ATP binding/hydrolysis by and Phosphorylation of Peroxisomal ATP-binding Cassette Proteins PMP70 (ABCD3) and Adrenoleukodystrophy Protein (ABCD1) *

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The 70-kDa peroxisomal membrane protein (PMP70) and adrenoleukodystrophy protein (ALDP), half-size ATP-binding cassette transporters, are involved in metabolic transport of long and very long chain fatty acids into peroxisomes. We examined the interaction of peroxisomal ATP-binding cassette transporters with ATP using rat liver peroxisomes. PMP70 was photoaffinity-labeled at similar efficiencies with 8-azido-[γ-32P]ATP and 8-azido-[γ-32P]ATP when peroxisomes were incubated with these nucleotides at 37 °C in the absence Mg2+ and exposed to UV light without removing unbound nucleotides. The photoaffinity-labeled PMP70 and ALDP were co-immunoprecipitated together with other peroxisomal proteins, which also showed tight ATP binding properties. Addition of Mg2+ reduced the photoaffinity labeling of PMP70 with 8-azido-[γ-32P]ATP by 70%, whereas it reduced photoaffinity labeling with 8-azido-[γ-32P]ATP by only 20%. However, two-thirds of nucleotide (probably ADP) was dissociated during removal of unbound nucleotides. These results suggest that ATP binds to PMP70 tightly in the absence of Mg2+, the bound ATP is hydrolyzed to ADP in the presence of Mg2+, and the produced ADP is dissociated from PMP70, which allows ATP hydrolysis turnover. Properties of photoaffinity labeling of ALDP were essentially similar to those of PMP70. Vanadate-induced nucleotide trapping in PMP70 and ALDP was not observed. PMP70 and ALDP were also phosphorylated at a tyrosine residue(s). ATP binding/hydrolysis by and phosphorylation of PMP70 and ALDP are involved in the regulation of fatty acid transport into peroxisomes.

ATP-binding cassette (ABC)1 superfamily proteins are composed of two homologous halves, each of which typically contains six transmembrane α helices and a nucleotide binding fold (NBF). Prominent members of eukaryotic ABC superfamily proteins, such as the multidrug efflux pump MDR1 (ABCB1) and the cystic fibrosis transmembrane conductance regulator CFTR (ABCC7), are full size and contain 12 transmembrane α helices and 2 NBFs. On the other hand, most of the organelle ABC superfamily proteins, such as antigen transporters TAP1 (ABCB1) and TAP2 (ABCB2) on endoplasmic reticulum membranes and peroxisomal ABC proteins, are half size and contain six transmembrane α helices and one NBF.

To date, four peroxisomal ABC proteins have been identified in mammalian peroxisomes: the 70-kDa peroxisomal membrane protein (PMP70, ABCD3), adrenoleukodystrophy protein (ALDP, ABCD1), ALDP-related protein (ALDRP, ABCD2), and PMP70-related protein (P70R, ABCD4) (1–7). These half-size ABC proteins are supposed to work after dimerization. Disruption of the half-size ABC protein gene of Saccharomyces cerevisiae PAT1 (Pxa1) or PAT2 (Pxa2), whose products were identified on peroxisomes, resulted in impaired growth in oleic acid medium, suggesting that Pat1p and Pat2p function as heterodimers (8–11). Indeed, Liu et al. (12) have shown that homo- and heterodimerization occurred among the ALDP, ALDRP, and PMP70 by using the yeast two-hybrid system and co-immunoprecipitation.

Because the defect of the ALDP gene resulted in impaired peroxisomal β-oxidation and accumulation of very long chain fatty acids (VLCFAs) (13–17), and because overexpression of PMP70 in Chinese hamster ovary cells increased β-oxidation of palmitic acid (18), ALDP and PMP70 are suggested to be involved in ATP-dependent import of VLCFAs and long chain fatty acids (LCFAs) into peroxisomes. Contreras et al. (19) reported that the NBF of ALDP was located on the cytoplasmic surface of peroxisomal membranes, and that the NBF released from peroxisomes by protease treatment bound to ATP (19). The NBFs of ALDP and PMP70, fused with the maltose-binding protein, were reported to be photoaffinity-labeled with 8-azido-[γ-32P]ATP and showed ATPase activities (20). However, the ATP binding and hydrolysis properties of isolated NBF do not necessarily represent those of complete native proteins. In this study, we analyzed the interaction of peroxisomal ABC transporters with ATP using rat liver peroxisomes. We found that ATP bound to PMP70 and ALDP tightly and that PMP70 and ALDP formed a stable complex with other peroxisomal membranes proteins, which also showed tight ATP binding properties. Furthermore, ATP occluded to homo- and heterodimer of PMP70 and ALDP in the absence of Mg2+ was
shown to be hydrolyzed in the presence of Mg^{2+}. PMP70 and ALDP were also phosphorylated at a tyrosine residue(s).

