Homo- and Heterodimerization of Peroxisomal ATP-binding Cassette Half-transporters*

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Mammalian peroxisomal proteins adrenoleukodystrophy protein (ALDP), adrenoleukodystrophy-related protein (ALDRP), and 70-kDa peroxisomal protein (PMP70) belong to the superfamily of ATP-binding cassette (ABC) transporters. Unlike many ABC transporters that are single functional proteins with two related halves, ALDP, ALDRP, and PMP70 have the structure of ABC half-transporters. The dysfunction of ALDP is responsible for X-linked adrenoleukodystrophy (X-ALD), a neurodegenerative disorder in which saturated very long-chain fatty acids accumulate because of their impaired peroxisomal β-oxidation. No disease has so far been associated with mutations of adrenoleukodystrophy-related or PMP70 genes. It has been proposed that peroxisomal ABC transporters need to dimerize to exert import functions. Using the yeast two-hybrid system, we show that homo- as well as heterodimerization occur between the carboxyl-terminal halves of ALDP, ALDRP, and PMP70. Two X-ALD disease mutations located in the carboxyl-terminal half of ALDP affect both homo- and heterodimerization of ALDP. Co-immunoprecipitation demonstrated the homodimerization of ALDP, the heterodimerization of ALDP with PMP70 or ALDRP, and the heterodimerization of ALDRP with PMP70. These results provide the first evidence of both homo- and heterodimerization of mammalian ABC half-transporters and suggest that the loss of ALDP dimerization plays a role in X-ALD pathogenesis.

The peroxisomal membrane is equipped with four ABC-transporting cassette (ABC)1 transporters, which include the adrenoleukodystrophy protein (ALDP) (1), the adrenoleukodystrophy-related protein (ALDRP) (2), the 70-kDa peroxisomal membrane protein (PMP70) (3), and the PMP70-related protein (4, 5). Typical mammalian ABC transporters, like the multidrug-resistant P-glycoprotein, are single functional proteins with two related halves comprised of one hydrophilic transmembrane domain and one hydrophilic nucleotide-binding fold (NBF) (6). In contrast, the peroxisomal ABC transporters, as well as TAP1/TAP2 (7) and ABC7 (8), which are respectively located within the endoplasmic reticulum or mitochondria, are half-transporters with only one hydrophilic domain and one NBF.

X-linked adrenoleukodystrophy (X-ALD) is the only genetic disease known to result from a peroxisomal ABC transporter gene defect. This neurodegenerative disorder is characterized by progressive demyelination within the central nervous system, adrenal insufficiency, and accumulation of very long-chain fatty acids because of an impaired peroxisomal β-oxidation (9–12). Although it is firmly established that the loss of ALDP function is responsible for the abnormality in VLCFA metabolism, its precise role is unknown. Similarly, no precise function has been assigned to ALDRP, PMP70, or PMP70-related proteins. ALDRP, PMP70, and PMP70-related protein present 66, 38, and 27% amino acid identity with ALDP, respectively, suggesting functional similarity of these four transporters. These proteins display specific but sometimes overlapping patterns of expression in different cell types (13–15). Because it is likely that half-transporters need to dimerize to exert their function (6), this raises the possibility that different types of peroxisomal ABC dimers could allow the import of distinct substrates. Few ABC transporters are known to dimerize. Genetic evidence suggests that the bacterial hemolysin transporter B ABC half-transporter forms homodimers (16), whereas the Drosophila white, brown, and scarlet gene products form heterodimers (17). Heterodimerization of ABC transporters has also been reported for the transporters of antigenic peptides, TAP1 and TAP2 (18), and the two yeast peroxisomal ABC proteins, Pxa1 and Pxa2 (19, 20).

This study used the yeast two-hybrid assay to show that the carboxyl-terminal half of ALDP, ALDRP, and PMP70 can engage in homo- and heterodimerization. These two processes were confirmed by co-immunoprecipitation methods. We examined the effect of four different ALD patient mutations upon these interactions and attempted to map the carboxyl-terminal subdomains of ALDP allowing dimerization of the protein.

