-HA were mostly localized to peroxisomes and partly in the cytosol. Moreover, ZP164 cells were transfected with PEX1-HA to assess the requirement of Pex6p for peroxisomal localization of Pex1p. Punctate staining pattern of Pex1p-HA was detectable upon co-expression of Pex6p (Fig. 4A, e), consistent with our earlier observation (7) and in a merged view with Pex14p-positive peroxisomes (data not shown), while it was diffused in the cytosol in ZP164 (c). Similarly, PEX6-HA was introduced into ZP107 cells. The peroxisomal localization of Pex6p-HA was re-established by co-expression of Pex1p (Fig. 4A, j), whereas it remained in the cytosol in the absence of Pex1p (Fig. 4A, f). These results confirmed that Pex1p-HA is localized to peroxisomes in a Pex6p-dependent manner. Peroxisomal localization of Pex6p-HA apparently requires its interaction with Pex1p in vivo, although Pex6p showed binding to Pex26p in the absence of Pex1p by in vitro pull-down assay (7). Collectively, it is more likely that the binding of Pex1p and Pex6p is essential for their localization to peroxisomes.

We determined intracellular localization of the Walker motif mutants of Pex1p and Pex6p by expressing in CHO-K1 cells. Pex1pB2(D940N)-HA was detected in a punctate staining pattern as wild-type Pex1p-HA (Fig. 4B, d), coincided with Pex1p-positive peroxisomes (data not shown), whilst Pex1pA1(K476E)-HA, Pex1pA2(K750E)-HA, and Pex1pB1(D662N)-HA, and Pex1p(A2K887E)-HA were diffused in the cytosol (Fig. 4B, a-c). Pex1pL664P-HA and Pex1pA2(K887E)-HA were also localized in the cytosol when expressed in ZP107 (Fig. 4C, a and b). Taken together, it is apparent that peroxisomal localization of Pex1p and its variants correlate well with their activity in binding to Pex6p. With respect to Pex6p, Pex6pB2(D803N)-HA was localized to punctate-stainable structures as the wild type (Fig. 4B, g), merged with Pex1p-positive peroxisomes (data not shown), whilst Pex6pA1(K476E)-HA and Pex6pA2(K750E)-HA were detectable in the cytosol (Fig. 4B, e and f). D532N-mutation in Pex6p did not affect the peroxisomal localization of Pex6p (data not shown). Therefore, Peroxisomal localization of Pex1p and Pex6p very likely requires their heteromeric interaction mediated by ATP binding of respective D1 and D2 domains. A Pex6p truncation mutant, Pex6p(1-882)-HA, was localized to peroxisomes in ZP164 (Fig. 4C, c), as assessed by dual-staining of HA with Pex1p (data not shown), whereas Pex6p(1-876)-HA and Pex6p(385-980)-HA were apparently in the cytosol (Fig. 4C, c and d). The data described here were summarized in Fig. 4D, suggesting that peroxisomal localization of Pex6p requires two distinct regions to be localized to peroxisomes, the domain interacting with Pex1p and the N-terminal region including a putative site for binding to Pex26 (see Fig. 2D).

