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Functional Characterization of the Atypical Integral Membrane Lipid Phosphatase PDP1/PPAPDC2 Identifies a Pathway for Interconversion of Isoprenols and Isoprenoid Phosphates in Mammalian Cells*

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The polyisoprenoid diphosphates farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP) are intermediates in the synthesis of cholesterol and related sterols by the mevalonate pathway and precursors for the addition of isoprenyl anchors to many membrane proteins. We developed tandem mass spectrometry assays to evaluate polyisoprenoid diphosphate phosphatase activity of an unusual integral membrane lipid enzyme: type 1 polyisoprenoid diphosphate phosphatase encoded by the *PPAPDC2* gene (PDP1/PPAPDC2). *In vitro*, recombinant PDP1/PPAPDC2 preferentially hydrolyzed polyisoprenoid diphosphates, including FPP and GGPP over a variety of glycerol- and sphingo-phospholipid substrates. Overexpression of mammalian PDP1/PPAPDC2 in budding yeast depletes cellular pools of FPP leading to growth defects and sterol auxotrophy. In mammalian cells, PDP1/PPAPDC2 localizes to the endoplasmic reticulum and nuclear envelope and, unlike the structurally related lipid phosphate phosphatases, is predicted to be oriented with key residues of its catalytic domain facing the cytoplasmic face of the membrane. Studies using synthetic isoprenols with chemical properties that facilitate detection by mass spectrometry identify a pathway for interconversion of isoprenols and isoprenoid diphosphates in intact mammalian cells and demonstrate a role for PDP1/PPAPDC2 in this process. Overexpression of PDP1/PPAPDC2 in mammalian cells substantially decreases protein isoprenylation and results in defects in cell growth and cytoskeletal organization that are associated with dysregulation of Rho family GTPases. Taken together, these results focus attention on integral membrane lipid phosphatases as regulators of isoprenoid phosphate metabolism and suggest that PDP1/PPAPDC2 is a functional isoprenoid diphosphate phosphatase.

Farnesyl diphosphate (FPP)[2] is a key intermediate in the mevalonate pathway for synthesis of cholesterol and related sterols (1). FPP and its derivative geranylgeranyl diphosphate (GGPP) are also precursors for the addition of isoprenoid anchors to many membrane proteins (2). The mevalonate pathway makes these polyisoprenoid diphosphates without synthesizing the corresponding isoprenols. However, isoprenols such as farnesol and geranylgeranol can be detected in cells and tissues where their production has been speculated to involve either sequential enzymatic dephosphorylation of their diphosphate derivatives or degradation of isoprenylated proteins (3). Statins are hydroxymethylglutaryl-CoA reductase inhibitors that attenuate synthesis of not only cholesterol but also isoprenoid diphosphates. Indications that many of the beneficial cardiovascular effects of statins are unrelated to their cholesterol-lowering actions suggest that effects on polyisoprenoid diphosphate-dependent protein isoprenylation may be involved (4). This evidence includes demonstrations that exogenously provided isoprenols can reverse inhibition of protein isoprenylation and deleterious effects of statins on cultured cells and can be concurrently incorporated into sterols and proteins (5–8). Although phosphatase activities that can dephosphorylate isoprenoid diphosphates have been reported, the one or more identities of the enzymes responsible have not been established (9, 10). Similarly, although kinase activities that convert isoprenols to their mono- and diphosphate derivatives have been convincingly demonstrated in plants and some microorganisms, direct evidence for their existence in mammalian cells is unconvincing (11). An integral membrane protein with a conserved lipid phosphatase catalytic motif, type 1 polyisoprenoid diphosphate phosphatase encoded by the PPPAPDC2 gene (PDP1/PPPAPDC2), was recently shown to