EXPERIMENTAL PROCEDURES

Materials—8-Azido-[α-^32P]ATP and 8-azido-[γ-^32P]ATP (18-22 Ci/mmol) were purchased from Affinity Labeling Technologies (Lexington, KY). [γ-^32P]ATP (800 Ci/mmol) was obtained from ICN Biomedicals (Costa Mesa, CA). H-89 and CTKl-7 were from Seikagaku Co. (Tokyo, Japan). Protein G-agarose, H-7, and lavendustin A were from Sigma. Rabbit anti-PMP70 antibody was raised against the COOH-terminal 15 amino acids of rat PMP70 (21). Rabbit anti-ALDP antibody raised against the COOH-terminal 24 amino acids of human ALDP (22) was kindly provided by Dr. T. Yamada (Kyushu University).

Preparation of Rat Liver Peroxisomes—Peroxisomes were purified from rat liver by differential centrifugation in a buffer containing sucrose followed by isopycnic centrifugation in Nycodentz (23) with some modifications. About 1.0 ml of a light mitochondrial fraction was layered onto a 10 ml linear Nycodentz gradient (density span, 1.15–1.25 g/ml) in a Beckman NVT65T rotor (Beckman, Fullerton, CA). The gradient rested on a 0.5 ml cushion of 1.3 g/ml Nycodentz. All solutions contained 0.25 M sucrose, 1 mM EDTA, 0.1% (v/v) ethanol, and 5 mM Hepes-KOH, pH 7.4. The centrifugation was carried out at 51,200 rpm (193,000 × g) for 120 min at 4 °C. Fractions of ~1.0 ml were collected in preweighed microtubes, and the density of each fraction was determined by refractometry. The peroxisomal fraction was identified by the density and the distribution of catalase (18).

Photoaffinity Labeling of Peroxisome Proteins with 8-Azido-[α-^32P]ATP and 8-Azido-[γ-^32P]ATP—Rat liver peroxisomes (30 μg) were incubated with 50 μM 8-azido-[α-^32P]ATP or 8-azido-[γ-^32P]ATP, 2 mM ouabain, 0.1 mM EGTA, and 40 mM Tris–Cl, pH 7.5, in a total volume of 6 μl for 10 min at 37 °C in the presence of 3 mM MgSO₄ or EDTA. After unbound nucleotides were removed before UV irradiation, 500 μl of ice-cold TEM buffer (40 mM Tris–HCl, pH 7.5, 0.1 mM EDTA, and 1 mM MgSO₄) or TEE buffer (40 mM Tris–HCl, pH 7.5, 0.1 mM EDTA, and 1.5 mM EDTA) was added to the samples, and the supernatants were removed from peroxisome pellets after centrifugation (15,000 × g, 10 min, 4 °C). This wash step was done again, and then the peroxisomes were resuspended in 8 μl of TEM buffer and exposed to UV light for 3 min (at 254 nm, 5.5 mW/cm²) on ice. The peroxisomes were solubilized, and PMP70 and ALDP were immunoprecipitated as described in the following section. Samples were electrophoresed on a 7% SDS-polyacrylamide gel and autoradiographed. 8-Azido-[α-^32P]ATP bound to PMP70 and ALDP was measured by scanning with a radioimaging analyzer (LAS2000; Fuji Photo Film Co.). Experiments were carried out at least four times.

