Activation of Polyisoprenyl Diphosphate Phosphatase 1 Remodels Cellular Presqualene Diphosphate[†]

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ABSTRACT: Polyisoprenyl diphosphates play diverse and vital roles in cell function in health and disease. The counter-regulatory lipid signaling molecule, presqualene diphosphate (PSDP), is rapidly converted to its monophosphate form (PSMP) upon cell activation [Levy, B. D., Petasis, N. A., and Serhan, C. N. (1997) *Nature 389*, 985−990]. The first PSDP phosphatase was recently identified and named polyisoprenyl diphosphate phosphatase 1 (PDP1) [Fukunaga, K., et al. (2006) *J. Biol. Chem. 281*, 9490−9497]. Here, we present evidence that PDP1 displays properties of a lipid phosphate phosphatase/phosphotransferase with distinct substrate preference for PSDP. Cell activation with PMA increased PSDP phosphatase activity in a concentration-dependent manner, and Western analysis suggested that PDP1 is directly phosphorylated by protein kinase C. Cellular PSDP phosphatase activity was also induced by the receptor-mediated agonists insulin and TNF-α. To address PDP1's contribution to cellular PSDP phosphatase activity, HEK293 cells were established that stably expressed PDP1 siRNA, leading to a 60% decrease in the level of PDP1 RNA, and concomitant decreases in PDP1 protein and PMA-initiated PSDP phosphatase activity. HEK293 cells harboring the PDP1 siRNA construct also displayed a marked decrease in the extent of PMA-initiated conversion of cellular PSDP to PSMP. Together, these findings are the first to indicate that PDP1 is activated during cell responses to soluble stimuli to convert PSDP to PSMP. Moreover, they provide evidence that PDP1 can serve as a new checkpoint for polyisoprenyl phosphate remodeling during cell activation.

Lipid mediators regulate cell responses to inflammatory stimuli (*1*–*3*). Lipid phosphate phosphatase/phosphotransferase (LPT)[1] proteins, such as lipid phosphate phosphatases (LPPs), sphingosine-1-phosphate phosphatases (SPPs), and sphingomyelin synthases, comprise a family of membrane proteins that play critical roles in lipid metabolism and signaling in organisms ranging from yeast to humans (*4*–*8*). The newest member of this family to be characterized is polyisoprenyl diphosphate phosphatase 1 (PDP1) (also known as PPAPDC2, GenBank accession number AK027568.1) (*9*). Originally identified as a type 2 candidate sphingomyelin synthase (CSS2) that lacked sphingomyelin synthase activity, this protein has a consensus lipid phosphate phosphatase domain (*10*) and was recently found to convert polyisoprenyl diphosphates to their monophosphate forms (*9*). Some members of the LPT family can utilize short isoprenyl diphosphates, such as farnesyl diphosphate (FDP), as substrates (*11*), but PDP1 is the first phosphatase identified that can convert presqualene diphosphate (PSDP) to presqualene monophosphate (PSMP) (*9*).

When cells are exposed to provocative stimuli, signaling pathways are activated to initiate functional responses. To regulate these responses, cells employ both "go" and "stop" signals (*12*). For example, the pro-inflammatory agonists leukotriene B_4 and formyl-methionyl-leucyl-phenylalanine trigger neutrophil NADPH oxidase assembly and reactive oxygen species generation that is activated within seconds but limited so that superoxide anion generation usually stops after several minutes (*13*). The membrane remodeling that accompanies cell activation and deactivation provides a rich source for bioactive lipid mediators (*14*). Presqualene diphosphate is present in cell membranes in nanomolar quantities (*15*). In response to stimuli, PSDP levels in PMN decrease by approximately 30% within seconds and return to baseline within a few minutes (*15*). There is an equally rapid and reciprocal increase in the level of PSMP. Of interest, counter-regulatory autacoids, such as lipoxins, which block cell activation, also block agonist-triggered PSDP

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[1] Abbreviations: CSS2, candidate sphingomyelin synthase type 2; FDP, farnesyl diphosphate; G6P, glucose 6-phosphate; LPP, lipid phosphate phosphatase; LPT, lipid phosphate phosphatase/phosphotransferase; LPR/PRGs, lipid phosphatase-related proteins/plasticity-related genes; LPA, lysophosphatidic acid; PA, phosphatidic acid; PAP2A, phosphatidic acid phosphatase 2A; PPAPDC2, phosphatidic acid phosphatase type 2 domain containing 2; PDP1, polyisoprenyl diphosphate phosphatase 1; PSDP, presqualene diphosphate; PSMP, presqualene monophosphate; PKC, protein kinase C; RT-PCR, reverse transcriptase polymerase chain reaction; S1P, sphingosine 1-phosphate; SPP, sphingosine-1-phosphate phosphatase.

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remodeling (*16*). PSDP and PSDP structural mimetics inhibit two pivotal signaling molecules in cell activation, namely, phosphatidlyinositol 3-kinase and phospholipase D, a property not shared by PSMP (*13, 16, 17*). Taken together, PSDP's ability to inhibit important enzymes for cell activation and its rapid remodeling to PSMP upon exposure of the cell to provocative stimuli suggest the presence of a regulated PSDP phosphatase that controls cellular PSDP and PSMP levels. The results from these experiments are the first to demonstrate agonist-initiated PDP1 activation for PSDP remodeling.