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[2] The abbreviations used are: FPP, farnesyl diphosphate; PSDP, presqualene diphosphate; GGPP, geranygeranyl diphosphate; AGOH, anilinogeraniol; AGPP, anilinogeraniol diphosphate; ESI, electrospray ionization; GFP, green fluorescent protein; d5-AGPP, ; LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem MS; HPLC, high-performance liquid chromatography; HA, hemagglutinin; DAPI, 4′,6-diamidino-2-phenylindole; LPP, lipid phosphate phosphatase; ER, endoplasmic reticulum; d5-AGPP, anilinogeraniol diphosphate (deuterium 2,3,4,5,6-aniline); PA, phosphatidic acid; LPA, lysophosphatidic acid; S1P, sphingosine 1-phosphate.

dephosphorylate certain isoprenoid diphosphates *in vitro* (12). We present evidence that this enzyme is a functional isoprenoid diphosphate phosphatase and use a chemical biological approach that takes advantage of isoprenol analogs with chemical properties that facilitate their detection by electrospray ionization (ESI) mass spectrometry and selective antibodies to demonstrate effects of manipulations of PDP1/PPPAPDC2 activity on cellular levels of isoprenoid diphosphates and isoprenylated proteins. These experiments reveal the existence of a pathway that interconverts isoprenols with their diphosphate derivatives in mammalian cells and support the idea that PDP1 is a functional component of one arm of this pathway.

EXPERIMENTAL PROCEDURES

General Methods and Reagents—Details of methods used in this report can be found in previous publications from our laboratory (12–17). Western blotting was performed using the Odyssey Infra Red Imaging System (Licor Inc.). The PDP1/PPPAPDC2-selective antibody was generated by immunization of rabbits with a conjugated peptide corresponding to residues 55–69 of human PDP1/PPPAPDC2. Affinity-purified material (1 mg/ml) was used at a 1:1000 dilution for Western blotting. Glycero- and sphingo-phospholipid substrates were obtained from commercial sources (Sigma-Aldrich, Avanti Polar Lipids, Echelon Research Laboratories, Isoprenoids Inc., American Radiolabeled Chemicals, and Amersham Biosciences) or synthesized as described below. The anilinogeraniol-selective antibody has been described previously (18).

Expression Constructs—A cDNA containing the complete human PDP1/PPPAPDC2 sequence was obtained from a publically accessible cDNA collection. Manipulations to generate plasmid and viral expression vectors (in some cases appending epitope tags) for transient or inducible expression in mammalian and yeast cells employed standard approaches using the Gateway cloning system (Invitrogen, Inc). Site-directed mutants were generated using the QuikChange protocol (Stratagene). Lentiviruses for expression of PDP1/PPPAPDC2 in mammalian cells were generated using the Virapower system from Invitrogen. The HuK4 cDNA was obtained from a publically accessible cDNA collection and expressed with a C-terminal GFP tag using pEGFPN1 (Clontech).

Expression of PDP1/PPPAPDC2 in Insect Cells—PDP1/PPPAPDC2 was expressed in insect cells using baculovirus vectors and a total membrane fraction purified from these cells and washed by repeated resuspension and centrifugation as described for other enzymes of this type (19).

Synthesis of Isoprenoid Diphosphates—Isoprenoid diphosphates and isoprenols used in this study, including anilinogeraniol (AGOH), were synthesized and purified as described previously (18, 20). d5-AGPP was prepared by the same procedures using 2,3,4,5,6-d5 aniline as a starting material. Structures were verified by ^{1}H NMR, and compounds were quantitated by elemental analysis or accurate mass measurements. Compounds were stored at −20 °C in 25 mM NH_4HCO_3, pH 8.0. Presqualene diphosphate (PSDP), prepared as described previously (12), was generously provided by Bruce Levy and Troy Carlo, Harvard Medical School, Boston, MA.