EXPERIMENTAL PROCEDURES

Antibodies, Yeast Strains, and Cell Lines—Monoclonal antibody 1D6 against human ALDP (hALDP) and polyclonal antibodies 1664 and 7373 raised against mouse ALDP (mALDP) and mouse ALDRP (mALDRP), respectively, have been described previously (13, 15, 21). Polyclonal antibody 1690 against hALDP was raised against the last 19 amino acids of this protein. Anti-mouse PMP70 (mPMP70) antibody was a gift from Dr. David Valle (Johns Hopkins University, Baltimore, MD). All these antibodies show specific protein affinities except the anti-mALDP 1664 antibody, which recognizes both human and mouse ALDP.

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The two-hybrid assays were performed in the yeast strains HF7c (MATa, ura3–52, his3–200, ade2–101, lys2–801, trp1–901, leu2–3, 112, can1–42, gal1–542, gal80–538, URA::Gal4 binding sites-CYC1-laCZ, LYS2::Gal1-HIS3) and L40 (MATa, trp1, leu2, his3, LYS2::lexA-HIS3, URA3::lexA-laCZ). M48-hALD and MFG-mALDR-producer Psi-CRIP cells were described previously (22, 23). They are amphotropic packaging cell lines derived from 3T3 cells that express hALDP and mALDRP.

**Plasmid Constructions and Mutagenesis**—The cDNA fragments encoding the cytoplasmic domain of hALDP (hALDPc, residues 361–745) (1), mALDRP (mALDRPc, residues 374–742) (2), and human PMP70 (hPMP70c, residues 338–660) (24) were amplified by PCR and cloned into pGBT9, pLEX9 (Invitrogen), and pGADGE yeast expression vectors. The cDNA fragments encoding the carboxyl-terminal subdomains of hALDP (N, residues 361–506; N+NBF, residues 361–630; C, residues 631–745; and NBF+C, residues 507–745) were amplified by PCR and cloned into pLEX9. The integrity of cloned PCR products was confirmed by DNA sequencing.

ALD point mutations (R389H, R401Q, P484R, and R591Q) were individually introduced into pGBT- and pLEX-hALDP by site-directed mutagenesis using “overlap extension PCR” with Pfu polymerase (Stratagene) and appropriate primers (25). Mutated inserts were completely sequenced.

**Yeast Two-hybrid Assay**—HF7c or L40 yeast reporter strains containing Gal4- or LexA-inducible genes, HIS3 and LacZ, were cotransformed with pGBT9/pLex9 and pGADGE vector plasmids and plated on selective medium lacking tryptophan and leucine. Double transformants were patched on the same medium and replica-plated on selective medium lacking tryptophan, leucine, and histidine for histidine auxotrophy analysis and on Whatman 40 filter for β-galactosidase assay. Yeast double transformants expressing wild type or mutated ALDPc hybrids were lysed in SDS-polyacrylamide gel electrophoresis sample buffer and analyzed by Western blotting with anti-hALDP antibody 1693.

**Co-immunoprecipitation Experiments**—20 × 10⁶ M48-hALD, MFG-mALDRP-producer Psi-CRIP cells, or normal 3T3 cells were lysed in 1 ml of binding buffer (25 mM HEPES, pH 7.4, 150 mM KCl, 5 mM EDTA, and 0.1% Triton X-100) containing a protease inhibitors mixture (Roche Molecular Biochemicals). Lysates were incubated overnight at 4 °C with either anti-hALDP (ID6, diluted 1:150), anti-mALDRP (1664, diluted 1:250), or anti-mPMP70 (diluted 1:500) antibodies. Protein A-Sepharose beads (PAS) were then added for 1 h at 4 °C. After three washes with binding buffer, PAS-bound fractions were resuspended in SDS-polyacrylamide gel electrophoresis sample buffer and analyzed by Western blotting.

**Western Blot Analysis**—Yeast transformant or fibroblast lysates were electrophoresed on a 7.5% SDS-polyacrylamide gel and then transferred onto a polyvinylidene difluoride membrane (Amersham Pharmacia Biotech) in a semi-dry blotter. Proteins were labeled with appropriate antibodies as commonly described. Antigen-antibody complexes were detected using ECL system (Amersham Pharmacia Biotech).