### Homologous Interaction of AAA Peroxins

AAA proteins generally assemble into homooligomers such as a hexamer to be a biologically active form (14). However, we could not show such homo-oligomerization of Pex1p and Pex6p in the yeast two-hybrid assays. We performed here essentially the same assays using a mammalian two-hybrid system. ACT and BIND domains were each fused to N- or C-terminus of Pex1p and expressed in Pex6p-deficient ZP164 cells (Figs. 5A and 5B). Co-expression or separate expression of ACT-Pex1p and BIND-Pex1p showed barely detectable luciferase activity, indicative of neither interaction nor autoactivation (Fig. 5B; see Fig. 2B). Alternative combination of Pex1p-ACT with BIND-Pex1p resulted in strong interaction, whilst ACT-Pex1p with Pex1p-BIND and Pex1p-ACT with Pex1p-BIND showed weaker binding (Fig. 5B). Pex1p-ACT and Pex1p-BIND showed no autoactivation. Thus, it is likely that ACT and BIND domains fused to N-terminus of Pex1p spatially hinder the homo-oligomerization. In contrast, co-expression with Pex6p appeared to significantly prevent homomeric-interaction of Pex1p (Fig. 5B), thereby suggesting that Pex6p disrupted homooligomeric conformation of Pex1p or formed a
hetero-oligomer with Pex1p. Co-expression with Pex6p and Pex26pN enhanced homo-interaction between ACT-Pex1p and BIND-Pex1p, apparently representing a ternary complex on peroxisomes, while ACT-Pex1p and BIND-Pex1p showed nearly no interaction (Fig. 5B). In the binding assays for Pex6p in Pex1p-deficient ZP107 cells, ACT-Pex6p, Pex6p-BIND, Pex6p-ACT , and BIND-Pex6p showed no autoactivation (Fig. 5C; see Fig. 2B). Interaction between Pex6p-ACT and Pex6p-BIND was distinct, while ACT-Pex6p and Pex6p-BIND weakly interacted (Fig. 5C). Addition of Pex1p to Pex6p-ACT with Pex6p-BIND and ACT-Pex6p with Pex6p-BIND reduced the Pex6p interaction, implying that Pex1p may regulate the Pex6p interaction. Expression of ACT-Pex6p with BIND-Pex6p and Pex6p-ACT with BIND-Pex6p resulted in barely detectable interaction. Taken together, Pex1p likely forms two distinct types of oligomers, a homo-oligomer in the cytosol and a hetero-oligomer with Pex6p and Pex26p on peroxisome membrane, both possibly functioning in peroxisome biogenesis.

In Vivo Homo-oligomer of Pex1p

The intracellular localization of AAA peroxins, Pex1p, is still controversial despite the studies in several species of organisms. We attempted to determine the intracellular localization of Pex1p by morphological and biochemical methods. In HEK293 cells stained with antibodies to Pex1p and Pex14p, Pex1p was discernible mostly in a punctate-staining pattern and in a manner superimposable with Pex14p-positive peroxisomes (Fig. 6A, a and b). Cytosolic staining of Pex1p was barely visible. Next, we performed subcellular fractionation of HEK293 cells. Following centrifugation, PNS fraction was separated into cytosolic and organelle fractions. Endogenous Pex1p was detected by immunoblot in the organelles and less in the cytosol (Fig. 6B, lane 1 and 2). In contrast, Pex6p was detected mostly in organelle fraction and barely in the cytosol (Fig. 6B, lane 3 and 4). Peroxisomal membrane peroxins, Pex14p and Pex26p, were exclusively present in the membrane fractions, not in cytosol (Fig. 6B, lane 5-8), thereby indicating adequate separation of organelle and cytosol fractions. Together, we interpret the results to mean that Pex1p is partly present in cytoplasm without forming a complex with Pex6p and Pex26p.

To define the molecular properties of the cytosolic Pex1p, we analyzed the cytosolic fraction from HEK293 cells by Blue Native-PAGE and immunoblot using anti-Pex1p antibody. Pex1p was detected as two distinct protein complexes, one with 450 kDa and the other less in amount with 900 kDa (Fig. 6C, lane 1). These two bands were separated by SDS-PAGE. Pex1p was detected as a 150-kDa band from the 450-kDa complexes, indicating that Pex1p was a constituent (Fig. 6C, lane 2). However, Pex1p was hardly detectable from the 900-kDa complexes, presumably under the detection level with the antibody used. Pex6p was not detectable in these two forms of complexes with anti-Pex6p antibody (data not shown). Together, it is likely that cytosolic Pex1p is in two sizes of homo- or hetero-oligomers.