EXPERIMENTAL PROCEDURES

Materials. PSDP was isolated from human neutrophils as described in ref *15*. PMA, glucose 6-phosphate (G6P), and FDP were purchased from Sigma Chemical Co. (St. Louis, MO). The pSilencer 3.1 neo system was from Ambion, Inc. (Austin, TX), Biomol green reagent from BIOMOL International, LP (Plymouth Meeting, PA), and phospho-(Ser) protein kinase C (PKC) substrate antibody from Cell Signaling Technology (Danvers, MA). Sphingosine 1-phosphate (S1P), lysophosphatidic acid (LPA, C18:1), and phosphatidic acid (PA, C10:1) were from Avanti Polar Lipids, Inc. (Alabaster, AL). The PSDP structural mimetic, a PSDP amino bisphosphonate analogue, and the (*Z*)-PSDP isomer were prepared by the organic synthesis core of NIH Grant P50-DE016191 (*13, 17*). All synthetic compounds were characterized by NMR spectroscopy. Rabbit anti-human phosphatidic acid phosphatase domain containing 2 (PPAPDC2) polyclonal antibody was from Exalpha Biologicals, Inc. (Maynard, MA).

In Vitro Phosphatase Assay. For recombinant human PDP1 (rhPDP1) experiments, 2 μg of recombinant protein, isolated as described in ref *9*, was exposed to substrate (0−60 μM) and subjected to rotational mixing (20 min at room temperature). The reaction (total volume of 50 μL) was allowed to proceed for 30 min at 37 °C. The amount of free phosphate was determined using malachite green as described in ref *9*.

For insect cell extract experiments, Sf21 cells were infected (5:1 MOI) with hPDP1 cDNA-expressing baculovirus. Forty-eight hours post-infection, infected and mock-infected cells were harvested by scraping and cells pelleted by centrifugation (100*g* for 10 min at 4 °C). After centrifugation, cells were washed to remove residual medium in calcium- and magnesium-free HBSS and then resuspended in lysis buffer [50 mM Hepes (pH 7.4), 80 mM KCl, 3 mM EDTA, 4 mM DTT, and 1× Complete protease inhibitors (Roche Diagnostics, Indianapolis, IN)]. Cells were disrupted by sonication (three pulses of 10 s) with a Fisher model 100 sonic dismembrator (Fisher Scientific, Pittsburgh, PA). Remaining intact cells and debris were pelleted by centrifugation (100*g* for 10 min at 4 °C). The resulting supernatant was centrifuged at 10000*g* (60 min at 4 °C). The pellet was resuspended in lysis buffer (lacking protease inhibitors) supplemented with 0.1% Triton X-100. One microgram of extract was combined with lipid, and phosphatase activity (30 min at 37 °C) was measured as described above.

For HEK293 cell extracts (see below), 2 μg of the extract and 0−60 μM test compound (PSDP, LPA, PA, FDP, G6P, or S1P) were incubated in a reaction buffer consisting of 50 mM Hepes (pH 7.4), 80 mM KCl, 3 mM EDTA, 4 mM DTT, and 0.1% Triton X-100. After gentle mixing (20 min at room temperature), the phosphatase reaction was allowed to proceed for 30 min at 37 °C.

Lipids. All lipids were maintained dissolved in a $CHCl_3$/MeOH mixture (2:1) at −20 °C. The required amount of lipid was brought to dryness under a gentle stream of nitrogen gas. Material was resuspended in Triton X-100-containing buffer by sonication (1 min at room temperature). The molar ratio of detergent to lipid was kept at a constant 12.5:1 for the Sf21 extract experiments. For all other experiments, the detergent:lipid ratio is described elsewhere.

Constructs. DNA oligonucleotides were cloned into the pSilencer 3.1 neo vector to create transfectable plasmids. The sequences of the oligonucleotides used were GATCCAATATTA and AGCTTAATATTGG (control), and GATCCAGCCAACTTGCTTAAAGAGTTCAAGAGACTCTTTAAGCAAGTTGGCTTTTTTTGGAAA and AGCTTTTCCAAAAAAAGCCAACTTGCTTAAAGAGTCTCTTGAACTCTTTAAGCAAGTTGGCTG (hPDP1 siRNA). After DNA sequencing confirmed each construct, the DNA was transfected into HEK293 cells using SuperFect Transfection Reagent (Qiagen, Valencia, CA). Transfectants were selected in medium containing 800 μg/mL G418 from Sigma Chemical Co. for 4 weeks, and the resultant polyclonal cell lines were maintained in medium containing 100 μg/mL G418.

RT-PCR. Total RNA was isolated using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA), and residual DNA was removed by DNase I (Invitrogen Life Technologies). Ready-To-Go Beads (Amersham Biosciences, Piscataway, NJ) were used to create cDNA from total RNA (2 μg/reaction). PCR (35 cycles) using primers specific to human PDP1 (*9*), two related genes [phosphatidic acid phosphatase 2a (PAP2A) (*9*) and CSS2α (forward, ATGGAGTGATGAACTCGGAAAT; reverse, ACAAAGGAATCTTGCCAGTGAT)], or β-actin (*9*) was performed using cDNA (1 μg/reaction).

Activation of Cellular PSDP Phosphatase. HEK293 cell lines were grown to approximately 80% confluence (5×10^6 cells) in DMEM with 10% FCS and then were exposed to PMA (10^{-8}−10^{-6} M) or vehicle (0.1% EtOH) for 10 min at 37 °C. For experiments with TNF-α and insulin, cells were serum-starved overnight and then exposed to TNF-α, insulin, or vehicle (PBS) for 10 min at 37 °C. After incubation, cells were collected by scraping and extracts prepared as described above for Sf21 cell extracts.