Quantitation of Isoprenoid Diphosphates by Tandem Mass Spectrometry—LC-MS/MS analyses of isoprenoid phosphates were performed using an ABI 4000-Qtrap hybrid linear ion trap triple quadrupole mass spectrometer in multiple reaction monitoring mode. Isoprenoid phosphates were separated using a Nucleodur C8 gravity column, 5 μm, 125 × 2 mm (Macherey-Nagel). The mobile phase consisted of 25 mM ammonium acetate with 0.1% triethylamine as solvent A and acetonitrile/25 mM ammonium acetate (4/1) with 0.1% triethylamine as solvent B. Separation was achieved using a linear gradient of 0 to 100% solvent B in 10 min. The flow rate was 0.5 ml/min with a column temperature of 30 °C. The sample injection volume was 10 μl. Unless noted otherwise, the mass spectrometer was operated in negative electrospray ionization mode. Optimal ion source settings determined by infusion of synthetic standards were a declustering potential of −70 V, entrance potential of 10 V, collision energy of −54 V, collision cell exit potential of −1 V, curtain gas of 20 p.s.i., ion spray voltage of −4500 V, ion source gas of 40 p.s.i., and temperature of 550 °C. Multiple reaction monitoring transitions were as follows: AGPP: *m*/*z* 403.9/78.9 and 403.9/158.6; d5-AGPP: *m*/*z* 408.9/78.9 and 408.9/158.6; GGPP: *m*/*z* 449/78.8 and 449/158.7; GPP: *m*/*z* 312.7/79 and 312.7/158.7; FPP: *m*/*z* 381/79 and 381/159; GGP: *m*/*z* 369/79; GMP: *m*/*z* 233/79; AGP: *m*/*z* 323.9/78.9; and PSDP: *m*/*z* 585.207/204.9 and 585.207/132.9. AGPP was also detected in positive mode with instrument-optimized ion source settings by monitoring transitions *m*/*z* 406/228, 406/193, and 406/107. Peak identification and integration were accomplished using ABI Analyst Software. Calibration was accomplished by reference to standard curves obtained using material that was quantitated by digestion and phosphorous determination. AGPP was quantitated by stable isotope dilution using d5-AGPP. MS/MS analysis of AGPP formation in cells incubated with AGOH was accomplished by using the "information-dependent acquisition" capabilities of the instrument in which detection of AGPP-instructed MS/MS analysis of the M^+H^+ species to identify the AGOH moiety. In general, for analysis of compounds obtained from *in vitro* enzyme assays or extracted from cells, individual measurements were made using 10% of the sample.

MS Measurements of Glycero- and Sphingo-phospholipids—Measurements of phosphatidic acid (PA), lysophosphatidic acid (LPA), and sphingosine 1-phosphate (S1P) were accomplished using HPLC ESI tandem mass spectrometry assays that have been reported previously (17, 21).

Lipid Phosphatase Assays—Sources of enzyme activity were incubated with substrates in mixed micelles of Triton X-100. Concentrations of substrates (S1P, C18-LPA, or di16-PA were all from Avanti Polar Lipids) and detergent were varied as detailed under "Results." Incubations were at 37 °C in a final volume of 100 μl containing 25 mM HEPES, pH 7.4, 1 mM EDTA. Reactions were terminated by transfer to ice and addition of 0.3 ml of ice-cold 2-propanol/100 mM NH_4HCO_3, pH 8.0, and 50 pmol of d5-AGPP as an internal recovery standard. Samples were mixed by vortexing, and deproteination was accomplished by addition of 0.5 ml of acetonitrile. Samples were centrifuged at ~20,000 × *g* for 15 min at 4 °C, and the supernatants were transferred to a borosilicate glass vial for evaporation to dryness under a stream of N_2. The dried material

was resuspended in 100 μl of 25 mM NH_4HCO_3, pH 8.0, and transferred to a polypropylene autosampler vial for LC-MS/MS analysis. For assays in which levels of both glycerol- and sphingo-phospholipids and isoprenoid diphosphates were measured simultaneously, the reaction mixture was split into two 50-μl aliquots. Half of the sample was processed for measurement of isoprenoid diphosphates as explained above. The other half was extracted using acidified organic solvents with addition of C17 LPA and/or C17 S1P as a recovery standard (17). After evaporation to dryness, material was resuspended in 100 μl of MeOH for LC-MS/MS analysis.

