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Cell Surface Activities of the Human Type 2b Phosphatidic Acid Phosphatase[1]

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Several isozymes of mammalian type 2, Mg^{2+}-independent phosphatidic acid phosphatase (PAP-2) have recently been cloned, and they are predicted to have their catalytic sites exposed at the cell surface membranes. We investigated the mode of utilization of extracellular lipid substrates by the human PAP-2b expressed in HEK293 cells as a green fluorescent fusion protein. We first confirmed the plasma membrane localization of the expressed PAP-2b. PAP-2b actively hydrolyzed exogenously added lysophosphatidic acid and short-chain phosphatidic acid. In the case of dephosphorylation of lysophosphatidic acid, the reaction products, including inorganic phosphate and monoacylglycerol, were recovered exclusively in the extracellular medium. Interestingly, PAP-2b exhibited negligible activities toward long-chain phosphatidic acid either exogenously when added or generated within the membranes by treating the cells with bacterial phospholipase D. These findings indicate that PAP-2b acts at the outer leaflet of cell surface bilayers and can account for the *ecto*-PAP activities previously described for various types of cells.

Key words: HEK293, lysophosphatidic acid, phosphatidic acid phosphatase, phospholipase D.

Phosphatidic acid phosphatase (PAP); [EC 3.1.3.4] converts phosphatidic acid (PA) to diacylglycerol, and has long been known to occupy a central position in the classical glycerolipid biosynthetic pathway (*1*). Mammalian PAP comprises at least two classes of enzymes that differ from each other with respect to intracellular distribution and enzymological properties (*2*). Although no data are available concerning the molecular structure of the type 1, Mg^{2+}-dependent and soluble PAP (PAP-1), considerable information concerning the type 2, Mg^{2+}-independent and membrane-bound enzyme (PAP-2) has recently come to light. Already 8 species of PAP-2 have been cloned from different organisms (*3*) in addition to several alternatively spliced forms (*4*, *5*). The cloned PAP-2 gene products include the mouse 35 kDa PAP (*6*), human PAP-2a, 2b, and 2c (*7–9*), rat LPP-1 (*10*), rat Dri42 (Ref. *11*, later found to be a homolog of human PAP-2b), *Drosophila* Wunen (*12*), and yeast LPP-1 (*13*). The members of this well-conserved gene family are integral membrane proteins with 6 transmembrane regions and, in the case of the mammalian enzymes, have been shown to be *N*-glycosylated at a single conserved site (*6*, *7*). All of the cloned PAP-2 enzymes belong to a phosphatase superfamily possessing a common novel phosphatase sequence motif (*14*, *15*).

When assayed *in vitro* in the presence of Triton X-100, mammalian PAP-2s dephosphorylate a wide range of lipid phosphates derived from both glycero- and sphingolipid metabolism. Lipid phosphates reported as PAP-2 substrates include, in addition to PA, lyso-PA (*7*, *16*), sphingosine-1-phosphate (*7*, *16*), ceramide-1-phosphate (*7*, *16*), diacylglycerol pyrophosphate (*17*), and *N*-oleoyl ethanolamine phosphoric acid (*8*). Such broad substrate specificity of this class of enzyme has led Waggoner and Brindley to rename PAP-2 family members lipid phosphate phosphohydrolases (LPPs, Ref. *18*). PAP-2s are thus potentially involved in the control of cellular functions by metabolizing a variety of lipid phosphates with signaling functions. Indeed, the physiological importance of this novel class of enzymes has been suggested by the function of *Drosophila* Wunen (*12*), which regulates germ cell migration in the fly embryo.

An important question concerning the function of PAP-2 is whether the enzyme can participate in the metabolic processing of intracellular lipid phosphates. PAP-2 localized on plasma membranes has generally been thought to dephosphorylate PA generated by phospholipase D. To participate in the metabolism of cellular PA, PAP-2 at the plasma membrane should have its catalytic site oriented toward the inner leaflet of the bilayer or exposed on the cytoplasmic surface. However, the catalytic sites of the cloned PAP-2 have been predicted in several studies (*7*, *15*, *18*, *19*) to be located at the putative extracellular loops, and recent work has demonstrated cell surface PAP activities of human and mouse PAP-2a (*9*, *10*). These studies suggest that PAP-2

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Abbreviations: PAP, phosphatidic acid phosphatase; PA, phosphatidic acid; GFP, green fluorescent protein; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; MAPK, mitogen-activated protein kinase.

may degrade extracellular substrates and/or those contained in the outer leaflet of the membrane bilayer. This prediction suggests that the enzyme attenuates receptor-directed extracellular signals such as lyso-PA and sphingosine-1-phosphate rather than participating in the intracellular metabolism of PA and other lipid phosphates. Indeed, Jasinska *et al.* reported that the overexpression of mouse PAP-2a in Rat2 fibroblasts increases the hydrolysis of extracellular lipid phosphates, resulting in the attenuation of mitogen-activated protein kinase (MAPK) activation and DNA synthesis caused by exogenous lyso-PA (*10*). However, there are several reports describing the participation of PAP-2 in intracellular PA metabolism. Human endothelial cells expressing PAP-2a (*4*) and HEK293 cells expressing PAP-2b (*20*) show an increased conversion of intracellular PA to DG, suggesting that PAP-2 participates in the intracellular metabolism of PA.