Monitoring of Cellular Polyisoprenyl Phosphates. HEK293 cells (5×10^5) (37 °C and 5% CO_2) were plated in six-well dishes in medium containing 10% fetal bovine serum and allowed to attach to the plate for 2 h. After attachment, the cells were washed and incubated overnight in serum-free medium. The following day, cells were incubated (37 °C and 5% CO_2) for 2 h with [α-^{32}P]ATP (4×10^7 μCi/mL) (PerkinElmer, Waltham, MA) in HBSS with calcium and magnesium. Cells were then exposed (0−60 s at 37 °C) to PMA (2×10^{-6} M). Reactions were terminated by addition of an equal volume (500 μL) of 10% KOH in methanol. Cellular materials were saponified (37 °C for 30 min) as described in ref *15*. After saponification, 0.25 volume (125 μL) of acetone and 4 volumes (2 mL) of a $CHCl_3$/MeOH mixture (2:1, v/v) were added, and the mixture was kept at −20 °C overnight. Nonsaponifiable lipids were extracted as described in ref *15* and separated by TLC with

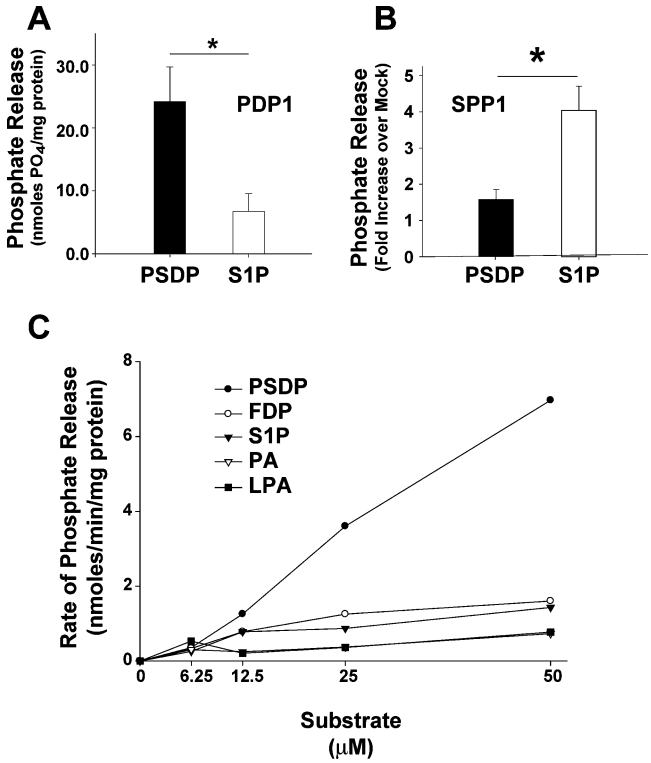

FIGURE 1: PDP1 and SPP1 substrate preferences. (A) rhPDP1 (2 μg) was exposed to PSDP (20 μM) or S1P (60 μM) for 30 min (37 °C) and phosphate release determined (see Experimental Procedures). (B) Cell extracts from HEK293 cells transfected with hSPP1 or a mock control were exposed (30 min at 37 °C) to either PSDP (20 μM) or S1P (60 μM), and phosphate release was assessed. (C) Concentration-dependent phosphate release was measured for rhPDP1 and control Sf21 extracts (1 μg) exposed to PSDP, FDP, S1P, PA, or LPA for 30 min (37 °C) at a constant detergent:lipid molar ratio (12.5:1). The net rate of phosphate release was determined for rhPDP1 Sf21 extracts (see Experimental Procedures). Values represent the mean ± the standard error (SE) ($n \geq 4$; *$P <$ 0.05).

a mobile phase of $CHCl_3$, MeOH, and H_2O (65:25:4, v/v) (45 min at room temperature) in a glass chamber with curtains (Schleicher & Schuell, Keene, NH). PSDP and PSMP were identified by R_f values. Amounts of radiolabeled materials were determined by phosphoimaging using a Molecular Dynamics Storm 840 phosphoimager and ImageQuant.

Statistical Analysis. Results are expressed as the mean ± the standard error of the mean (SEM). $P < 0.05$ was set as the level of significance as assessed by a Student's t test. One-way ANOVA analysis was used to determine differences between groups of three or more.

RESULTS

Substrate Preference of PDP1. Recently, PDP1 was shown, in vitro, to favor the polyisoprenyl phosphates PSDP and FDP over PA (*9*). To further characterize PDP1's substrate preference, we first prepared rhPDP1 and exposed PSDP (20 μM) or the LPT substrate sphingosine 1-phosphate (S1P) (60 μM) in 0.1% Triton X-100 to rhPDP1 (2 μg) for 30 min (37 °C). Phosphatase activity was monitored by phosphate release. Despite 3-fold higher concentrations of S1P, rhPDP1 released significant amounts of phosphate from only PSDP (Figure 1A). The enzyme was next exposed, in parallel, to equimolar amounts (20 μM) of several additional substrates, including LPA and G6P. Only PSDP led to significant phosphate release from rhPDP1 (32.8 nmol of phosphate/mg of protein) ($n = 3$; $P < 0.04$) with a structure−activity relationship for PDP1 that gave the following rank order preference: PSDP > S1P ≈ G6P ≈ LPA.

There are at least three classes of LPTs with phosphatase activity, namely, LPPs, SPPs, and CSS2s (*8*). Because PSDP is a poor substrate for the LPPs (*9*) and CSS2α lacks the amino acid residues predicted for phosphatase catalytic activity (*8*), we next determined if a representative SPP could utilize PSDP as a substrate. HEK293 cells were transiently transfected with an expression vector containing hSPP1 cDNA or an empty vector as a transfection control. SPP1 released substantial amounts of phosphate from S1P, but hSPP1-transfected cell extracts did not carry significant phosphatase activity for PSDP (Figure 1B). Together, published work (*9*) and these new results support a preferential relationship among the LPTs for PDP1 and PSDP.