DISCUSSION

In this study, we identified the regions involved in the interaction of human Pex1p and Pex6p. We showed by yeast two-hybrid analysis that two AAA-cassette structures (D1 and D2) of Pex1p and Pex6p are required for their association. To assess the role of ATP for Pex1p-Pex6p interaction, two different mutations were chosen to inactivate the D1 and D2 cassettes of Pex1p and Pex6p in analogy to the reported mutations of NSF (35), one preventing ATP-binding (A1 and A2), and the other preventing ATP-hydrolysis (B1 and B2). Further analysis using mammalian two-hybrid system elucidated the importance of ATP-binding to the two AAA-cassettes of Pex1p and Pex6p in analogy to the reported mutations of NSF (35), one preventing ATP-binding (A1 and A2), and the other preventing ATP-hydrolysis (B1 and B2). Further analysis using mammalian two-hybrid system elucidated the importance of ATP-binding to the two AAA-cassettes of Pex1p and Pex6p for their interaction (Fig. 7). Recently, Birschmann et al. (22) assigned the sites responsible for the interaction of Pex1p and Pex6p of Saccharomyces cerevisiae. They showed that the interaction of Pex1p and Pex6p involves the respective first AAA-cassette, and also demonstrated that ATP-binding but not ATP-hydrolysis in the second AAA-cassette (D2) of Pex1p is required for the Pex1p-Pex6p interaction. In contrast to this yeast system, the interaction of human Pex1p and Pex6p requires ATP binding to both D1 and D2 domains, suggesting that the interaction is enhanced by conformational changes in both D1 and D2 domains upon ATP binding.

We earlier reported that the dysfunction of PEX26 is responsible for PBD of CG8 (7), and Pex26p recruits Pex1p-Pex6p complexes to

- 8 -
peroxisomes via Pex6p (7). In this study, we verified the relationship between peroxisomal localization and the interaction of Pex1p-Pex6p hetero-oligomer. The data presented here demonstrate the importance of Walker motif A1, B1 and A2 in Pex1p, and Walker motif A1 and A2 in Pex6p, for their peroxisomal localization, in good agreement with the finding in binding assays of Pex1p-Pex6p hetero-oligomer. Additionally, we found by morphological analysis that neither Pex1p nor Pex6p was localized to peroxisomes in the absence of its mutual partner, suggesting that the localization of Pex1p and Pex6p on peroxisomes requires the formation of the ternary complexes in vivo. On the other hand, we also showed in our earlier report that the recombinant Pex6p bound to Pex26p fused to glutathione S-transferase in a pull-down assay (7). Furthermore, in the present work, we demonstrated in mammalian two-hybrid assays that Pex6p act as a bridge between Pex26p and Pex1p and that Pex26p bound to Pex6p in the absence of Pex1p. Similarly, Weller et al. (36) very recently showed that Pex26p tagged with nuclear localization signal bound to Pex6p in nucleus. These results raise several possibilities with respect to the relationship between the ternary complexes and peroxisomal localization. Pex1p may stabilize Pex6p by forming the ternary complex on peroxisomes. It is also possible that Pex1p and Pex6p may interact with other peroxins that regulate the subcellular localization and function of AAA peroxins.

Pex1p, Pex6p and Pex26p were localized predominantly to peroxisomes, upon coexpression in pex26 ZP167 cells, consistent with the results in immunolocalization study of these peroxins. Pex1p was also detected in the cytosolic fraction of HEK293 cells, suggesting that Pex1p is also in the cytosol without interacting with Pex6p and Pex26p. Based on the findings in the present study, it is more likely that Pex1p-Pex6p interaction is dispensable for their peroxisomal localization. Although Pex1p harboring mutations in the Walker motif A1 and B1 was not localized to peroxisomes owing to the impaired interaction with Pex6p, these Pex1p mutants partially restored peroxisomal import of PTS1 proteins and catalase, with nearly 50% efficiency as compared to the normal Pex1p. Recently, we reported several PBD patient-derived Pex26p mutants showing insufficient binding to Pex1p-Pex6p complexes (9), hence inferring that Pex1p was likely in the cytosol. Such Pex26p mutants were apparently competent in PTS1 import in the patients’ fibroblasts. Taken together, a part of Pex1p in the cytoplasm is responsible for the transport of PTS1 proteins, but not catalase. In regard to the second AAA-cassette (D2) of Pex1p, ATP-binding was dispensable for the transport of both PTS1 proteins and catalase, where ATP-hydrolysis was essential for import of catalase. We have previously reported that the import of PTS1 protein seemed to be normal in fibroblasts from PEX1-defective CG1 IRD patients, whilst the import of catalase and PTS2 proteins was impaired at normal temperature, 37°C (37,38). At permissive 30°C, catalase and PTS2 import was normalized. Thereby, it is likely that ATP-hydrolyzing activity of Pex1p variants differentiates such two distinct types of protein import.