In order to answer several of the above questions concerning the basic properties of cloned PAP-2s, we attempted to define the intracellular localization and mode of utilization of extracellular substrates by transfected human PAP-2b. During these attempts, we found that PAP-2b actively degrades extracellular lyso-PA and short-chain PA, but shows negligible activity toward long chain PA added exogenously or accumulated within membranes. We also obtained evidence to suggest that PAP-2b can dephosphorylate exogenous lyso-PA without prior binding to the cell surface.

EXPERIMENTAL PROCEDURES

Materials—The sources of most of the materials have been described previously (*6*, *7*). [γ-^{33}P]ATP, [^{14}C]myristic acid, and [^{3}H]lyso-PA(1-[9, 10-^{3}H]oleoyl type) were obtained from DuPont NEN. Radiolabeled PA, lyso-PA, and dioctanoylPA were prepared by incubating dioleoylglycerol, monoolein, and dioctanoylglycerol, respectively, with [γ-^{33}P]ATP and *Escherichia coli* diacylglycerol kinase as described previously (*6*, *7*). FuGENE 6, *Streptomyces chromofuscus* phospholipase D, and anti-green fluorescent protein (GFP) monoclonal antibody were purchased from Roche Diagnostics Corp. Rhodamine-conjugated concanavalin A was purchased from Molecular Probe.

Cell Culture and Transfection—HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum as described previously (*7*). An expression plasmid encoding human PAP-2b fused with GFP at the C-terminus (PAP2b-GFP) was constructed as described previously (*7*) using the plasmid pEGFP-3N (CLONTECH). For transient transfection into HEK293 cells, 0.6 μg per 35-mm dish each of plasmid DNA and FuGENE 6 was used according to the manufacturer's protocol. When approximately 70%-confluent cells were subjected to the transfection procedure, the transfection efficiency was always greater than 90% after 24 h as estimated by fluorescence microscopic observation of GFP-expressing cells.

PAP Assay—For enzyme assays using intact cells, HEK 293 cells transfected with PAP2b-GFP or the empty vector were used 24 h posttransfection. The transfected cells plated on 12-well dishes (approximately 1.4×10^6 cells per well) were starved for 24 h in DMEM containing 0.1% bovine serum albumin (BSA, fatty acid–free, Sigma). The cells were then incubated at 37°C with labeled lipid substrates (5 μM, 5,000–20,000 cpm/nmol) in 0.5 ml of DMEM containing 1 mg/ml of BSA. In this case, the labeled lipids in chloroform were dried under nitrogen, suspended in DMEM containing 1 mg/ml BSA, and dispersed by brief sonication. After incubation, the medium was rapidly aspirated and the cells were rinsed once with 0.5 ml of Tris-buffered saline (10 mM Tris-HCl, pH 7.2, 150 mM NaCl). Aliquots of the combined medium (250 μl) and the attached cells were extracted with 1 ml of chloroform/methanol (1:1, v/v) and 0.3 ml of 0.1 N HCl containing 0.5 M NaCl. After vigorous mixing, the two phases were separated by brief centrifugation. The radioactivity in the water-soluble fractions determined by scintillation counting was taken as the amount of substrate hydrolyzed. We found that 75–80% of the water-soluble radioactivity was recovered as the phosphomolybdate complex (*21*) when [^{33}P]lyso-PA was incubated for 10 min with control cells transfected with empty vector. We also confirmed that the increase in the water-soluble radioactivity caused by the expression of PAP-2b in HEK293 cells could be accounted for by the release of [^{33}P]Pi. In the assay using [^{3}H]lyso-PA, extracted lipids were analyzed by thin-layer chromatography (*6*, *7*, *22*).

For the enzyme assay using permeabilized cells, the starved cells were incubated with labeled lyso-PA in permeabilizing buffer (20 mM Hepes, pH 7.2, 135 mM KCl, 5 mM $NaHCO_3$, 5 mM EGTA, 4 mM $MgCl_2$, 1.5 mM $CaCl_2$, 5.6 mM glucose, 4 mM ATP, 1 mg/ml BSA, and 50 μM digitonin or 0.05% octylglucoside). Loss of membrane integrity was determined by the inability of cells to exclude the vital dye Trypan Blue. In this case HEK293 cells were incubated for 5 min at 37°C with the permeabilizing buffer. The cells were then trypsinized, suspended in phosphate-buffered saline (PBS), diluted 1:2 with 0.4% Trypan Blue, and counted. The percentage of trypan blue positive cells was more than 95% when permeabilized with digitonin or octylglucoside, while the score was less than 3% in the case of intact cells.