Phosphorylation of Peroxisomal Membrane Proteins—All procedures were carried out at 4 °C unless otherwise stated. Peroxisomes (200 μg) were suspended in 200 μl of a phosphorylation buffer (25 mM Tris–HCl, pH 7.5, 10 mM MgCl₂, 2 mM MnCl₂, 0.4 mM EDTA, 1 mM dithiothreitol, 2 mM orthovanadate, 10 mM NaF, 5 mM β-glycerophosphate, and 10 μM ATP) containing a protease inhibitor mixture (1 mM phenylmethylsulfonyl fluoride and 10 μg/ml antipain, chymostatin, leupeptin, and pepstatin A). 50 μCi of [γ-^32P]ATP was then added to the mixture and incubated for 20 min at 30 °C. In some experiments, protein kinase inhibitors at a concentration of 50 μM were preincubated for 10 min before the addition of [γ-^32P]ATP. After centrifugation at 15,000 × g for 5 min, the pellet was washed with the same buffer and suspended with 600 μl of RIPA buffer. Immunoprecipitates, prepared as described below, were analyzed on a 7–12% SDS-polyacrylamide gradient gel and autoradiographed.

Immunoprecipitation of PMP70 and ALDP—The peroxisome pellets were solubilized with 100 μl of RIPA buffer (20 mM Tris–HCl, pH 7.5, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 0.15 mM NaCl, 10 μg/ml leupeptin, and 100 μg/ml 1H-[2,3-3H]leucine) (1). The lysates were kept on ice or rotated for 1 h, and the debris was removed by centrifugation at 15,000 × g for 5 min. The supernatants were precleared with an appropriate amount of protein G-agarose that was prewashed in RIPA buffer. After centrifugation at 10,000 × g for 10 s, the supernatant was incubated with 5 μl of anti-PMP70 or anti-ALDP antibodies for 1 h and then incubated with 20 μl of a 50% (v/v) suspension of protein G-agarose for 1 h.

Other Methods—Protein and catalase were assayed as described previously (18, 21). Western blot analysis was done using ECL + Plus, a Western blotting detection system.

FIG. 1. Specific interaction of peroxisomal proteins with ATP. A, rat liver peroxisomes were incubated with 50 μM 8-azido-[α-^32P]ATP in the absence (lanes 1 and 4) or presence of 1 (lane 5), 5 (lane 6), or 10 μM ATP (lane 7) at 26 °C for 10 min and exposed to UV light after removing unbound nucleotide by washing with excess buffer. Western hybridization of the blot was run in parallel with anti-PMP70 (lane 2) and anti-ALDP (lane 3) antibodies. B, rat liver peroxisomes were incubated with 50 μM 8-azido-[γ-^32P]ATP at 37 °C for 10 min. UV irradiation was done after removing unbound nucleotides. Peroxisomes were solubilized with RIPA buffer, and immunoprecipitation was done with the preimmune antibody (lane 1), the anti-PMP70 antibody (lane 2), and anti-ALDP antibody (lane 3), respectively. Western hybridization of the blot was run in parallel with anti-PMP70 (lane 4) and anti-ALDP (lane 5) antibodies. The photoaffinity-labeled proteins were separated by electrophoresis on a 7% SDS-polyacrylamide gel and autoradiographed. Free 8-azido-[^32P]ATP was seen below the molecular mass marker of 50 kDa in A. The band of ~45 kDa in B seems to be nonspecific because it was also detected with preimmune serum.

RESULTS

Specific Interaction of Peroxisomal Proteins with ATP—To examine the interaction of peroxisomal membrane proteins with ATP, rat liver peroxisomes were incubated at 26 °C with 8-azido-[α-^32P]ATP. Then, the peroxisomes were exposed to UV light after removing unbound 8-azido-[α-^32P]ATP by washing with excess buffer. Several peroxisomal proteins between 60 and 100 kDa were found to be specifically photoaffinity-labeled (Fig. 1A, lane 1). The intensity of the bands decreased in the presence of an excess amount of ATP in a concentration-dependent manner (Fig. 1A, lanes 4–7), suggesting that ATP bound to these peroxisomal proteins specifically. Under these conditions, peroxisomal matrix proteins such as hydratase-dehydrogenase, acyl-CoA oxidase, catalase, and urate oxidase were not released from peroxisomal particles (data not shown). Indeed, all the photoaffinity-labeled proteins were recovered in the membrane pellet when the peroxisomes were separated into soluble and membrane fractions by the sodium carbonate procedure (24) (data not shown), suggesting that photoaffinity-labeled proteins were located on the peroxisomal membranes. Western hybridization of the blot, run in parallel, with anti-PMP70 or ALDP antibody showed a single band with a molecular mass of 65 or 75 kDa (Fig. 1A, lanes 2 and 3), which could correspond to one of the radiolabeled proteins.