We detected homomeric interaction of Pex1p in mammalian two-hybrid assays and Pex1p oligomers, maybe a trimer and a hexamer, in the cytosol of HEK293 cells. Similarly, Pex6p showed weak homo-binding. In two-hybrid system, we usually fuse the activation and DNA-binding domains to the N-terminus of target proteins. In this present study, we assessed homo-binding assay using various proteins by fusing to not only to the N-terminus but also to the C-terminus. Pex1p indeed showed high homo-binding activity by a combination of BIND-Pex1p and Pex1p-ACT. The interaction of N-terminally fused proteins, BIND-Pex1p and ACT-Pex1p, were enhanced by addition of Pex6p together with Pex26p, whereas homo-binding of BIND-Pex1p and Pex1p-ACT was disrupted by addition of Pex6p. These results suggested that Pex1p shows dynamic conformational changes in the presence or absence of Pex6p and Pex26p. Furthermore, Pex1p forms two distinct oligomeric structures on peroxisomal membrane or in the cytoplasm. As depicted by Blue-Native PAGE analysis of cytosolic Pex1p from HEK293 cells, Pex1p is mostly in a homo-trimer and less in a homo-hexamer. These results are similar to the findings in the structural study on NSF (39,40), another member of the AAA-protein family. NSF forms cylindrical homo-oligomeric complexes, of which ATP-hydrolytic activity is essential for the membrane fusion. Pex1p and Pex6p were shown to be required for the fusion of peroxisomal membranes in the yeast Yarrowia lipolytica (41).
With regard to Pex6p, the mutations at A1 and A2 inactivating ATP-binding activity, reduced the efficiency of binding to Pex1p, consistent with the lowered level of peroxisomal localization. In addition, Walker motif mutants, A1, A2 and B2, could not restore the import of matrix proteins, as assessed by expression in pex6 ZP164 cells. N-terminal region including amino-acid residues 207-292 is more likely involved in binding to peroxisomes, even though this deletion mutant is competent to bind to Pex1p. Furthermore, B2 mutation in Pex6p increased binding efficiency to Pex26p, implying that ATP-hydrolysis catalyzed by D2 domain is required to dissociate Pex6p from Pex26p. Taking together, it is more likely that N terminal part of Pex6p provides the binding site for Pex26p and that ATP-binding to D1 and D2 is required for stabilizing the interaction of Pex6p to Pex26p (Figs. 2D and 7). On the other hand, ATP-hydrolysis by D2 is indispensable for Pex6p to dissociate from Pex26p. Very recent reports (18,19) suggested that Pex1p and Pex6p are involved in the regulation of translocation cycle of Pex5p, including the export step from peroxisomes. Moreover, Pex26p may act as a scaffold protein to recruit the target proteins, if any, of ATP-hydrolysis by Pex1p and Pex6p. It is also possible that D2 domain of Pex6p provides a driving force for dissociation of protein complexes involved in a recycling step of Pex5p. Nevertheless, our findings suggested that there is a functional analogy between Pex1p/Pex6p and NSF at least in mammalian cells. The AAA-family peroxins likely play multiple roles in peroxisome biogenesis.

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FOOTNOTES

The abbreviations used are: ACT, Gal4 transactivation domain; BIND, Gal4 DNA binding domain; CG, complementation group; CHO, Chinese hamster ovary; HA, influenza virus hemagglutinin; NSF, N-ethylmaleimide-sensitive fusion protein; PBD, peroxisome biogenesis disorder; PNS, postnuclear supernatant; PTS1 and PTS2, peroxisome targeting signal types 1 and 2.

FIGURE LEGENDS

Fig. 1. Functional domain mapping of Pex1p and Pex6p. 