**RESULTS**

**Interactions of the Carboxyl-terminal Half of Human ALDP, Mouse ALDRP, and Human PMP70 in the Yeast Two-hybrid System**—hALDP, mALDRP, and PMP70 are peroxisomal membrane proteins with a carboxyl-terminal hydrophilic domain oriented toward the cytoplasm (1, 3, 26).2 To examine the possible interactions among ALDP, ALDRP, and PMP70, we generated constructs in which hALDPc, mALDRPc, and hPMP70c were fused to the Gal4 or LexA binding domain (BD) or the Gal4 activation domain (AD). Each hybrid protein was analyzed for protein-protein interactions with the others in the yeast strains containing Gal4- (HF7c strain) or LexA- (L40 strain) inducible reporter genes, HIS3 and LacZ. Protein-protein interaction was monitored by the ability of yeast patches to grow in the absence of histidine and by the β-galactosidase activity. The results obtained in the L40 yeast strain are shown in Fig. 1. Similar results were obtained in the HF7 yeast strain (data not shown). β-galactosidase activity and growth of yeast patches in the absence of histidine were observed when hALDPc or mALDRPc was present as both BD and AD hybrids (Fig. 1A, rows 1 and 3). Interaction was also detected between hALDPc fused to LexA-BD and mALDRPc fused to Gal4-AD (Fig. 1A, row 2). Identical results were obtained using symmetric hybrid proteins (not shown). The specificity of the interactions was demonstrated by the absence of β-galactosidase activity or growth in medium without histidine when hALDPc or mALDRPc was assayed with the irrelevant Raf serine/threonine kinase (Fig. 1A, rows 4 and 5). Interaction between Raf and p21ras (27) was used as a positive control (Fig. 1A, row 6).

Interactions were also observed between hPMP70c fused to LexA-AD and hPMP70c and between hALDPc or mALDRPc fused to Gal4-AD (Fig. 1B, rows 1, 3, and 5). No interaction could be observed between hPMP70c and the Raf protein or between Ras and hALDPc or mALDRPc (Fig. 1B, row 2, 4, and 6). These data demonstrate that the carboxyl-terminal halves of human ALDP, mouse ALDRP, and human PMP70 can interact with themselves and each other in the yeast two-hybrid system.

**Effects of X-ALD Mutations on the Interactions of hALDPc with Itself, mALDRPc, and hPMP70c**—We examined the effects of four naturally occurring X-ALD mutations upon interaction of hALDPc with itself, mALDRPc, or hPMP70c. These mutations (R389H, R401Q, P484R, and R591Q) were generated as described above and tested in two-hybrid assays. The P484R mutation leads to a decreased amount of ALDP in patient fibroblasts,3 whereas the three other mutations have no effect on ALDP stability in vivo (28–31). Our results show that the mutations R389H and R401Q had no effect on the interactions of hALDPc with itself (Fig. 2A, rows 1, 3, and 5), mALDRPc (Fig. 2B, rows 1, 3, and 5), or hPMP70c (Fig. 2C, rows 1, 3, and 5). In contrast, the P484R and R591Q mutations

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2 L. X. Liu, K. Janvier, V. Berteaux-Lecellier, N. Cartier, R. Benarous, and P. Aubourg, unpublished results.

3 J. Berger, personal communication.
decreased significantly the interaction of hALDPc with itself (Fig. 2A, rows 7 and 9) and abolished its interactions with mALDRPc (Fig. 2B, rows 7 and 9) and hPMP70c (Fig. 2C, rows 7 and 9). No interaction of wild type or the four mutated ALD hybrid proteins was observed with the irrelevant protein SNF4 (Fig. 2A, B, and C, rows 2, 4, 6, 8, and 10). Western blotting with anti-hALDP antibody 1693 revealed that the four mutated hALDPc hybrids were expressed in yeast at the same level as wild type hALDPc hybrid (Fig. 2D), ruling out that the lack of growth in the absence of histidine was because of the unstability of mutated ALD hybrid proteins. These results demonstrate that the residues Pro-484 and Arg-591 are important for the interaction of the carboxyl-terminal domain of human ALDP with itself, mALDRP, and hPMP70 and suggest that the loss of ALDP function in patients harboring either of these two mutations may result from an ALDP dimerization defect.