Identification of Photoaffinity-labeled ABC Proteins—PMP70 is a major constituent of peroxisomal membranes of normal rat liver. As compared with the amounts of PMP70, ALDP, ALDRP, and P70R on the membranes, ALDP was about one-seventh the amount of PMP70, and ALDRP and P70R were roughly less than one-tenth the amount of ALDP. To examine whether the photoaffinity-labeled 65- and 75-kDa proteins were PMP70 and ALDP, the proteins labeled with 8-azido-[α-^32P] were immunoprecipitated with antibodies against PMP70 and ALDP (Fig. 1B). Both antibodies precipitated mainly four photoaffinity-labeled proteins from 65 to 90 kDa. The anti-PMP70 antibody mainly precipitated a photoaffinity-labeled

2 D. Tomimoto, Y. Murasaki, M. Morita, and T. Imanaka, unpublished observations.
65-kDa protein. The photoaffinity-labeled 65-kDa protein was also precipitated by the anti-ALDP antibody, but less efficiently than by the anti-PMP70 antibody. The other three labeled proteins were precipitated at similar efficiencies by these antibodies. Western hybridization of the blot, run in parallel, with anti-PMP70 and anti-ALDP antibodies showed that photoaffinity-labeled 65- and 75-kDa proteins were PMP70 and ALDP, respectively (Fig. 1B, lanes 4 and 5). Photoaffinity labeling of these proteins was scarcely observed when peroxisomes were incubated with 8-azido-[α-32P]ATP at 0 °C and irradiated by UV light after removing unbound nucleotide (data not shown). These results suggest that PMP70 and ALDP bind to ATP tightly in a temperature-dependent manner and form a stable complex together with other peroxisomal membrane proteins, which also bind ATP.

**ATP Binding Properties of PMP70 and ALDP**—To examine the ATP binding properties of PMP70 and ALDP, peroxisomes were incubated with 8-azido-[α-32P]ATP or 8-azido-[γ-32P]ATP of the same specific radioactivities at 37 °C for 10 min in the presence or absence of Mg2+ and exposed to UV light before or after unbound nucleotides were removed by washing with excess buffer. Proteins were then immunoprecipitated with anti-PMP70 antibody. Free 8-azido-[32P]ATP was seen below the 50 kDa molecular mass marker.

No Vanadate-induced Nucleotide Trapping in PMP70 or ALDP—It is well known that MDR1 and MRP1 (ABCC1), the multidrug transporters, trap ADP in the presence of orthovanadate and Mg2+. As a result, the labeling intensity of these proteins was increased when membranes containing these proteins were reacted with the nucleotide in the presence of orthovanadate and Mg2+ (25-27). Based on this observation, we tried to examine vanadate-induced nucleotide trapping of PMP70 and ALDP. However, no increase of photoaffinity labeling of PMP70 or ALDP was observed when peroxisomes were incubated with 8-azido-[α-32P]ATP in the presence of orthovanadate and Mg2+ and exposed to UV light before (data not shown) or after unbound nucleotide was removed by washing with excess buffer (Fig. 4).

**Phosphorylation of PMP70 and ALDP**—Because PMP70 was labeled strongly with 8-azido-[γ-32P]ATP (Fig. 2) and because some members of the ABC superfamily have been reported to be phosphorylated, we examined whether PMP70 and ALDP were phosphorylated. Peroxisomes were incubated with [γ-32P]ATP for 20 min at 30 °C in the presence of Mg2+, and immunoprecipitation was performed with either anti-PMP70...
antibody or anti-ALDP antibody. As shown in Fig. 5A, both antibodies precipitated two phospho-proteins corresponding to PMP70 and ALDP (lanes 2 and 7). Co-precipitation of PMP70 and ALDP was revealed by immunoprecipitation followed by Western blot analysis. As shown in Fig. 5B, after immunoprecipitation with anti-PMP70 antibody, PMP70 and ALDP were detected in the immunoprecipitate by Western blotting of either anti-PMP70 antibody or anti-ALDP antibody (lane 2 of the left and right panels). Both proteins were also detected in the immunoprecipitate with anti-ALDP antibody (lane 3 of the left and right panels). In addition, the phosphorylation of PMP70 and ALDP was markedly inhibited in the presence of vanadate and then UV-irradiated after unbound nucleotides were removed by washing with excess buffer. Proteins were then immunoprecipitated with anti-PMP70 antibody.