A, schematic representation of truncation mutants of human Pex1p (*Hs* Pex1p, left panel). N, D1, D2, and C designate N-terminal region, D1 domain, D2 domain, and C-terminal region, respectively. The Walker motifs in D1 and D2 domains were indicated as A1/B1 and A2/B2, respectively. SRH, the second region of homology. Interaction of Pex1p with Pex6p was verified by yeast two-hybrid assay. Host strain MaV203 was transformed with two plasmids, one encoding the Pex1p mutants and the other coding for full-length Pex6p. Pex1p variants derived from PBD patient E-04 were also assessed.
The transformants were tested for growth on SC medium lacking leucine, tryptophan and histidine in the presence of 10 mM 3-amiotriazole (-His + 3AT) (right panel). Complementation of the impaired import of peroxisomal proteins, PTS1 and catalase, in CHO pex1 ZP107 were from assays as in panel D. B, interaction of human (Hs) Pex6p variants with Pex1p was likewise assessed as in panel A. N, D1, D2, and C are as in panel A. Restoration of import of peroxisomal proteins was assessed with CHO pex6 ZP164 cells as in panel D. C, minimal domains in Pex1p-Pex6p binding. Pex1p(552-1283) and Pex6p(385-980), apparently the shortest sequences of Pex1p and Pex6p shown in panels A and B, were similarly verified. D, mutants of Pex1p and Pex6p were assayed for peroxisome-restoring activity in ZP107 and ZP164 cells, respectively. Left, ZP107 cells were transfected with cDNAs each for Pex1p (a and b) and Pex1p(552-1283) (c and d) and stained with antibodies to PTS1 (a and c) and catalase (b and d). Right, Pex6p and Pex6p(385-980) were similarly verified in ZP164 cells. Bar, 20 μm.

**FIG. 2.** Ternary-complex formation of Pex1p, Pex6p, and Pex26p. A, schematic representation of mutation sites in Pex1p and Pex6p. N, D1, D2, and C are as in Fig. 1A. The B1 site of D1 domain in Pex6p was not conserved (T 529), where 3-amino-acid downstream D 532 was instead mutated to N, hence indicated as (B1). B, Pex6p aids Pex26p-Pex1p interaction. Mammalian two-hybrid interaction assays were performed. pG5 luc reporter plasmid was co-transfected into CHO pex6 ZP164 cells with pACT plasmid encoding ACT fused to Pex1p or Pex26p and pBIND coding for BIND fused to Pex6p or Pex1p, as indicated. -, plasmid encoding mock fusion protein. PEX6 and PEX1 in pCMVSPORT were transfected to ZP164 together with pACT-PEX26/pBIND-PEX1 and pACT-PEX26/pBIND-PEX6, respectively. Luciferase activity of cell lysates was determined at 2 d after the transfection and was expressed as relative light units (RLU) as described in Experimental Procedures. Values (RLU x 10^{-2}) are means ± standard deviations (S.D.) of three experiments. C, mutations in the Walker motif affect the Pex1p-Pex6p interaction. Binding of Pex1p and its Walker mutants to Pex6p was similarly verified in CHO pex1 ZP107 cells. Interaction with Pex1p of Pex6p Walker motif mutants was assessed in ZP164 cells. Luciferase activity was determined as in (A) and expressed as percentages of that of normal Pex1p and Pex6p. D, interaction of Pex1p with Pex26p is dependent on Pex6p. ZP164 was co-transfected with pACT-PEX26/pBIND-PEXI and pACT-PEX26/pBIND-PEX6, respectively. Luciferase activity of cell lysates was determined at 2 d after the transfection and was expressed as relative light units (RLU) as described in Experimental Procedures. Values (RLU x 10^{-2}) are means ± standard deviations (S.D.) of three experiments. E, mutation in the Walker B2 in D2 region enhances the Pex6p binding to Pex26p. Interaction between Pex6p mutants and Pex26p was verified in ZP164 and expressed as percentages of that of wild-type Pex6p and Pex26p.