For *in vitro* phosphatase assay, the total membrane fractions from HEK293 cells were obtained by subjecting the postnuclear fraction prepared as described previously (*23*) to centrifugation at 100,000 $\times g$ for 1 h. The membranes suspended in homogenization buffer (0.25 M sucrose, 1 mM $MgCl_2$ and 5 mM Hepes, pH 7.2) were preincubated with 4.2 mM *N*-ethylmaleimide at 37°C for 10 min (*24*) to inactivate type 1 PAP. The PAP assay using preincubated membranes was performed as described previously (*6*, *7*, *22*) in reaction mixtures (50 μl) containing 50 mM Tris-HCl (pH 7.5), 0.2 mM labeled lipids, 3.2 mM Triton X-100, 1 mM EDTA, 1 mg/ml BSA, and membranes (10 μg of protein).

Phospholipase D Treatment of Intact Cells and Isolated Membranes—HEK293 cells grown on 35-mm dishes were transfected with PAP2b-GFP and starved for 24 h as described above. The starved cells were radiolabeled with 0.2 μCi/ml of [^{14}C]myristic acid for 3 h in serum-free DMEM. After labeling, the cells were rinsed twice with PBS and incubated at 37°C for 10 min in serum-free DMEM in the presence or absence of bacterial phospholipase D (5 U/ml). The medium was then removed and the rinsed cells were further incubated for 20 min at 37°C in serum-free DMEM. In the case of phospholipase D treatment of isolated membranes, total membranes were obtained from radiolabeled cells by centrifuging the postnuclear fraction as described

above. The membranes were suspended (250 μg of protein/ml) in 10 mM phosphate buffer (pH 7.2) containing 150 mM NaCl, 5 mM sodium pyrophosphate, 1 mM EGTA, and 1 mM $ZnCl_2$. The membrane suspension was treated with phospholipase D (15 U/ml) for 10 min at 37°C with constant shaking. The membrane suspension was then treated with 10 mM iodoacetamide to inactivate phospholipase D, and the incubation was continued for another 20 min. Lipids extracted from the cells and membranes were analyzed by thin-layer chromatography developed by two runs in one dimension as described by van Dijk *et al.* (*25*). First run was developed in petroleum ether/diethylether/acetic acid (25:25:1, v/v), and stopped when the solvent reached the top of the plate. After the position of diacylglycerol was marked, the second run was developed in the upper phase of ethyl acetate/isooctane/acetic acid/H_2O (9:5:2:1, v/v) until the solvent reached the position of diacylglycerol. Various lipid standards were visualized by iodine vapor and the radioactivities of the separated lipids were analyzed with a BAS2000 image analyzer (Fuji).

Immunoblotting—Cell lysates (5 μg protein) were resolved in 12.5% SDS polyacrylamide gels, and transferred onto nitrocellulose membranes by electroblotting (*6*, *7*). The membranes were blocked with Block Ace (Dainippon Pharmaceutical, Tokyo) for 1 h at room temperature. Anti-GFP monoclonal antibody was used at 1:1,000 dilution for 1 h at room temperature in 10% Block Ace in PBS. The blots were washed with PBS containing 0.05% Tween-20, and incubated with secondary antibody for 30 min at a dilution of 1:10,000 in 10% Block Ace/PBS. The blots were washed and detected by chemiluminescence (SuperSignal, Pierce).

Fluorescence Microscopy—HEK293 cells grown on polylysine-coated coverglasses were transfected with PAP2b-GFP. At 24 h posttransfection, the cells were rinsed twice with PBS, and incubated with 5 μg/ml rhodamine-conjugated concanavalin A in PBS containing 1% BSA for 30 min at room temperature. The cells were rinsed once with PBS, and fixed in methanol for 10 min at –20°C. The cells were then washed three times with PBS and once with deionized water, sealed in the presence of Vectashield antifade (Vector Laboratories, CA), and examined using a Laser Scanning Confocal Imaging System MRC-1024 (Bio-Rad Laboratories) equipped with an Eclipse E600 microscope (Nikon). Digital images were acquired and processed using LaserSharp software (Bio-Rad Laboratories), Adobe Photoshop software (Mountain View, CA), and Canvas software (Deneba software, Miami, FL).