To determine PDP1 activity in cell extracts, we next expressed hPDP1 in Sf21 cells and exposed membrane extracts to increasing concentrations (0−50 μM) of candidate phosphorylated lipid substrates prepared in Triton X-100 mixed micelles (see Experimental Procedures). Because PDP1 converts PSDP to PSMP and not presqualene alcohol (*9*), we directly compared the amount of phosphate released from PSDP, S1P, FDP, LPA, and PA. The molar ratio (12.5:1) of detergent (Triton X-100) to lipid was kept constant for each lipid concentration. At concentrations greater than 6.25 μM, PSDP gave the highest rate of phosphatase activity (Figure 1C). At a lipid concentration of 50 μM, PDP1's rank order of substrate preference was as follows: PSDP > FDP ≈ S1P > LPA ≈ PA with rates of 6.9, 1.6, 1.4, 0.8, and 0.7 nmol mg^{-1} min^{-1}, respectively. A rate of 6.9 nmol mg^{-1} min^{-1} (50 μM) equates to 8.3% conversion of PSDP to PMSP during the 30 min experimental time frame. More complete enzyme kinetics were not performed with this phosphatase assay secondary to the limited availability of PSDP and the presence of contaminating phosphate in Triton X-100.

Agonist-Initiated PDP1 Activity. Because cell activation leads to PSDP remodeling (*15*), we next investigated the regulation of PDP1 in cells exposed to soluble stimuli. HEK293 cells were chosen as a model system because they express PDP1 (*9*), are readily available, and are more tractable to molecular manipulation than human neutrophils and neutrophil-like cell lines. HEK293 cells exposed to PMA (10^{-8}−10^{-6} M) gave a concentration-dependent increase in the level of release of phosphate from PSDP (20 μM) (Figure 2A). The hPDP1 amino acid sequence was analyzed at NetPhosK (http://www.cbs.dtu.dk/services/NetPhosK/) for phosphorylation sites and determined to contain several serine residues in sequence motifs that could serve as potential targets for direct PKC phosphorylation (Figure 2B). In addition to concentration-dependent increases in PSDP phosphatase activity, PMA also led to concentration-dependent increases in the level of PDP1 phosphorylation by PKC, as determined by Western analysis (see Experimental Procedures) with a phospho-(Ser) PKC substrate antibody (Cell Signaling Technology) that detects phosphorylated serine residues within the context of a PKC phosphorylation motif (Figure 2C). In addition to PMA, extracts of HEK293

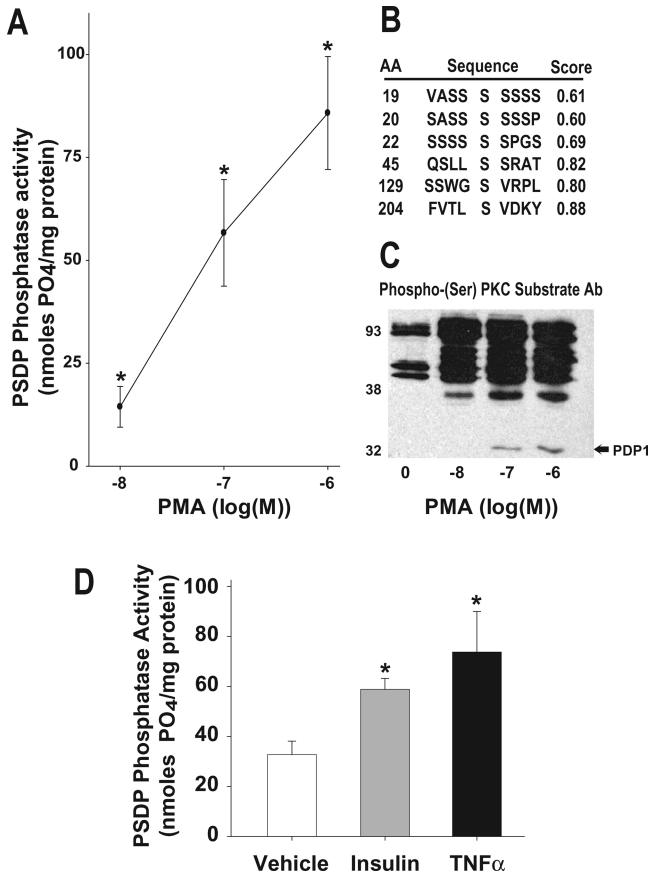

FIGURE 2: Agonist-induced increase in cellular PSDP phosphatase activity. HEK293 cells were exposed (10 min at 37 °C) to (A) PMA ($10^{-8}-10^{-6}$ M) or vehicle (0.1% EtOH) or (D) TNFα (10 ng/mL), insulin (25 μM), or vehicle (PBS), and cell extracts were assayed for PSDP (20 μM) phosphatase activity in 0.1% Triton X-100. Values represent the mean ± SEM ($n = 3$; $^*P < 0.05$ relative to vehicle). (B) Potential PKC phosphorylation sites in hPDP1 amino acid sequence motifs are indicated with a score generated by the NetPhos K 1.0 computer algorithm. (C) Western analysis of cell extracts from HEK293 cells exposed (10 min at 37 °C) to PMA ($10^{-8}-10^{-6}$ M) or vehicle (0.1% EtOH) probed with a phospho-(Ser) PKC substrate antibody (1:1000 dilution, Cell Signaling Technology). Molecular weight markers (left) and PDP1 (right) are indicated.

cells exposed to either the pro-inflammatory cytokine TNF-α (10 ng/mL) or insulin (25 μM) also led to significant increases in the level of release of phosphate from PSDP (20 μM) (Figure 2D). These data indicate that PDP1 phosphorylation and PSDP phosphatase activity in HEK293 cells are stimulated by soluble stimuli and are consistent with a putative role for PDP1 in the conversion of PSDP to PSMP by activated cells.