Cell Labeling with AGOH and Extraction of Isoprenoid Diphosphates—Monolayer cultures of cells at ~30% confluence were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum medium containing vehicle (0.03% DMSO) or vehicle containing a final concentration of 100 μM AGOH. Cells were treated as described previously for analysis of anilinogeraniol incorporation into proteins by Western blotting using an anilinogeraniol-selective antibody (18, 22). For analysis of isoprenoid diphosphate levels, culture medium was removed and cells were washed once in ice-cold phosphate-buffered saline and once in ice-cold freshly prepared 25 mM NH_4HCO_3, pH 8.0, and then scraped in 1 ml of ice-cold 2-propanol/100 mM NH_4HCO_3, pH 8.0, which was transferred to a 15-ml polypropylene tube. The dish was washed with 1 ml of 25 mM NH_4HCO_3, pH 8.0, which was combined with the first extraction. 50–500 pmol of d5-AGPP was added as a recovery standard or for quantitation of endogenous AGPP by stable isotope dilution. The mixture was vortexed for 5 min, sonicated in a water bath containing ice for 5 min, and then vortexed again for 5 min. 3 ml of acetonitrile was added, and the mixture was incubated on ice for 10 min followed by centrifugation at 20,000 × *g* for 15 min to precipitate proteins. The supernatant was transferred to a new 15-ml polypropylene tube and evaporated to dryness under N_2. The oily liquid obtained was resuspended in 100 μl of 25 mM NH_4HCO_3, pH 8.0, vortexed, centrifuged in a microcentrifuge at full speed for 10 min to remove any remaining particulate material, and the supernatant was transferred to a polypropylene autosampler vial for HPLC MS/MS analysis. Recovery of a d5-AGPP internal standard added at the initial extraction step was 30–50% through this entire extraction and sample preparation process.

Inducible Expression of PDP1/PPPAPDC2 in Yeast—Yeast strains harboring 2-μm plasmid vectors for expression of wild-type or catalytically inactive PDP1/PPPAPDC2 under control of the galactokinase promoter (pYES) were obtained by standard approaches. Cells were grown in liquid YEP medium (0.5% yeast extract, 0.5% bacteriological peptone, 0.3% KH_2PO_4, 0.3% $(NH_4)_2SO_4$, and 2% glucose or 2%galactose; all w/v), and cell growth was monitored by measuring optical density. For evaluation of growth on solid agar we used YEP medium, which was supplemented with 50 μg/ml ergosterol and 0.5% Tween 80, both w/v (23). Extractions of isoprenoid diphosphates from yeast followed the methods described for mammalian cells except that pellets of cells were resuspended in 2 ml of ice-cold 2-propanol/100 mM NH_4HCO_3, pH 8.0, and disrupted by grinding with glass beads in a "bead beater" for five 1-min cycles with cooling on ice between cycles. An aliquot of this material was removed for determination of total phospholipid phosphorous after extraction in organic solvents and digestion in perchloric acid (24). After allowing the beads to settle under gravity, the supernatant was removed and processed for LC-MS/MS analysis as detailed above for mammalian cell extracts.