RESULTS

Since the subcellular localization of PAP-2 is of critical importance for understanding the function of this class of enzyme, we first attempted to define the cellular localization of human PAP-2b. For this purpose we expressed PAP-2b fused with GFP (PAP2b-GFP) in HEK293 cells and examined the cells by confocal microscopy (Fig. 1). The expressed PAP-2b localized mainly at the cell surface membrane and colocalized completely with concanavalin A used as an established marker for cell surface glycoproteins. In addition, a variable portion of the expressed enzyme was reproducibly detected in intracellular compartments, and this portion remained unidentified. These findings are consistent with the result recently obtained by indirect immunofluorescence analysis of wild-type PAP-2b expressed in HEK293 cells (*20*).

Recently, mouse PAP-2a (lipid phosphate phosphohydrolase-1) expressed in Rat2 fibroblasts has been shown to exhibit cell surface activities degrading various lipid phosphates (*10*). After confirming the localization of PAP-2b in plasma membranes, we investigated whether human PAP-2b also possesses cell surface activities. For this purpose, we transiently expressed PAP2b-GFP in HEK293 cells and examined the mode of hydrolysis of exogenously added lipid phosphates. HEK293 cells were selected as a model system because of their high cDNA transfection efficiency (more than 90%) consistently achieved and also for their relatively low endogenous PAP-2 enzyme activity compared to NIH3T3 cells (data not shown). The expression of PAP2b-GFP was confirmed by Western blot analysis (Fig. 2A). Anti-GFP antibody detected two species of GFP-fusion protein (68 and 60 kDa) and a 31-kDa GFP in extracts of HEK293 cells transfected with the cDNA for PAP2b-GFP and the control vector, respectively. The two immunoreactive species observed for PAP2b-GFP may correspond to the glycosylated and non-glycosylated forms, as previously described for wild-type (*20*) and epitope-tagged PAP-2b (*7*).

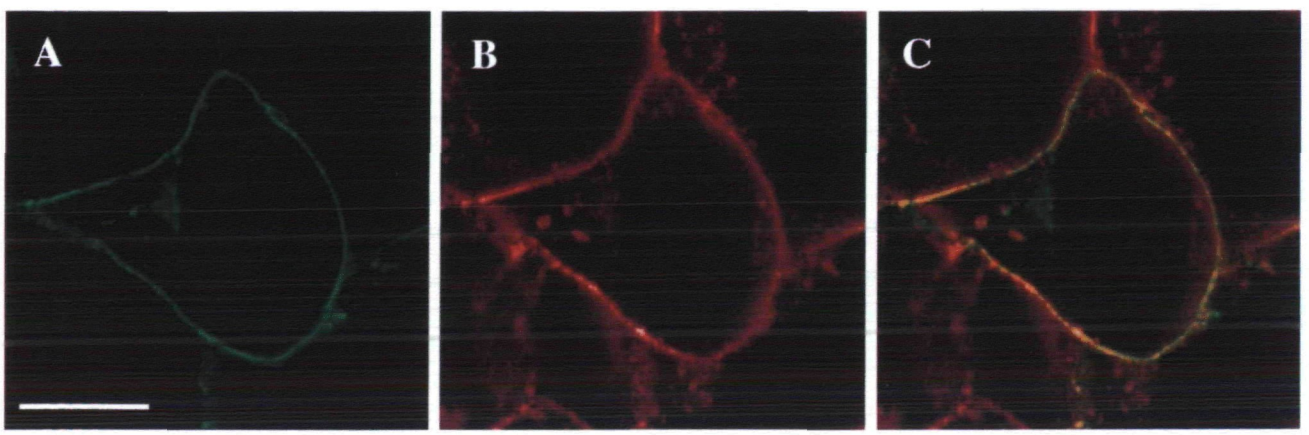

Fig. 1. **Plasma membrane localization of PAP2b-GFP expressed in HEK293 cells.** HEK293 cells expressing PAP2b-GFP (A) were labeled with rhodamine-conjugated concanavalin A (B) and analyzed using a laser scanning confocal microscope. Panel C is a merged image. The bar represents 10 μm.