PDP1 Functions as a PSDP Phosphatase. To determine the quantitative contribution of PDP1 in PMA-initiated PSDP phosphatase activity (Figure 2), we established stable polyclonal cell lines harboring either an siRNA construct designed to reduce the level of PDP1 expression (PDP1 siRNA) or a control construct (mock). Introduction of the siRNA construct reduced PDP1 mRNA levels approximately 60%, as assayed by semiquantitative RT-PCR (Figure 3A,B). To control for off-target effects, expression levels of closely related LPT genes CSS2α and LPP1 were determined. Neither CSS2α, LPP1, nor β-actin was affected by expression of PDP1 siRNA (Figure 3B). To determine protein expression in the PDP1 siRNA cells, blots of extracts from PMA (10^{-7} M)-

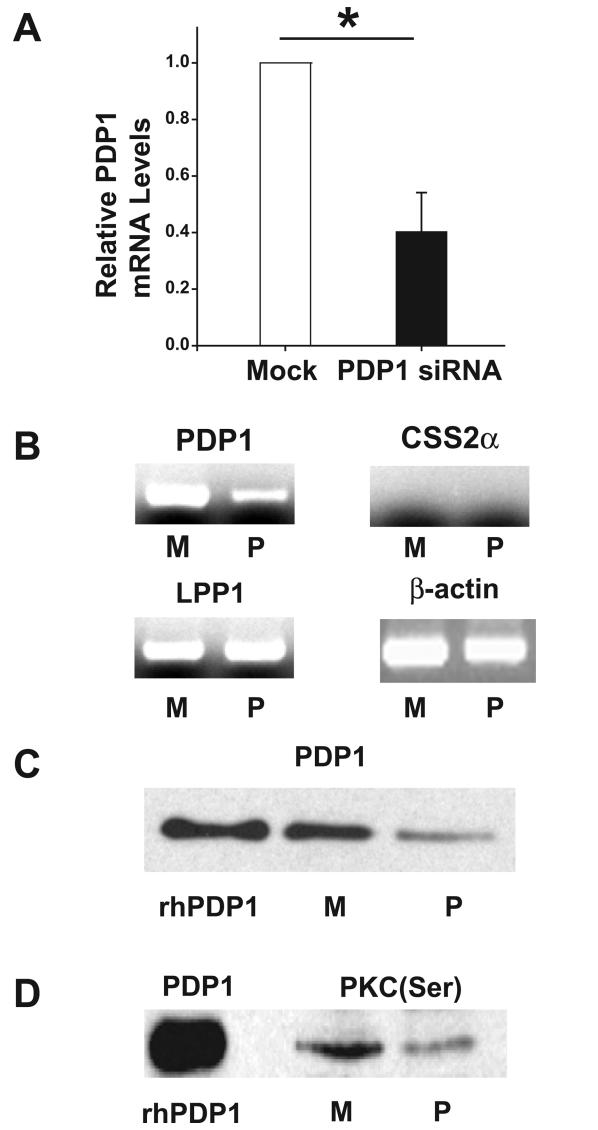

FIGURE 3: Decreased level of cellular PDP1 expression by siRNA. HEK293 cells were stably transfected with either a control vector (Mock or M) or a PDP1 siRNA construct (P) (see Experimental Procedures). (A) PDP1 RNA levels, relative to β-actin, were measured by semiquantitative RT-PCR and densitometry. Values for Mock were arbitrarily set at 1.0. Values for PDP1 siRNA cells represent the mean ± SEM ($n = 3$; $^*P < 0.05$). (B) Expression of the closely related LPT genes, CSS2α, and LPP1 as well as β-actin was assessed by RT-PCR. (C) Protein levels were determined by Western analysis of rhPDP1 (1 μg) or protein extracts from Mock (M) or PDP1 siRNA (P) transfected cells (60 μg) using PDP1 specific antisera (see Experimental Procedures). Representative of $n = 4$.

treated mock and PDP1 siRNA cells were probed with either a PDP1 specific antibody (1:800 dilution) or a phospho-(Ser) PKC substrate antibody (1:1000 dilution). In parallel with the decreased level of RNA expression, PDP1 siRNA cells also expressed approximately 60% less PDP1 protein as measured by Western analysis using both PDP1 specific and phospho-(Ser) PKC specific antibodies (Figure 3C,D).