Expression of PDP1/PPPAPDC2 in Mammalian Cells—Immortalized cell lines used in this study were obtained from ATCC or Invitrogen. HeLa cell lines expressing PDP1/PPAPDC2 under control of the tetracycline-inducible promoter were generated by stable transfection of tetracycline repressor-expressing HeLa cells (T-Rex HeLa cells, Invitrogen), which were propagated according to the manufacturer's instructions and selected using Geneticin with an expression vector for HA-tagged PDP1/PPPAPDC2, containing a hybrid CMV/Tet Operator promoter (derived from pT-Rex DEST30, Invitrogen). Several clonal lines of cells with undetectable expression of PDP1/PPPAPDC2 in the absence of the inducer were obtained. Induction of PDP1/PPPAPDC2 expression was accomplished by addition of 1 μM tetracycline to the culture medium. Mouse aortic vascular smooth muscle cells were isolated and cultured as described previously (25, 26). Cells were transfected using plasmid vectors and Lipofectamine (Invitrogen) or infected with lentivirus vectors at a multiplicity of infection of ~5. For Western blotting studies, cells were harvested and lysed, and membrane fractions were prepared as described previously (26). For studies of the susceptibility of the PDP1/PPPAPDC2 C-terminal GFP tag to proteolysis, cells were washed in phosphate-buffered saline and then permeabilized on ice by treatment with 0.3 M sucrose, 0.1 M KCl, 2.5 mm $MgCl_2$, 1 mM EDTA, 10 mM HEPES, pH 7.2, containing 50 μg/ml saponin (Sigma) for 10 min using methods adapted from (27). Trypsin (Worthington Chemical) was added to a final concentration of 10 μg/ml, and the cells were incubated for a further 20 min. Soybean trypsin inhibitor (Worthington Chemical) was added to a final concentration of 100 μg/ml, and the cells were harvested for preparation of membrane fractions, which were analyzed by Western blotting.

Immunofluorescence Microscopy—Cells were plated on serum-coated coverslips and transfected with the indicated plasmid expression constructs using Lipofectamine (Invitrogen). Cells were processed for visualization of filamentous actin, nuclei, and recombinantly expressed or endogenous proteins by staining with rhodamine phalloidin, DAPI, or suitable primary antibodies (14). Images were obtained using a Nikon TE200 fluorescence microscope equipped with a Cascade charge-coupled device camera, acquired using Metamorph software, and processed using Adobe Photoshop. Antibodies for detection of the GFP, Myc, and HA epitope tags and other markers used in this study were from previously described sources (14).

Analysis of Rho Family GTPases—Cells were washed twice with ice-cold phosphate-buffered saline and scraped into lysis buffer containing a mixture of protease inhibitors (100 mM HEPES, pH 7.3, 100 mM KCl, 3.5 mM $MgCl_2$, 3 mM NaCl, 10 mg/ml pepstatin, 10 mg/ml leupeptin, and 100 mM phenylmethylsulfonyl fluoride). Lysates were centrifuged at 800 × *g* for 5 min, and the pellets were discarded. The resulting supernatants were recentrifuged at 100,000 × *g* for 1 h at 4 °C; this pellet provided the membrane fraction, and the supernatant,