Mapping of ALDP Cytosolic Subdomains Involved in Carboxyl-terminal Domain-Domain Interactions—To determine which region of hALDPc is necessary and sufficient for its interactions with itself, mALDRPc, and PMP70c, we constructed four deletants of hALDPc that were analyzed in the yeast two-hybrid system for their ability to interact with hALDPc, mALDRPc, or hPMP70c (Fig. 3). Based on histidine auxotrophy and β-galactosidase activity assays, none of these deletants interacted with their partners as strong as the full-length hALDPc. When tested against hPMP70c, only background level was detected for all these deletants. The C (residues 631–745) deletant reacted against the irrelevant proteins Ras or Raf.

![Fig. 2. Effects of X-ALD mutations on the interactions of human ALDPc with itself (A), mouse ALDRPc (B), and human PMP70c (C). A, HF7c yeast strains expressing wild type or mutated (R389H, R401Q, P484R, and R591Q) ALDPc fused to Gal4-BD were analyzed for histidine auxotrophy (left panel, medium with histidine; right panel, medium without histidine). The background levels of wild type or mutated ALDPc hybrid proteins were evaluated by testing against the irrelevant proteins SNF1 and SNF4. B and C, interactions of wild type and mutated (R389H, R401Q, P484R, and R591Q) ALDPc with mALDRPc and hPMP70c. D, expression levels of wild type and mutated LexA BD-ALDPc hybrid proteins in yeast transformants assayed by Western blot with anti-hALDP antibody 1693. Lane 1, yeast co-expressing LexA BD-SNF1 and Gal4 AD-PMP70c; lane 2, yeast co-expressing wild type LexA BD-ALDPc and Gal4 AD-PMP70c; lane 3, yeast co-expressing LexA BD-ALDPc; lane 4, yeast co-expressing LexA BD-hALDPc; lane 5, yeast co-expressing LexA BD-hALDPc; lane 6, yeast co-expressing LexA BD-hALDPc; lane 7, yeast co-expressing LexA BD-hALDPc; lane 8, yeast co-expressing LexA BD-hALDPc; lane 9, yeast co-expressing LexA BD-hALDPc; lane 10, yeast co-expressing LexA BD-hALDPc.]

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![Fig. 3. Mapping of the subdomains of ALDPc involved in domain-domain interactions. The different subdomains of hALDPc are represented on the left by boxes with corresponding amino acid positions of hALDP. The gray boxes indicate the position of the NBF. The interactions of these deletants and full-length ALDPc with ALDPc, ALDRPc, and PMP70c were analyzed by histidine auxotrophy and β-galactosidase activity assays in L40 reporter strain. The strength of interaction was scored by +, ++, or +++ (corresponding to increasing intensity of interaction), whereas — represents the background level when the interaction was assayed against the irrelevant proteins Ras or Raf.]

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Interactions of Peroxisomal ABC Transporters

Fig. 4. Co-immunoprecipitation experiments demonstrating the interactions of human ALDP, mouse ALDP, mouse ALDRP, and mouse PMP70 in 3T3 fibroblasts. A, mouse PMP70 was co-immunoprecipitated with human ALDP by anti-hALDP monoclonal antibody 1D6 in 3T3 cells expressing human ALDP but not in 3T3 cells expressing mouse ALDP. PAS-bound fractions were analyzed by immunoblotting with anti-hALDP (upper panel) or anti-hPMP70 (lower panel) antibodies. B, heterodimerization between mouse ALDP and ALDRP. Immunoprecipitation using anti-mALDP antibody was performed in 3T3 cells expressing either mouse ALDRP or human ALDP. PAS-bound fractions were analyzed by immunoblotting with anti-mALDP (top panel), anti-mALDRP (middle panel), and anti-hALDP (bottom panel) antibodies. C, co-immunoprecipitation of human and mouse ALDP. Immunoprecipitation was carried out in 3T3 cells expressing human ALDP and in normal 3T3 cells with anti-hALDP 1D6 in 3T3 cells expressing human ALDP but not in 3T3 cells expressing mouse ALDP. As expected, neither the hALDP nor the mPMP70 band could be detected (Fig. 4A, lane 1, and lower panel). Heterodimerization of mALDP with mALDRP was demonstrated by the co-immunoprecipitation of these two proteins using anti-mALDP antibody in 3T3 cells expressing mALDRP (Fig. 4B, lane 2, top and middle panels). A control experiment was performed in 3T3 cells expressing hALDP but not mALDRP. Because the antibody against mALDP cross-reacts with hALDP, immunoprecipitation with this antibody in 3T3 cells expressing hALDP resulted in the presence of both mouse and human ALDP bands in the PAS-bound fraction as revealed by immunoblotting with anti-mALDP (Fig. 4B, lane 1, top panel) and specific anti-hALDP antibody (Fig. 4B, lane 1, bottom panel). However, no band was detected in the PAS-bound fraction with the antibody against mALDRP (Fig. 4B, lane 1, middle panel), indicating that the signal detected in cells expressing mALDRP is specific to mouse ALDRP (Fig. 4B, lane 2, middle panel).