**FIG. 4.** No vanadate-induced nucleotide trapping in PMP70 or ALDP. Rat liver peroxisomes were incubated with 8-azido-[γ-32P]ATP at 37 °C for 10 min in the presence of 3 mM MgCl2 and 1 mM sodium vanadate and then UV-irradiated after unbound nucleotides were removed by washing with excess buffer. Proteins were then immunoprecipitated with anti-PMP70 antibody.

**FIG. 5.** Phosphorylation of PMP70 and ALDP. A, rat liver peroxisomes were incubated with 8-azido-[γ-32P]ATP at 30 °C for 20 min in the presence of MgCl2. After solubilization with RIPA buffer, proteins were immunoprecipitated with preimmune antibody (lane 1), anti-PMP70 antibody (lanes 2–6), and anti-ALDP antibody (lane 7), and labeled proteins were analyzed by SDS-PAGE followed by autoradiography. Protein kinase inhibitors (50 µM) H-7 (lane 3), H-89 (lane 4), lavendustin A (lane 5), and CKI-7 (lane 6) were preincubated for 10 min and included in the reaction mixture for the labeling period. B, co-immunoprecipitation of PMP70 and ALDP. Immunoprecipitates with preimmune antibody (lane 1), anti-PMP70 antibody (lane 2), and anti-ALDP antibody (lane 3) as described in A were electrophoresed and subjected to immunoblotting using anti-PMP70 antibody (left panel) or anti-ALDP antibody (right panel).

**DISCUSSION**

Peroxisomes are involved in oxidative degradation of LCFA and VLCFA. LCFA are known to be activated into CoA derivatives outside of peroxisomes by a LCFA-CoA synthetase before peroxisomal β-oxidation (28). Recently, we demonstrated that overexpression of PMP70 in Chinese hamster ovary cells increased the rate of palmitic acid β-oxidation into peroxisomes by 2- to 3-fold and that the peroxisomes prepared from these cells stimulated pulmityl-CoA β-oxidation. These results suggest that PMP70 is involved in the transport of LCFA-CoA across peroxisomal membranes (13–18). ATP was shown to bind to the NBF of ALDP released from peroxisomes by trypsin digestion (19). It was also reported that ATP bound to and was hydrolyzed by the recombinant NBFs of PMP70 and ALDP (20). However, the properties of these proteins against ATP in native states have not yet been characterized. In the present study, we have used photoaffinity labeling of peroxisomes with 8-azido-[α-32P]ATP and 8-azido-[γ-32P]ATP under different conditions, and we have found the following characteristics of native PMP70 and ALDP. (i) 8-azido-ATP is occluded in PMP70 and ALDP in the absence of Mg2+ and is hydrolyzed in the presence of Mg2+. (ii) The hydrolysis product, 8-azido-ADP, is retained in PMP70 and ALDP before removing the excess nucleotides. (iii) PMP70 does not form a stable inhibitory complex E-MgADP-Vi after hydrolysis in the presence of orthovanadate to trap 8-azido-ADP. (iv) PMP70 and ALDP exist as homo- or heterodimer and form a stable complex together with other peroxisomal membrane proteins, which also showed tight ATP binding properties. (v) PMP70 and ALDP are tyrosine-phosphorylated by some protein kinase(s) on peroxisomal membranes.

ABC proteins have divergent functions and can be classified as transporters, channels, and regulators, although their predicted domain structures and amino acid sequences of their NBFs in a region of about 200 amino acids are well conserved (29,30). ABC proteins show various properties in ATP binding and hydrolysis, which might explain the divergent functions of ABC proteins. We compared the nucleotide binding/hydrolysis properties of PMP70 and ALDP with those of other eukaryotic ABC proteins.