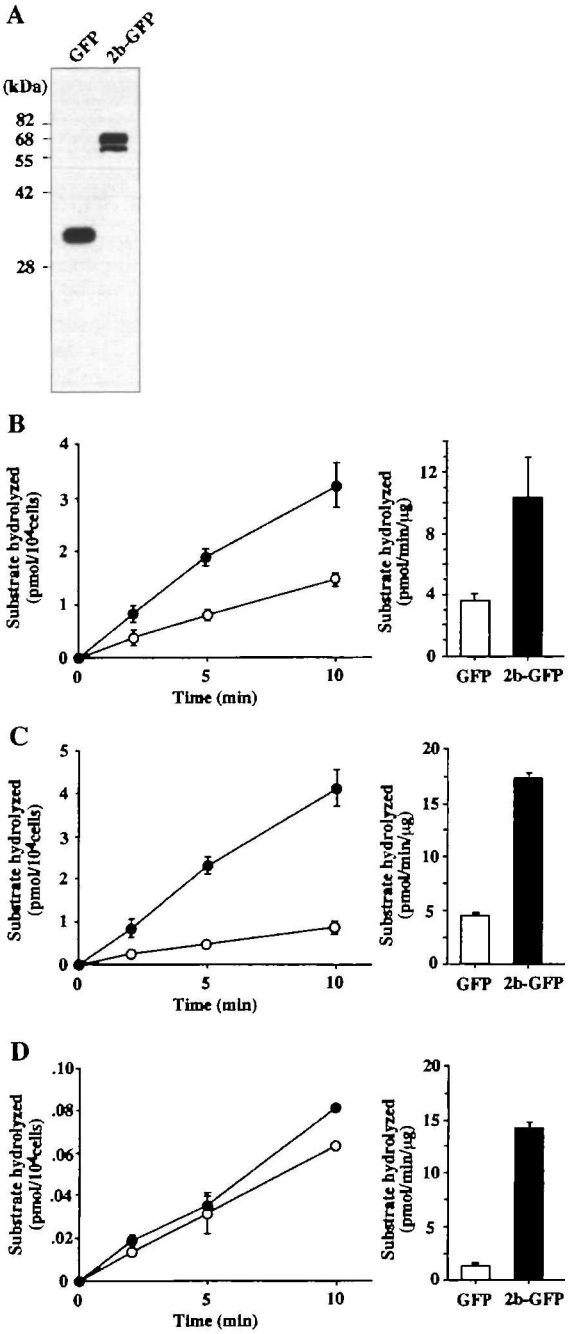

Fig. 2. **Dephosphorylation of exogenous lipid phosphates by intact HEK293 cells expressing PAP2b-GFP.** A, the expression of PAP2b-GFP was confirmed by Western blot analysis. The lysates from HEK293 cells expressing PAP2b-GFP and GFP were analyzed using anti-GFP antibody as described under "EXPERIMENTAL PROCEDURES." The positions of the molecular size markers are shown. B–D, intact HEK293 cells expressing PAP2b-GFP (●) or GFP alone (○) were serum-starved for 24 h, and assayed for PAP activity in Hepes-buffered DMEM containing 1 mg/ml of BSA and 5 μM each of radioactive lyso-PA (B), dioctanoylPA (C), and dioleoylPA (D). After incubation for the indicated periods, the radioactivity of the water-soluble fractions was determined by lipid extraction of the extracellular medium (left panel). In this case very little radioactivity was detected in the water-soluble fractions obtained from the cells. Note the different scales of the *Y*-axis in D. In parallel experiments, *in vitro* PAP activity towards each substrate was determined in the presence of Triton X-100, and the results are given as bars (right panel). All data shown are means ± SD of triplicate determinations.

We also confirmed that GFP fused at the N- or C-terminus of PAP-2b did not affect enzyme activities measured in intact cells and cell lysates.

In the experiments shown in Fig. 2, B, C, and D, cells transfected with the empty vector in parallel were used as controls, and the degradation of lyso-PA, dioctanoylPA and dioleoylPA was measured in intact cells and in *in vitro*

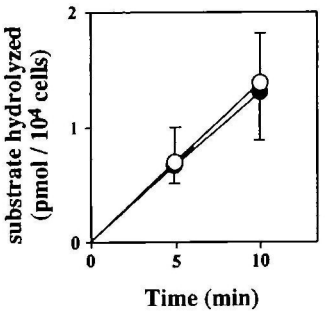

Fig. 3. **Effects of cell permeabilization on the hydrolysis of exogenous lyso-PA by PAP2b-GFP.** HEK293 cells expressing PAP2b-GFP were incubated for varying periods in DMEM containing 1 mg/ml of BSA and 5 μM lyso-PA with (○) or without (●) 50 μM digitonin. The PAP-2b activity was estimated by subtracting the activity obtained for the control, GFP-expressing cells incubated in the same experiments. Data are means ± SD of triplicate determinations.

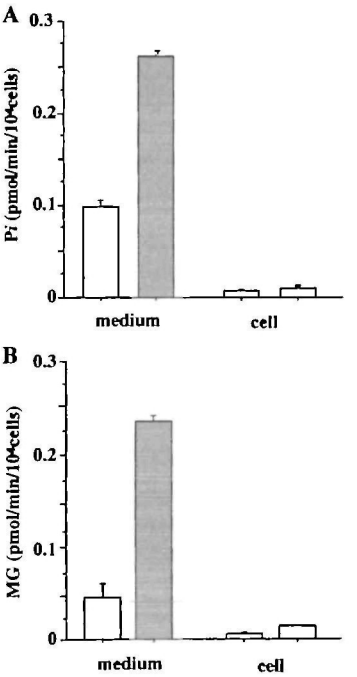

Fig. 4. **Release into the extracellular medium of the reaction products from the hydrolysis of exogenous lyso-PA by PAP2b-GFP.** HEK293 cells expressing PAP2b-GFP (filled bars) or GFP alone (open bars) were incubated for 10 min in Hepes-buffered DMEM containing 1 mg/ml of BSA and 5 μM each of [^{33}P]lyso-PA (A) or [^{3}H]lyso-PA (B). After incubation, the medium and cells were collected separately and subjected to lipid extraction. In A, [^{33}P]P_i was measured as the phosphomolybdate complex, and in B, [^{3}H]monoacylglycerol (MG) produced was separated by thin-layer chromatography, scraped, and assayed for radioactivity. Data are means ± SD of triplicated determinations.