To determine the impact of PDP1 on agonist-initiated PSDP phosphatase activity, PDP1 siRNA and mock-transfected cells were exposed to PMA (10^{-7} M at 37 °C for 10 min) and the baseline activity was determined in nontransfected cells exposed to vehicle (0.1% EtOH). Incubation (37 °C for 30 min in 0.1% Triton X-100) of cell extracts (2 μg) with PSDP (20 μM) led to significant increases in the levels

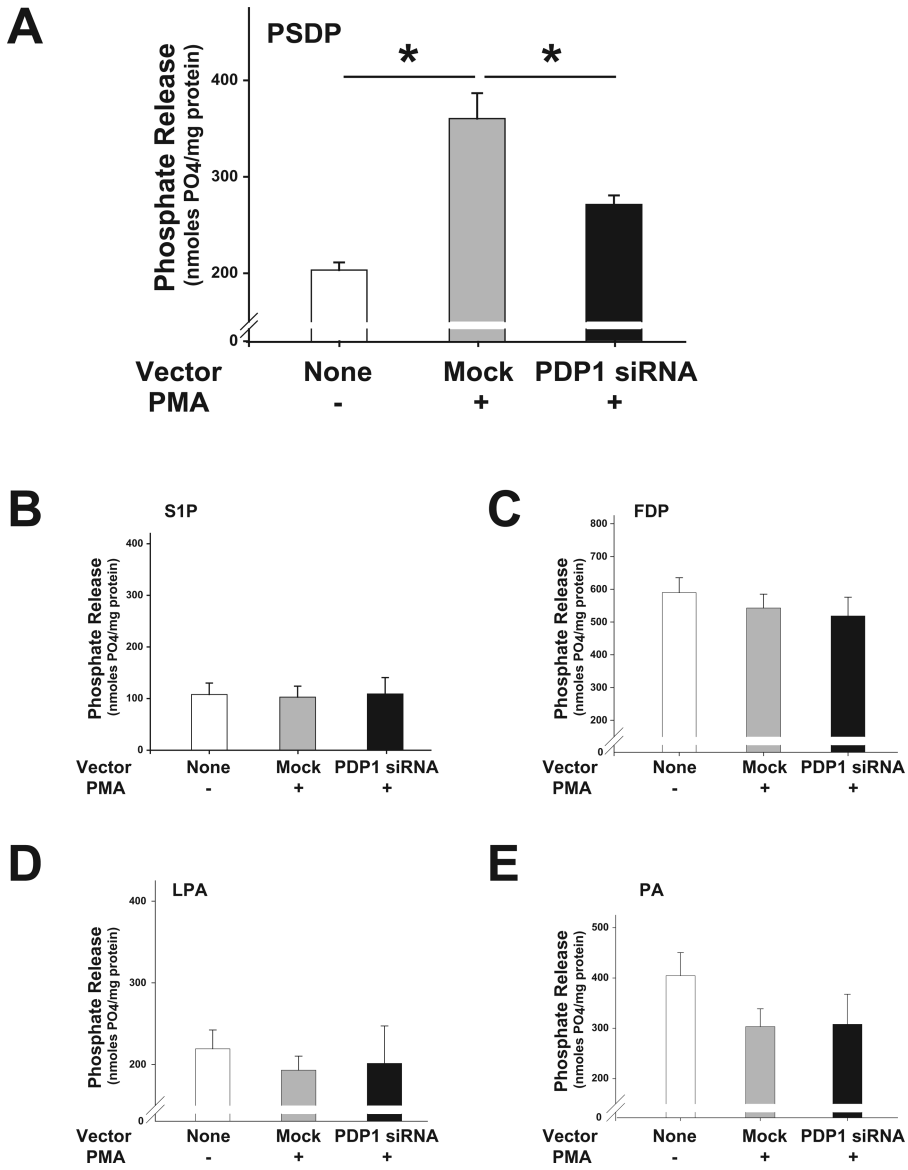

FIGURE 4: Decreased level of cellular expression of PDP1 blocks agonist-induced PSDP phosphatase activity. The level of release of phosphate from (A) PSDP (20 μM), (B) S1P (60 μM), (C) FDP (20 μM), (D) LPA (20 μM), or (E) PA (20 μM) was determined in cell extracts from untransfected HEK293 cells not exposed to PMA (None) or PMA-activated (10^{-7} M for 10 min at 37 °C) cells that were stably transfected with either a mock vector (Mock) or hPDP1 siRNA. Values represent means ± SEM ($n \geq 3$; *$P < 0.01$).

of release of phosphate by the PMA-activated mock-transfected cells (360 ± 83 nmol/mg) compared to nontransfected control cells (203 ± 19 nmol/mg) ($n \geq 3$; $P < 0.01$) (Figure 4A). In the PDP1 siRNA-transfected cells, decrements were present in PMA-initiated phosphate release from PSDP (271 ± 30 nmol/mg) ($n = 4$; $P < 0.01$ vs mock cells exposed to PMA) (Figure 4A) that paralleled the decrements in PDP1 RNA and protein expression (Figure 3). Despite the capacity of rhPDP1 to release phosphate in vitro from S1P, FDP, LPA, and PA (Figure 1), there was no substantial effect of PMA or PDP1 siRNA on agonist-initiated release of phosphate from these potential lipid substrates for cellular PDP1 (Figure 4B−E). Of note, basal levels of phosphatase activity were seen for FDP and PA in these cell extracts, yet in contrast to PSDP phosphatase activity, PMA did not increase the level of release of phosphate from these phosphorylated lipids.

PDP1 Regulates Cellular PSDP Remodeling. To determine if PDP1 remodels polyisoprenyl phosphates in intact cells, endogenous PSDP and PSMP levels were monitored before and after cell activation. HEK mock and PDP1 siRNA stably transfected cells were exposed (37 °C for 0−60 s) to PMA (2 μM). Immediately after cells were stimulated, lipids were extracted and analyzed by TLC (see Experimental Procedures). The amount of materials with the R_f values of PSDP and PSMP was calculated by phosphoimaging (Figure 5). Both PSDP and PSMP were present in cells at baseline (time zero). PMA triggered decrements in PSDP and a significant increase in PSMP in mock cells at 60 s [32.4 ± 13.4% increase compared to baseline ($n = 3$; $P < 0.05$)]. In the PDP1 siRNA cells, both PSDP and PSMP were still detected, but in sharp contrast to mock cells, PMA-initiated PSDP remodeling was completely blocked in the PDP1 siRNA line (Figure 5A,B). These results demonstrate a critical role for PDP1 in the agonist-initiated conversion of cellular PSDP to PSMP and PSDP remodeling occurs in a time frame and amount consistent with a role in signal transduction.