**FIG. 3.** Mutation of the Walker motif affects the Pex1p and Pex6p function in matrix protein import. A, Walker motif mutants of PEX1 and PEX6 were verified for peroxisome-restoring activity by expressing in pex1 ZP107 (upper panels) and pex6 ZP164 (lower panels), respectively. Cells were cultured for 3 d at 37°C and were stained with antibodies to PTS1 and catalase. Immunofluorescent pictures for catalase in ZP107 cells transfected with PEX1A2 or PEX1A1mutant (upper panels) were taken after a longer exposure, where in addition to cytosolic staining an apparently punctate staining pattern was partly discernible but distinct from that of Pex14p-positive structures (not shown). Magnification 630 x; bar = 10 μm. B, results obtained in panel A were represented as percentages of the import-restoring activities of wild-type Pex1p in ZP107 and Pex6p in ZP164. The number of peroxisome-positive cells was the mean of total cells number counted in three separate areas, where the deviation was within ±15% of the respective mean values. In the cells transfected with the wild-type Pex1p and Pex6p, 70-80% cells were complemented.

**FIG. 4.** Intracellular localization of Pex1p and Pex6p. A, human Pex1p and Pex6p were tagged with an epitope HA at the C-terminus. pex1 ZP107 cells were transfected with PEX1-HA (a and b), PEX6-HA (f), or PEX6-HA plus PEX1 (j). Transfection to pex6 ZP164 cells was likewise done with PEX1-H4 (c), PEX6-H4 (g and h), or PEX1-H4 plus PEX6 (e). As a control, CHO-K1 cells were transfected with PEX1-HA (d) and PEX6-HA (i). Cells were stained with antibodies to HA (a, c-g, i, and j) and Pex14p (b and h). Magnification 630 x; bar = 10 μm. B, Pex1p-HA and Pex6p-HA, each harboring point mutations in Walker motifs A and B as indicated at the top, were expressed in ZP107 (upper panels) and ZP164 (lower panels), respectively.
Pex6p with D532N mutation downstream of the non-conserved B1 was not included. Cells were stained with anti-HA antibody. Bar, 10 μm. C, PBD patient PBDE-04-derived PEX1L664P-HA (a) and PEX1del634-690-HA (b) were expressed in ZP107. Truncated PEX6 variants, including PEX6(1-882)-HA (c), PEX6(1-676)-HA (d), and PEX6(385-980)-HA (e), were expressed in ZP164. Cells were stained as in panel A. Bar, 10 μm. D, several variants of Pex1p and Pex6p were verified for Pex1p-Pex6p interaction and binding to Pex26p.

FIG. 5. Homologous interaction of AAA peroxins. 
A, schematic diagram of the fusion constructs of Pex1p and Pex6p with ACT and BIND domains used for mammalian two-hybrid assays. B, Pex1p forms homo-oligomer and ternary complexes with Pex6p and Pex26p. Two-hybrid assays were done in pex6 ZP164, as in Fig. 2B. Values are means ± S.D. from three experiments. C, homo-interaction of Pex6p was similarly analyzed in pex1 ZP107.

FIG. 6. In vivo homo-oligomer of Pex1p.
A, HEK293 cells were dual-stained with antibodies to Pex1p (a) and Pex14p (b). Bar = 10 μm. B, cytosolic (Cyt) and organelle (Mb) fractions from PNS fraction of HEK293 cells (2 x 10⁶) were analyzed by SDS-PAGE and immunoblotting with antibodies to Pex1p (lanes 1 and 2), Pex6p (lanes 3 and 4), Pex26p (lanes 5 and 6), and Pex14p (lanes 7 and 8). Arrowheads indicate respective peroxins. Dots designate nonspecific bands (7). Molecular mass markers are on the left. C, cytosolic fraction was separated by Blue-Native PAGE. In the first dimension, protein complexes were detected by immunoblotting with anti-Pex1p antibody (lane 1). Molecular mass markers are at the top. In the second, denaturing gel-dimension, the lane of the Blue-Native PAGE gel containing the sample was cut into a strip of gel slice, transferred into a slot of SDS-PAGE 7.5% gel, and electrophoresed. Protein band was detected by immunoblotting with anti-Pex1p antibody (lane 2). Molecular markers are on the left.