assay using Triton X-100. In intact cells, the transfection of PAP-2b markedly increased the dephosphorylation of exogenously added lyso-PA and dioctanoylPA, which could account for the fold-increase of the *in vitro* PAP-2 activity measured for each substrate simultaneously. As will be shown later, the released P_i was exclusively recovered in the extracellular medium. On the other hand, dephosphorylation of dioleoylPA was extremely low in intact cells and increased to a negligible extent by transfection with PAP-2b despite its high activity in *in vitro* assay. Although the small increment of dioleoylPA degradation caused by PAP-2b transfection was observed reproducibly, the hydrolysis of dioleoylPA by PAP-2b in intact cells was estimated to be less than 1% of the activity toward lyso-PA or dioctanoylPA.

In order to confirm that the dephosphorylation of the lipid substrates occurs at the cell surface, we tested the effects of cell permeabilization on the enzyme activities. As shown in Fig. 3, P_i released from lyso-PA into the extracellular medium, which was ascribed to the expressed PAP-2b, was not significantly affected by cell permeabilization with 50 μM digitonin or 0.05% octylglucoside (not shown). The extremely low activity toward long-chain PA also remained unaffected in permeabilized cells (not shown). These results support the notion that lipid dephosphorylation occurs at the cell surface.

As previously described for various *ecto*-PAP activities, P_i liberated from lyso-PA in the control and PAP2b-transfected cells was recovered almost exclusively in the extracellular medium (Fig. 4). Interestingly, the lipid product, monoacylglycerol, was also released into the medium containing 0.1% BSA used throughout these experiments. It is known that in cells tested in the absence of BSA, lipid products liberated by dephosphorylation at the outer bilayer of the cell surface from lyso-PA (*26*), fluorescently labeled PA (*27*), or octadecylacetylglycerol phosphate (*28*) are rapidly transported across the bilayer to be further metabolized. It is also known that various lipid phosphates bind rapidly to the cell surface, and that before dephosphorylation they can be back-extracted with 2% BSA (*28*) or with liposomes (*27*). At least in neutrophiles, 0.1% BSA inhibits the binding of platelet activating factor and other PA-related substances to the cell surface by as much as 90% (*28*). The present results obtained in the presence of 0.1% BSA thus suggest that the dephosphorylation of exogenous lipid phosphates by PAP-2b may not require their binding to the cell surface and that the liberated lipid products are

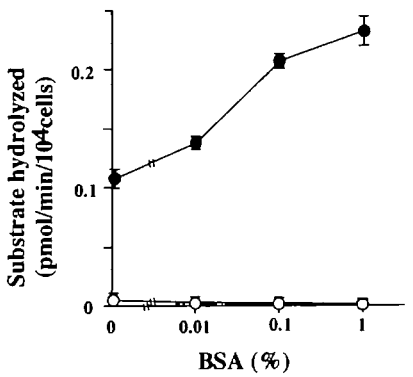

Fig. 5. **Effects of BSA on the hydrolysis of exogenous substrates by PAP2b-GFP.** HEK293 cells expressing PAP2b-GFP were incubated for 10 min in DMEM containing varying concentrations of BSA and 5 μM of radioactive lyso-PA (●) or dioleoylPA (○). The PAP-2b activity was estimated by subtracting the activity obtained for the control, GFP-expressing cells incubated in the same experiments. Data are means ± SD of triplicate determinations.