PDP1 Dephosphorylation of PIPPs and Bioactive PSDP Mimetics. Structural mimetics of PSDP have been prepared, and structure−activity relationships indicate that a (*Z*)-PSDP

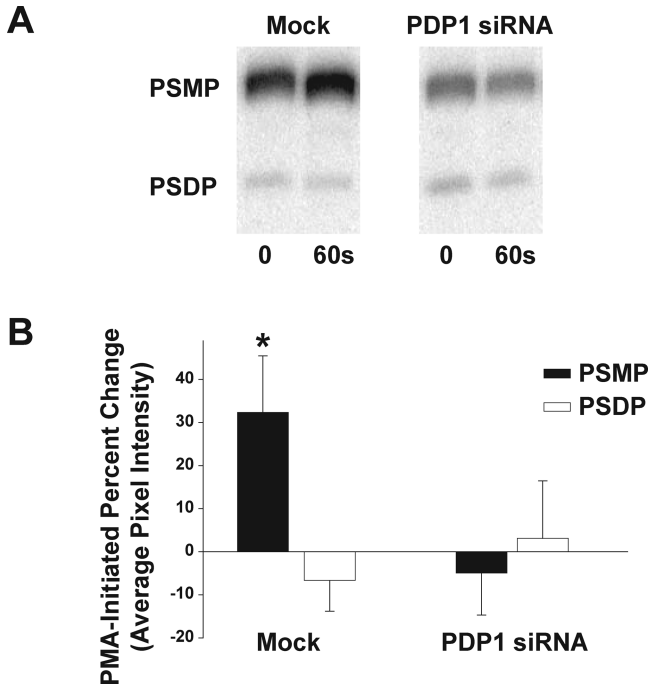

FIGURE 5: Decreased level of cellular expression of PDP1 blocks agonist-induced PSDP dephosphorylation to PSMP. Mock and PDP1siRNA cells were exposed (0−60 s at 37 °C) to PMA (2 μM). Lipids were extracted and analyzed by TLC (see Experimental Procedures). Materials with the same R_f values as PSDP and PSMP were quantitated by phosphoimaging. (A) Image of ^{32}P-labeled PSDP and PSMP. Representative of $n = 3$. (B) PMA-initiated change in ^{32}P-labeled PSDP and PSMP amounts as measured by phosphoimaging. Values represent means ± SEM ($n = 3$; *$P < 0.05$).

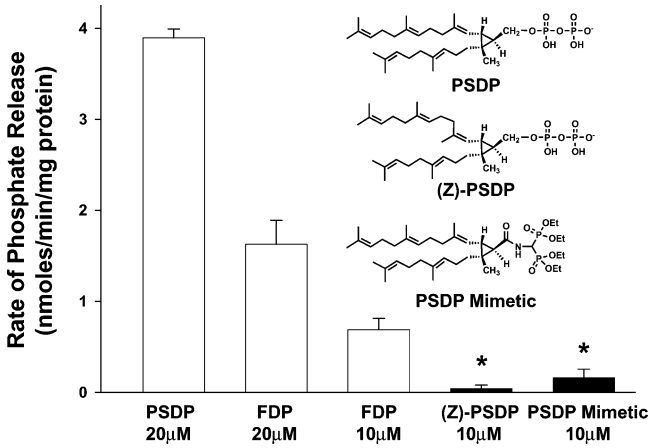

FIGURE 6: PSDP structural mimetics resist PDP1 phosphatase activity. rhPDP1 (2 μg) was exposed to PSDP (20 μM), FDP (10−20 μM), or either the (*Z*)-PSDP or PSDP amino bis-phosphonate structural mimetic (inset) (10 μM) in 0.1% Triton X-100 for 30 min (37 °C) and the level of release of phosphate determined (see Experimental Procedures). Values represent the means ± SE ($n = 3$; *$P < 0.04$ vs PSDP and both 10 and 20 μM FDP).

isomer with a change in double bond geometry and a PSDP amino bis-phosphonate analogue with a modified diphosphate unit (Figure 6, inset) display counter-regulatory properties for human neutrophil activation in vitro and acute inflammation in vivo (*13, 17*). To determine if these bioactive PSDP mimetics are substrates for rhPDP1, each mimetic (10 μM) was exposed (37 °C for 30 min) to rhPDP1 (2 μg) and phosphatase activity measured. While rhPDP1 released substantial amounts of phosphate from native (*E*)-PSDP and FDP, no significant phosphate was released from either the (*Z*)-PSDP isomer or the PSDP amino bis-phosphonate analogue (Figure 6). In addition, neither mimetic inhibited rhPDP1's ability to release phosphate from PSDP (data not shown). These results indicate that the mimetics' protective in vitro and in vivo actions may result from their capacity to resist PDP1-mediated inactivation to their corresponding monophosphates.