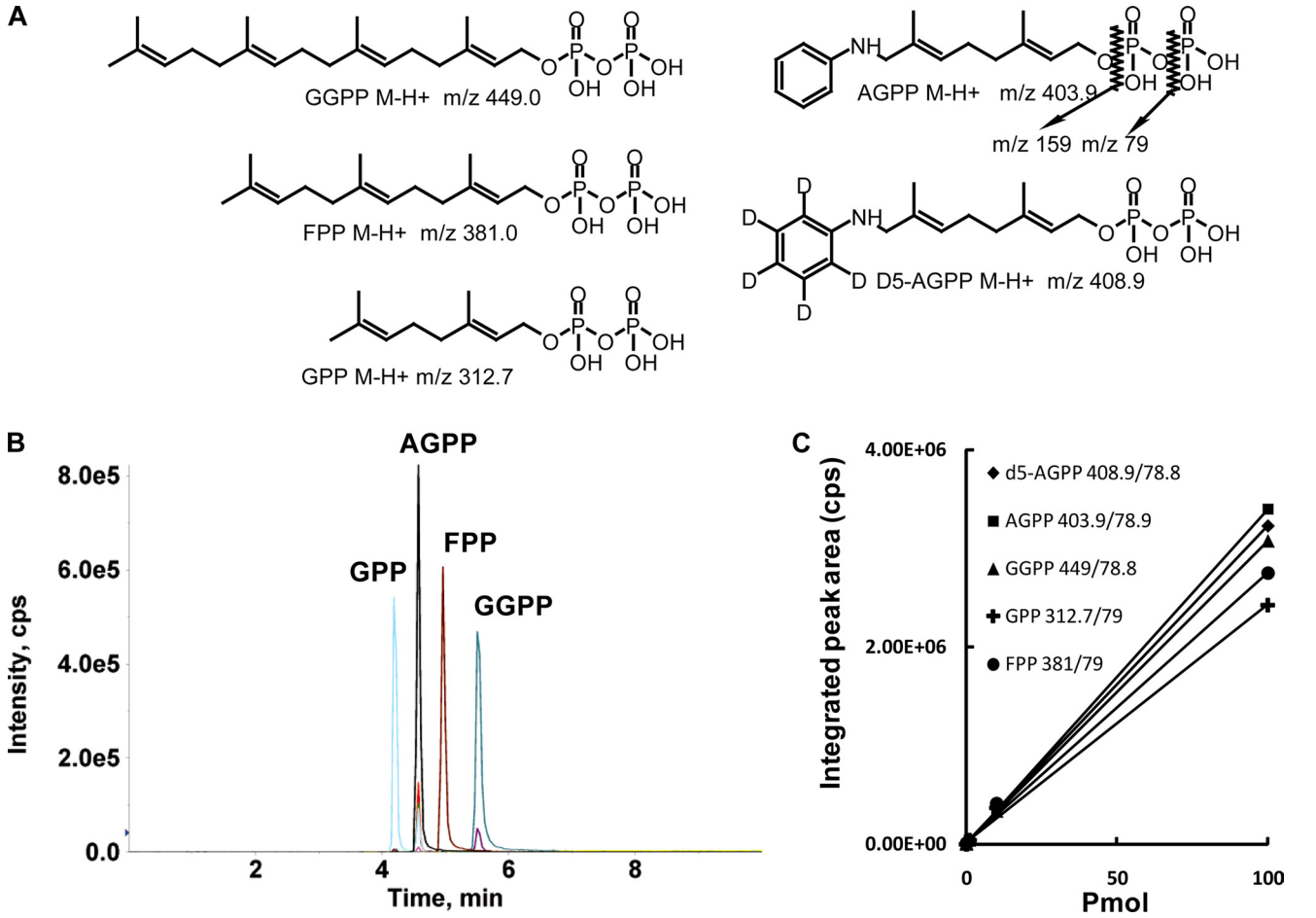

FIGURE 1. **Quantitation of polyisoprenoid diphosphates by HPLC ESI tandem mass spectrometry.** *A*, structures of natural and unnatural polyisoprenoid diphosphates. *B*, extracted ion chromatogram of selective reaction monitoring mode detection of the indicated isoprenoid diphosphates. For each analyte the two coincident peaks represent monitoring the mono- and diphosphate product ions. Detection of all analytes was most sensitive when monitoring the monophosphate product ion. *C*, calibration curve for quantitation of the indicated isoprenoid diphosphates.

provided the cytosolic fraction. The membrane pellet was resuspended in lysis buffer (containing 1% Triton X-100), sonicated for 30 s, and centrifuged at 800 × *g* for 10 min at 4 °C. The supernatant obtained was used as the "membrane fraction" for analysis of RhoA. Measurement of GTP-bound Rho was performed using a Rho activation assay kit (Upstate Biotechnology), following the manufacturer's instructions. Briefly, the RhoA-binding domain of rhotekin expressed as a glutathione *S*-transferase fusion protein was used to affinity-precipitate GTP-bound Rho from the cell membrane and cytosolic fractions. Precipitated Rho-GTP was then detected by immunoblot analysis, using a polyclonal anti-RhoA antibody (Upstate Biotechnology) (26).