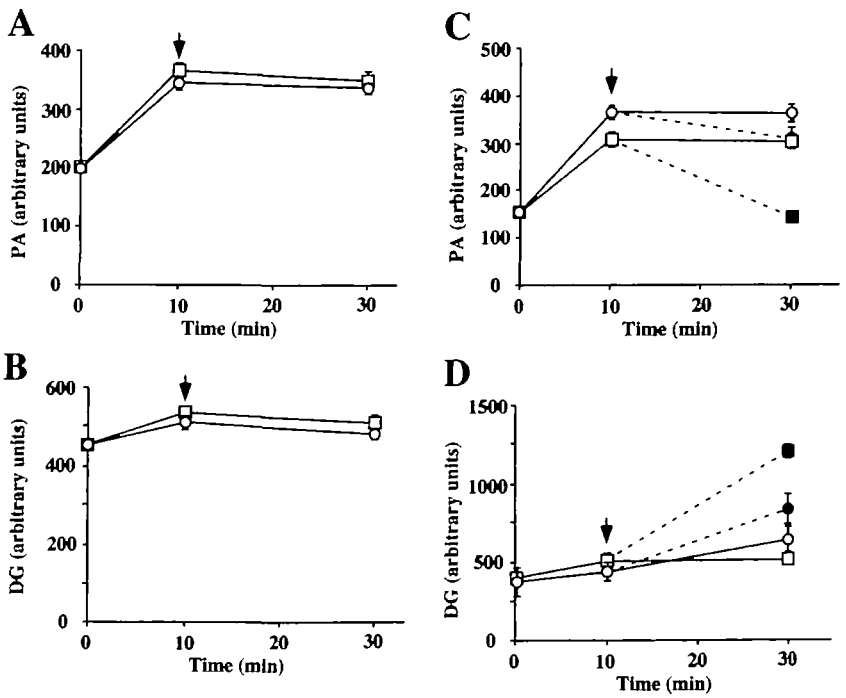

Fig. 6. **PAP2b-GFP fails to dephosphorylate PA generated in intact cells and isolated membranes by treatment with bacterial phospholipase D.** HEK293 cells expressing PAP2b-GFP (□) or GFP alone (○) were serum-starved for 24 h and then labeled for 3 h with 0.2 μCi/ml of [^{14}C]myristic acid. In experiments using intact cells (A and B), the cells were first incubated for 10 min with bacterial phospholipase D (5 U/ml). The medium was then replaced with DMEM *without* phospholipase D (arrows), and the incubation was continued for another 20 min. At the indicated time points, the cells were harvested and crude membranes were prepared as described under "EXPERIMENTAL PROCEDURES." Lipids were extracted from the membranes, separated by thin-layer chromatography, and the signal intensities of PA (A) and diacylglycerol (DG, B) were determined by densitometry using BAS-2000. In the case of isolated membranes (C and D), the membranes (0.25 mg/ml) were incubated with phospholipase D (15 U/ml) for 10 min. Iodoacetamide (10 mM) was then added (arrows) and the mixtures were further incubated for 20 min. In parallel experiments, 1% Triton X-100 was added together with iodoacetamide after 10 min of phospholipase D treatment, and the lipids were analyzed after 20 min incubation (closed symbols). In these experiments, the *in vitro* PAP-2 activities toward dioleoylPA were similar to those given in Fig. 2D for both control and PAP2b-GFP–expressing cells. The data shown are means ± SD of triplicate determinations.

trapped by BSA before being internalized across the cell surface bilayers. In order to see whether the cell surface binding of lipid phosphates is required for PAP-2b activity, we studied the effects of varying concentrations of BSA on cellular activity. As shown in Fig. 5, PAP-2b shows negligible activity toward dioleoylPA even in the absence of BSA, indicating that the inhibition of the cell-binding of long-chain PA by BSA cannot explain the low activity toward this substrate. Furthermore, increasing concentrations of BSA stimulate PAP-2b activity to lyso-PA, and the presence of BSA concentrations as high as 1% fails to inhibit PAP-2b activity. This suggests that PAP-2b can dephosphorylate exogenous lyso-PA without prior binding to the cell surface.

The extremely low activity of PAP-2b toward long-chain PA might have been caused by the limited accessibility due to its hydrophobic property. However, as shown in Fig. 6, the cell surface PA generated by treating intact cells with bacterial phospholipase D failed to be dephosphorylated as no significant changes in the amounts of PA and diacylglycerol were observed during prolonged incubation of control and PAP-2b–transfected cells. Similarly, PA generated in isolated membranes failed to be utilized by the expressed PAP-2b, which became activated upon the addition of Triton X-100. In this case the change in diacylglycerol caused by Triton X-100 did not quantitatively match that of PA, suggesting that diacylglycerol is also produced by unknown detergent-activated processes other than PAP-2b activity. These findings strongly suggest that PAP-2b, and possibly other PAP-2s, have an intrinsic property in intact membranes of being essentially incapable of significantly dephosphorylating long-chain PA either added exogenously or generated at cell surface membranes.