DISCUSSION

PSDP is rapidly and transiently remodeled to PSMP during cell activation (*15–17*). Here, we provide several lines of evidence that PDP1 serves as a pivotal PSDP phosphatase. This LPT displays a unique preference for PSDP as a substrate, even when compared to closely related phosphorylated lipids that can serve as substrates for other LPTs. Cellular PSDP phosphatase activity is present in resting HEK293 cells and markedly upregulated by both receptor-mediated agonists and PMA in a concentration-dependent manner. A decreased level of expression of PDP1 blocked agonist-initiated PSDP phosphatase activity, and bioactive structural mimetics of PSDP resist metabolic inactivation by PDP1. Together, these findings implicate PDP1 as a regulated PSDP phosphatase and are the first to demonstrate modulation of PDP1 expression can change endogenous PSDP and PSMP levels in activated cells.

The LPT family of proteins has been segregated by sequence homology into five distinct classes, including the LPPs, SPPs, sphingomyelin synthases, lipid phosphatase-related proteins/plasticity-related genes (LPR/PRGs), and the CSS2s with more distant active site homology with glucose 6-phosphatase (*8*). LPPs are phosphatases that hydrolyze a large range of phosphorylated lipids, including LPA, PA, and S1P, with similar levels of activity toward each phospholipid (*18, 19*). All three LPPs were recently tested for PSDP phosphatase activity, and despite their broad range of substrate utilization, they were not able to efficiently utilize PSDP as a substrate (*9*). In contrast to the LPP wide spectra of substrates, the SPPs are reported to selectively dephosphorylate one principle substrate, S1P (*20*), and here extracts from cells transfected with SPP1 released substantial amounts of phosphate from S1P, but not from PSDP. Sphingomyelin synthases serve as lipid phosphotransferases, not lipid phosphatases, catalyzing the transfer of a phosphocholine group to ceramide to create sphingomyelin (*21*). LPR/PRGs lack the consensus amino acids required for phosphatase enzymatic activity and do not appear to function as either lipid phosphatases or sphingomyelin synthases (reviewed in ref *8*). To date, two CSS2s have been identified, CSS2α and PDP1 (formerly CSS2β). While these proteins lack sphingomyelin synthase activity (*21*), PDP1 carries a lipid phosphate phosphatase domain and activity for PIPPs (*9*). Recently, cell lysates with an increased level of expression of PDP1 were reported to have phosphatase activity for PA (C16:0) (*22*). Of note, CSS2α, similar to the LPR/PRGs, lacks consensus sequences predicted to be critical for lipid phosphatase activity. PDP1 is predicted to have a core protein structure consisting of six transmembrane α-helices with a defined active site split between two extramembranous loops (*8*). Here, we provide further evidence that, in vitro, rhPDP1

was able to utilize a wide variety of substrates yet displayed distinct substrate preference for PSDP, confirming that PDP1 is a member of the LPT family as a PSDP phosphatase that is functionally distinct from even the closely related lipid phosphatases, SPP1, LPP1, LPP2, and LPP3. In future studies, as more becomes known about PDP1's full function, other possible substrates in addition to PSDP may be identified.

Isoprenoids play diverse roles as molecular signals in the regulation of cell function in health and disease, including, but not limited to, isoprenylation, formation of ubiquinone and dolichols, and modification of tRNA (*23–26*). Structure−activity relationships have determined that the diphosphate moiety is important for PSDP's bioactivity as an inhibitor of neutrophil activation and signaling (*13, 15–17*). Consistent with a role in cellular activation, levels of PSDP rapidly and transiently decrease and PSMP levels increase within seconds after cells encounter agonist, concurrent with activation and deactivation of functional responses (*15, 16*). Upon neutrophil activation by leukotriene B_4, PSDP directly interacts with phosphoinositol 3-kinase to potently inhibit its activity and further superoxide anion generation (*17*). These kinetics suggest a PSDP phosphatase as a critical point of regulation for the rapid activation of cells by soluble stimuli. Here, cell activation with PMA led to concentration-dependent increases in both PSDP phosphatase activity and phosphorylation of a 32 kDa protein with properties determined by Western analysis consistent with PDP1. In addition, the expression of PDP1 siRNA in HEK293 cells led to concomitant decreases in PDP1 RNA, protein, and phosphorylation as well as decreased PSDP phosphatase activity, indicating that PDP1 plays a pivotal role in PMA-initiated PSDP remodeling. Of interest, unbiased approaches to exploring cell signaling networks have also uncovered stimulus-triggered phosphorylation of PDP1 after exposure to epidermal growth factor (*27*). Together with prior reports (*9, 13, 15–17, 28*), these findings support roles in addition to cholesterol biosynthesis for PSDP in signal transduction and point to PDP1 as a pivotal checkpoint for these PIPP remodeling events in cell activation.

PSDP structural mimetics have been prepared and screened for bioactivity by inhibition of in vitro cell activation (*13, 17*). These bioactive mimetics also potently block acute inflammation associated with peritonitis and acute lung injury (*13, 17*). The PIPP analogues most active in vivo are a (*Z*)-PSDP isomer and a PSDP amino bis-phosphonate analogue. Characterization of PDP1 as a PSDP phosphatase enabled determination of the capacity of these PIPP mimetics to resist phosphatase-based inactivation, and these PSDP mimetics proved to be neither PDP1 substrates nor inhibitors. Together, these results suggest that these bioactive mimetics can resist metabolic inactivation by PDP1 in vivo, providing a rationale for their superior counter-regulatory properties.

In summary, rhPDP1 displayed catalytic properties of an LPT with a distinct substrate preference for PSDP, and cell activation was associated with PDP1-dependent PIPP remodeling of PSDP to PSMP. These results are the first evidence that PDP1 is critical to agonist-initiated PIPP remodeling in cells and provides a potential new counter-regulatory signaling checkpoint in cell activation.

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