Immunoprecipitation with anti-hALDP antibody in 3T3 cells expressing hALDP and in normal 3T3 cells demonstrated the dimerization of hALDP with mALDRP. Using anti-hALDP antibody, Western blotting of PAS-bound fractions (Fig. 4C, lanes 1 and 2) and cellular lysates (Fig. 4C, lanes 3 and 4) showed a specific hALDP band in cells expressing hALDP (Fig. 4C, lanes 1 and 3, upper panel). No hALDP band was detected in normal 3T3 cells (Fig. 4C, lanes 2 and 4, upper panel). Reprobing with anti-mALDP antibody revealed that in a PAS-bound fraction and cellular lysate of cells expressing hALDP a hALDP band accounted for the cross-reaction of anti-mALDP antibodies with hALDP and a fainter and higher band that corresponded to mALDP (Fig. 4C, lanes 1 and 3, lower panel). No mALDP band was detected in a PAS-bound fraction of normal 3T3 cells (Fig. 4C, lane 2, lower panel), although it could be revealed in cellular lysate prior to immunoprecipitation (Fig. 4C, lane 4, lower panel).

Finally, immunoprecipitation with antibodies against mPMP70 in 3T3 cells expressing hALDP or mALDRP cells demonstrated heterodimerization of mPMP70 with hALDP (Fig. 4D, lane 1, middle panel) and mALDRP (Fig. 4D, lane 2, bottom panel). Altogether, our results demonstrate that human ALDP dimerizes with mouse PMP70, mouse ALDP with human ALDP and mouse ALDRP, and mouse PMP70 with mouse ALDRP.

DISCUSSION

The high degree of homology among ALDP, ALDRP, and PMP70 together with their expression pattern suggest that these ABC half-transporters may therefore assume a specific function in a particular cell type or have similar/redundant functions in the same cell type. Although the exact function of ALDP is unknown, it is involved in the metabolism of VLCFA in peroxisomes. The oxidation of VLCFA is initiated by a VLCFA acyl-CoA synthetase (VLACS), which activates VLCFA into acyl-CoA derivatives. The oxidation of VLCFA is reduced by 60–80% in ALD fibroblasts because of a deficiency in VLACS activity (11, 12). Studies of the two yeast peroxisomal ABC transporters, Pxa1p and Pxa2p, provided clues about the putative function of ALDP (19, 20, 32). In yeast, long-chain fatty acids (LCFA) are activated into CoA derivatives within the cytosol by a LCFA-CoA synthetase and then transported across the peroxisomal membrane by Pxa1p/Pxa2p heterodimers. Similarly, ALDP could import VLCFA-CoA into peroxisomes following dimerization with itself or a partner.
Whether the active site of the mammalian VLACS is located on the cytoplasmic or luminal side of peroxisomes remains unclear (33, 34). If it is located within peroxisomes, this would rather support that ALDP transports VLCFA or a substrate necessary to VLACS activation. It is unlikely that ALDP transports the VLACS enzyme across the peroxisomal membrane because VLACS is normally expressed in the liver from ALDP-deficient mice and normally localized in peroxisomes (35).

Because overexpression of ALDRP corrects the accumulation of VLCFA in ALD fibroblasts (23, 36, 37), it is possible that ALDRP has a substrate specificity overlapping with ALDP. Overexpression of PMP70 in Chinese hamster ovary cells increases by 2–3-fold the rate of palmitic acid β-oxidation into peroxisomes, suggesting that PMP70 is involved in the metabolic transport of LCFA across peroxisomal membrane (38). ALDRP and PMP70 could also import fatty acids with other chain lengths as well.