FIG. 7. Mechanistic model for Pex1p-Pex6p interaction and ternary complexes with Pex26p. Cytosolic Pex1p is predominantly in homo-trimer and partly hexamer. Pex1p oligomer is assembled to oligomer complexes with Pex6p. Walker motifs (A1, B1, and A2) of AAA domain are required for the interaction of Pex1p and Pex6p and their localization to peroxisomes (+), whilst the motif (B2) is dispensable (-). In contrast, ATP hydrolysis by B2 of Pex1p is essential for catalase import and that by Pex6p B2 initiates the release of Pex6p from Pex26p.
**A**  
*Hs Pex1p*

|  | N | D1 | D2 | C | Binding to Pex6p | PTS1/cat. import |
|---|---|----|----|---|------------------|-----------------|
| 1 | A1B1 A2B2 SRH | - |  | + | + |
| (PBDE-04) | L664P |  |  | - | - |
| 1 | 633 691 |  |  | - | - |
| (PBDE-04) | 1216 |  |  | +/- | - |
| 1 | 552 1283 |  |  | + | - |

**B**  
*Hs Pex6p*

|  | N | D1 | D2 | C | Binding to Pex1p | PTS1/cat. import |
|---|---|----|----|---|------------------|-----------------|
| 1 | A1B1 A2B2 SRH | - |  | + | + |
| 1 | 676 |  |  | - | - |
| 1 | 385 980 |  |  | + | - |
| 1 | 593 980 |  |  | +/- | - |

**C**  
*Hs Pex1p*  
*Hs Pex6p*

|  | -His+3AT  
(10 mM) | Binding to Pex1p | PTS1/cat. import |
|---|----------------|------------------|-----------------|
| 1 | 552 1283 |  | + |
| 1 | 385 980 |  | + |

**D**  
*pe1 ZP107*  
*pe6 ZP164*

|  | αPTS1 | αcatalase |
|---|--------|-----------|
| Pex1p | c | b |
| Pex6p | a | a |

**Fig. 1**
Fig. 2

A

Hs Pex1p

Hs Pex6p

B

(pex6 ZP164)

Luciferase activity (RLU x 10^-2)

0 20 40 60 80 100 120

pACT 1 1 1 1 1 1 1

pBIND 6 6 6 6 6 6 6

Pex1p - - - - - - -

Pex6p - - - - - - -

C

(pex1 ZP107) (pex6 ZP164)

Pex1p/6p binding (%)

0 20 40 60 80 100 120

pACT1 + + + + + + +

pBIND6 + + + + + + +

Pex1p - - - - - - -

Pex6p - - - - - - -

D

(pex6 ZP164)

Pex1p/26p binding (%)

0 20 40 60 80 100 120 140 160 180 200

pACT26 + + + + + + +

pBIND1 + + + + + + +

Pex6p - - A1(B1) A2 B2

Pex1p - - - - - - -

E

(pex6 ZP164)

Pex6p/26p binding (%)

0 20 40 60 80 100 120 140 160 180 200

pACT26 + + + + + + +

pBIND6 + A1(B1) A2 B2

Pex6p - 1-882 1-682 del207-292

Pex1p - - - - - - -
Fig. 3
Fig. 4
Fig. 5
Fig. 6

A

HEK293

\(\alpha\text{Pex1p}\) \(\alpha\text{Pex14p}\)

\[\text{a} \quad \text{b}\]

B

Cyt Mb

Pex1p

1 2

207 117 95

(kDa)

Cyt Mb

Pex1p

3 4

51

(kDa)

Cyt Mb

Pex1p

5 6

35 51

(kDa)

Cyt Mb

Pex1p

7 8

88 51

(kDa)

C

Blue Native-PAGE

669 440 232 140 (kDa)

1

207

(kDa)

117

(kDa)

Fig. 6
Fig. 7

Pex1p homo-oligomer in the cytoplasm
association with Pex6p
Pex1p-Pex6p interaction
Ternary-complex formation with Pex26p

dissociation

catalase import

N
C

Pex1p
Pex6p
Pex26p

cytosol
peroxisome

A1 B1 A2 B2 A1 A2 B2

trimer > hexamer
Dynamic and functional assembly of the AAA peroxins, Pex1p and Pex6p, and their membrane receptor Pex26p
Shigehiko Tamura, Shinobu Yasutake, Naomi Matsumoto and Yukio Fujiki

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