DISCUSSION

In the present work, we transiently expressed human PAP-2b fused with GFP in HEK293 cells, and demonstrated that PAP-2b exhibits cell surface activities that dephosphorylate extracellular substrates. There are several reports available concerning the existence of *ecto*-PAP activity residing in the outer layer of cell surface membranes (*21*, *29*, *30*). These *ecto*-PAPs have been described to dephosphorylate exogenous lyso-PA and short-chain PA actively with much less, but variable, activities toward long-chain PA (*21*, *30*). The *ecto*-PAP activities were studied especially in neutrophiles, which respond actively to exogenous PA by generating superoxide (*21*, *30*). In this case, protein kinase C activation by diacylglycerol generated at the cell surface by *ecto*-PAP was, at least in part, responsible for the neutrophile response (*21*). Recently, it was shown that almost all of the PAP-2 activities in neutrophiles can be accounted for by the *ecto*-PAP activities (*30*). In the case of PAP-2, all of the cloned enzymes are predicted to have their catalytic sites exposed at the cell surface, suggesting that PAP-2s may be the molecular entities of the *ecto*-PAPs so far described. Indeed, the cell surface activities have been observed in Rat2 cells expressing mouse PAP-2a (*10*) and insect cells expressing human PAP-2a (*9*). Together with the present results, it is likely that all of the cloned PAP-2 isozymes can hydrolyze extracellular lipid phosphates.

Using HEK293 cells expressing PAP-2b, we showed that this enzyme actively degrades exogenous lyso-PA and short-chain PA, but with negligible activity to long-chain PA when added in the extracellular medium or generated at the cell surface. This property of the expressed PAP-2b is in general agreement with recent results obtained for mouse PAP-2a (*10*) and neutrophile *ecto*-PAP activities (*30*). So far, the release of P_i into the extracellular medium has been used as an important criterion with which to distinguish *ecto*-PAP activity (*21*, *29*, *30*). In the present study, not only P_i, but also monoacylglycerol liberated from lyso-PA, was recovered exclusively in the extracellular medium containing 0.1% BSA. The mode of cell binding of extracellular lipid phosphates has been studied in detail in fibroblasts (*27*) and neutrophiles (*28*). It is commonly recognized that various PA-related lipids bound at the cell surface are rather slowly internalized, and that these lipid phosphates remain in the outer leaflet of the bilayers, since they can be back-extracted by BSA (*28*) or liposomes (*27*). It is also known that once dephosphorylated at the outer cell surface by PAP, the dephosphorylated lipid products are rapidly internalized by transbilayer movement to be further metabolized (*27*, *28*). The present work shows that monoacylglycerol liberated at the cell surface is trapped by BSA before being transported across the cell membrane, suggesting that the binding of lyso-PA to the cell surface may not be required for the action of PAP-2b. Indeed, increasing concentrations of BSA, a compound known to have a potent inhibitory effect on the cell-binding of octadecylacetylglycerol phosphate (structural analog of lyso-PA, Ref. *28*), were found to stimulate rather than inhibit the dephosphorylation of lyso-PA by PAP-2b. It is thus quite likely that PAP-2b participates in the metabolism of extracellular lyso-PA, which is physiologically present in a BSA-bound form, without its prior binding to the cell surface. Lyso-PA is now established as an extracellular growth signal acting through specific G-protein–linked receptors (*31*). Jasinska *et al.* showed that PAP-2a expression in fibroblasts attenuates the activation of MAPK and DNA synthesis by exogenous lyso-PA (*10*). In repeated experiments, however, we could not obtain reproducible results concerning the attenuating effects of PAP-2b expression on lyso-PA–induced MAPK activation, which is known to occur in HEK293 cells (*32*).

The most puzzling result obtained in the present study is the apparent inability of PAP-2b to dephosphorylate long-chain PA despite its high activity when measured in the presence of Triton X-100. This finding can not be explained by the insolubility of long-chain PA, since PA generated at the cell surface or within membranes also failed to react significantly with the expressed PAP-2b. Indeed, it was previously shown that PA generated by phospholipase D treatment of rat pancreatic islet cells is not converted to diacylglycerol during incubation (*33*). At present, we conclude that such an inability of PAP-2b, and possibly other PAP-2s, to dephosphorylate long-chain PA may be due to an intrinsic property of the enzymes when they are present in intact membranes. However, PAP-2b was recently shown to hydrolyze PA generated by phospholipase D when HEK293 cells were treated with phorbol ester (*20*). In this case such a functional link between PAP-2b and phospholipase D appears to exist only in caveolin-1–enriched detergent insoluble microdomains of the plasma membrane. It is therefore possible that PAP-2b operating at the cell surface can still participate in the control of intracellular signal transduction processes. In this respect, PAP-2a has similarly been

shown to act at the outer surface of plasma membranes, whereas this isozyme has also been reported to lower the steady state concentration of intracellular PA in endothelial cells (*4*). Apparently, more work is needed to understand the mechanisms by which PAP-2 can participate in both extracellular and intracellular lipid metabolism. In any case, the present work shows that PAP-2b participates in the metabolism of extracellular lyso-PA and other lipid phosphates. In view of the potent biological activities of lyso-PA, PAP-2b would control cell migration and other functions that depend on extracellular lipid signals, as has been suggested for the function of *Drosophila* Wunen (*12*).

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