Using the yeast two-hybrid system, we observed that the carboxyl-terminal half of ALDP, ALDRP, and PMP70 can form homo- or heterodimers, a novel function of the nucleotide binding domain of ABC transporters. ALDP homo- and heterodimerization are altered by two disease-associated mutations, suggesting that ALDP homo- and/or heterodimerization are necessary for its function. Interestingly, the P484R mutation results in an unstable protein in vivo. Analysis of ALD mutations showed that 62.5–66% of ALD gene missense mutations lead to an unstable protein (10, 39). Our results demonstrate that ALDP instability in vivo may directly result from dimerization deficiency. In contrast, the R591Q disease mutation, which alters the dimerization of ALDP in the yeast two-hybrid assays, does not lead to ALDP instability in vivo. The stability of ALDP is therefore not entirely dependent on the dimerization of the carboxyl-terminal domains. This is supported by the observation that several ALD missense mutations (in particular R518W and P566L) can lead to either a normal, decreased level or absence of ALDP in ALD fibroblasts.

The specific carboxyl-terminal subdomain region of ALDP responsible for dimerization could not be defined. Western blot analysis indicated, however, that all subdomains were correctly expressed. This suggests that in the absence of the neighboring protein sequence, the subdomain involved in dimerization was probably incorrectly folded and failed to interact with its partners. Alternatively, the integrity of the carboxyl-terminal domain may be required for the interactions.

Taking advantage of the high homology between mouse and human ALDP, ALDRP, and PMP70, we demonstrate that dimerization occurs between human ALDP and mouse PMP70, between mouse ALDP and human ALDP or mouse ALDRP, and between mouse PMP70 and mouse ALDRP using the co-immunoprecipitation method. Although we did not study the homodimerization of PMP70 and ALDRP, our results in the yeast two-hybrid system suggest strongly that these two ABC half-transporters can also form homodimers. A summary of our data is that each peroxisomal ABC half-transporter can dimerize with itself or with a related partner.

Regarding Drosophila, the uptake of precursors for the synthesis of red and brown pigments is controlled by various combinations of the ABC half-transporters scarlet, brown, and white, resulting in different substrate specificities (17). Our results raise the possibility that different combinations of peroxisomal dimers perform the import of different substrates, including fatty acids of different lengths. If confirmed, it may have important implications for the pathogenesis and phenotypic heterogeneity of ALD. The inability of a mutated ALDP to heterodimerize with other partners might result in the accumulation of metabolites other than VLCFA. This may modulate the functions of cells directly affected by the ALD mutations and eventually influence the clinical manifestations of the disease. PMP70 being involved in the peroxisomal β-oxidation of LCFA (38), different amounts of ALDP and PMP70 homo- and heterodimers could determine the oxidation rate of VLCFA and LCFA in response to substrate availability and/or cell-specific expression of ALDP and PMP70. Imanaka et al. (38) showed that overexpression of PMP70 in 3T3 cells reduced the oxidation rate of VLCFA by 30–40%. One explanation could be that the overexpression of PMP70 competes with ALDP for the targeting to the peroxisomal membrane. It is also possible that overexpression of PMP70 leads to the increased formation of ALDP-PMP70 heterodimers and a decrease of ALDP homodimers. However, the overexpression of wild type or functionally defective PMP70 have similar effects on VLCFA β-oxidation (38). Thus ALDP-PMP70 heterodimers may not be directly involved in the β-oxidation of VLCFA or LCFA but rather constitute a reservoir of ALDP and PMP70 molecules that contribute to the maintenance of the appropriate ratio of ALDP and PMP70 homodimers within the peroxisomal membrane. This hypothesis is consistent with the observations that PMP70 levels are reduced in ALD fibroblasts lacking ALDP (40) and that ALDP overexpression in 3T3 cells increases the amount of PMP70.

Our results may have implications for gene therapy approach aiming at targeting the ALD gene into hematopoietic cells or oligodendrocytes (10, 41). Because ALDP, ALDRP, and PMP70 form a different combination of dimers, one should ensure that artificial expression of ALDP does not disrupt significantly the balance between the physiological dimers in transduced cells.

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