RESULTS

Tandem Mass Spectrometry Assays for Isoprenoid Phosphates—We developed HPLC ESI multiple reaction monitoring mode tandem mass spectrometry assays for the mono- and diphosphate derivatives of farnesol, geraniol, and geranylgeraniol and for the squalene synthase intermediate PSDP. Quantitation was accomplished by reference to calibration curves generated using synthetic standards that were independently determined by elemental analysis or in some cases by stable isotope dilution using deuterated variants of some of the molecules of interest. AGOH is a synthetic analog of farnesol that has been used extensively as a probe for monitoring protein isoprenylation. The diphosphate derivative of this unnatural isoprenol, AGPP, is a substrate for farnesyl transferases, and the widely observed incorporation of this probe into cellular proteins when AGOH added to intact cells implies prior conversion to AGPP (18). To enable quantitation of AGPP by stable isotope dilution we synthesized a deuterium-substituted variant of AGPP, d5-AGPP, which we also used as a recovery standard to monitor stability of these labile molecules during extraction and sample preparation for analysis. For studies of the dephosphorylation of AGPP we also developed a positive-mode HPLC ESI tandem mass spectrometry assay for AGOH. Finally, we adapted and validated methods for extraction of isoprenoid phosphates from cells and enzyme assays. Fig. 1 shows the structures of the natural and unnatural isoprenoid diphosphates used in this study along with representative extracted ion chromatograms and calibration curves for some of these assays.

Substrate Selectivity of Recombinant PDP1/PPPAPDC2—PDP1/PPPAPDC2 is an integral membrane protein containing a conserved lipid phosphatase catalytic motif found in the related lipid phosphate phosphatase (LPP) enzymes. PDP1/PPPAPDC2 exhibits Mg^{2+}-independent polyisoprenoid diphosphate, PA, and S1P (12, 28, 29) activities, although a direct quantitative comparison of these substrates has not been