It has been reported that 8-azido-ATP is occluded in the absence Mg2+ in NBF1 of CFTR (ABCC7) (31–33). This binding is mediated by minimal Mg2+-dependent hydrolysis and retention of the hydrolysis product, 8-azido-ADP, but orthovanadate does not stabilize 8-azido-ADP binding. 8-azido-ATP oucellation in the absence of Mg2+ was also found in NBF1 of SUR1 and SUR2 (ABCC8, 9) (34–36), channel regulatory subunits of the ATP-sensitive K+ channel. However, it has not been clear whether NBF1 hydrolyzes ATP because NBF1 was photoaffinity-labeled with both 8-azido-[α-32P]ATP and 8-azido-[γ-32P]ATP in the presence of Mg2+ under the conditions examined (35, 37). These observations suggest that the nucleotide binding/hydrolysis properties of PMP70 and ALDP resemble those of NBF1 of CFTR and partially resemble those of NBF1 of SUR.

Although the nucleotide binding/hydrolysis properties of PMP70 and ALDP resemble those of the NBF1s of CFTR and SUR1, PMP70 and ALDP are different in the combination of their properties with those of other eukaryotic ABC proteins.
ately (35, 37). Thus, CFTR and SUR have two NBFs with different nucleotide binding/hydrolision properties in a molecule. However, PMP70 works as a homodimer, and PMP70/ALDP works as a heterodimer that has two NBFs with similar nucleotide binding/hydrolision properties.

The prominent eukaryotic ABC protein, which has two NBFs with similar nucleotide binding/hydrolision properties, is MDR1, a primary active xenobiotics efflux pump. Both NBFs of MDR1 hydrolize ATP and trap ADP in the presence of orthovanadate equivalently. One ADP-Vi has been reported to be trapped in a molecule (38), therefore the NBF at which the initial hydrolision occurs is supposed to be chosen randomly. Two NBFs of PMP70 homodimer and PMP70/ALDP heterodimer would hydrolize ATP equivalently. This combination of two NBFs may cause PMP70/PMP70 and PMP70/ALDP to be primary active transporters like MDR1, but not ion channels like CFTR. Recently, Kashiwayama et al. (39) reported that the cycle of ATP binding and hydrolision induced conformational changes in PMP70.

The properties of ATP binding and the kinetics of its hydrolision might be important to the transport of substrates by PMP70 and ALDP. Recently, we succeeded in purifying ABCA1, which is suggested to be involved in cholesterol homeostasis, and found that purified ABCA1 showed ATPase activity.3 ABCA1 shows 8-azido-ATP occlusion, but no vanadate-included nucleotide trapping was observed (40).4 The kinetics of ATP hydrolision of ABC proteins involved in lipid homeostasis, such as PMP70, ALDP, and ABCA1, could be different from those of MDR1, a xenobiotic transporter.

With regard to phosphorylation of ABC proteins, CFTR, channel gating itself, is known to be regulated by phosphorylation. Both TAP1 and TAP2, which are involved in major histocompatibility complex class I antigen processing and presentation, are phosphorylated under physiological conditions, and their function is modulated by reversible TAP phosphorylation (41). ATPase activities of CFTR have also been reported to be regulated by protein phosphorylation (42). Interestingly, ATP hydrolysis of ABC proteins involved in lipid homeostasis, such as PMP70, ALDP, and ABCA1, could be different from those of MDR1, a xenobiotic transporter.

Another important point addressed in the present study is that PMP70 and ALDP formed a stable complex with each other and formed stable protein complexes in the peroxisomal membranes (43). The complex solubilized by the detergents showed a molecular mass of ~400 kDa on sucrose gradient and ~700 kDa on blue-native PAGE. Previously, we found that PMP70 solubilized with peroxisomal membranes with 0.5% C12E6 showed two peaks with molecular mass of ~500 kDa and 160–200 kDa by gel filtration on superose 6 prep HR15/50 column (44). It is possible that the former is a complex of several proteins including PMP70, ALDP, and other proteins and that the latter is homo- or heterodimer of PMP70 and ALDP.

In this study, we found that PMP70 and ALDP formed a stable complex together with some peroxisomal proteins and bound to and hydrolized ATP with a unique manner. Furthermore, we found that PMP70 and ALDP were tyrosine-phosphorylated. Formation of the complex and phosphorylation of PMP70 and ALDP are expected to be involved in the regulation of fatty acid transport into peroxisomes.

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