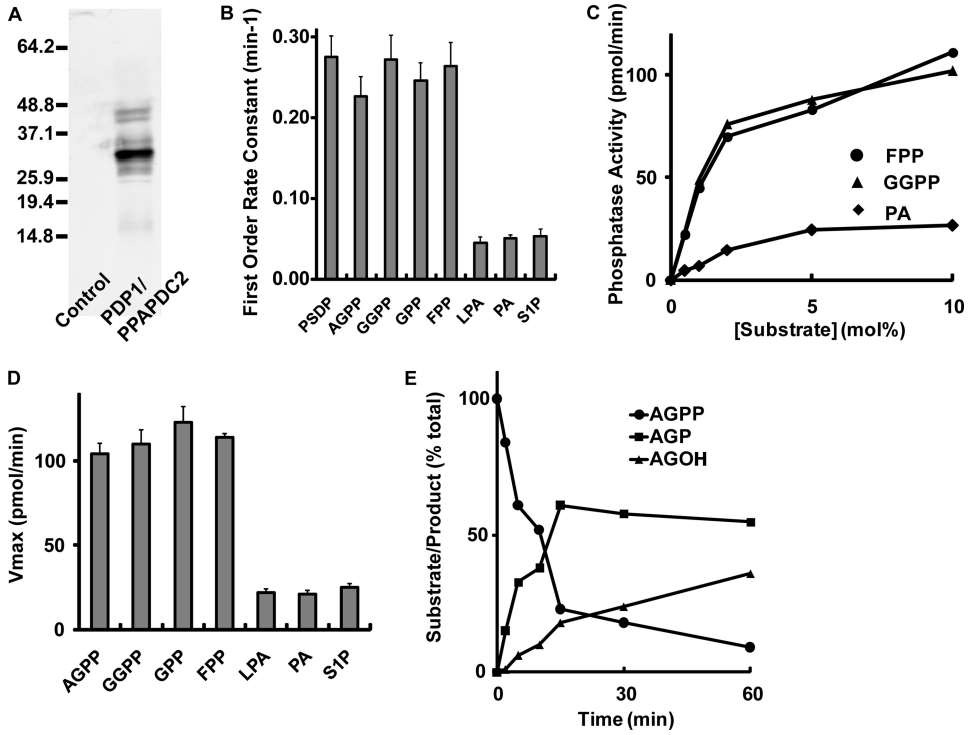

FIGURE 2. **Substrate specificity of PDP1.** *A*, PDP1/PPPAPDC2 or an irrelevant control protein were expressed in Sf9 cells using a recombinant baculovirus vector. Equal quantities of total membrane proteins were analyzed by SDS-PAGE and Western blotting using a PDP1-selective antibody. *B*, detergent-extracted membrane proteins were used as a source of PDP1/PPPAPDC2 activity in assays containing the indicated substrates at a concentration of 1 μM solubilized in 3.2 mM Triton X-100. Data show means ± S.D. ($n = 3$) from a representative experiment. Phosphatase activity associated with an equivalent quantity of detergent-extracted membrane proteins from control cells was <1% of that observed with material from PDP1/PPPAPDC2-expressing cells. *C*, substrate concentration dependence of PDP1/PPPAPDC2 phosphatase activity against the indicated substrates. Data shown are means of duplicate determinations from a representative experiment. *D*, phosphatase activity of PDP1/PPPAPDC2 was determined at a concentration of 100 μM of the indicated substrates solubilized in 3.2 mM Triton X-100. Data shown are means ± S.D. ($n = 3$) from a representative experiment. *E*, sequential dephosphorylation of AGPP by PDP1/PPPAPDC2 was monitored by measuring time-dependent changes in AGPP, AGP, and AGOH in incubations containing 100 μM AGPP and 3.2 mM Triton X-100. Data shown are means of duplicate determinations.

reported. We expressed PDP1/PPPAPDC2 in insect cells and used membranes prepared from these cells as a source of enzyme activity (Fig. 2*A*). PSDP has been suggested to be a preferred PDP1/PPPAPDC2 substrate (12). Because our supplies of PSDP were limited we initially conducted experiments to compare the rate of dephosphorylation of a series of candidate substrates in incubations containing 3.2 mM Triton X-100 and 1 μM of each lipid phosphate substrate. At this low concentration, substrates were dephosphorylated with apparent first order kinetics. Relative activities were compared by calculating first order rate constants. The rate of PDP1/PPPAPDC2-catalyzed dephosphorylation of the isoprenoid diphosphate substrates was ~4-fold higher than for the glycero- and sphingophospholipid substrates. At these low substrate concentrations PSDP was dephosphorylated at the same rate as other polyisoprenoid diphosphates (Fig. 2*B*). To examine the kinetic basis of the apparent preference of PDP1/PPPAPDC2 for isoprenoid diphosphates, we examined the dependence of PDP1/PPPAPDC2 activity on substrate concentration by increasing the molar fraction of substrates in Triton X-100-mixed micelles. As reported for LPPs (19), PDP1/PPPAPDC2 activity was dependent on the surface concentration of substrate. The apparent K_m values for the substrates tested were comparable. By contrast V_{max} values for the isoprenoid diphosphate substrates were ~4-fold higher than for glycerol- or sphingo-phospholipid substrates suggesting that this is the basis for the preference of PDP1/PPPAPDC2 for isoprenoid diphosphate substrates observed at low substrate concentrations (Fig. 2, *C* and *D*). Neutral isoprenols cannot be readily detected by ESI mass spectrometry. The synthetic isoprenoid diphosphate, AGPP, is an excellent PDP1/PPPAPDC2 substrate (Fig. 2) and has chemical properties that facilitate detection of the corresponding isoprenol, AGOH, by positive-mode ESI mass spectrometry (see Fig. 6). Studies using this substrate revealed that complete dephosphorylation of isoprenoid diphosphates by PDP1/PPPAPDC2 proceeds sequentially. The rate of hydrolysis of the monophosphate AGP was slower than that of the diphosphate (Fig. 2*E*). PDP1/PPPAPDC2 contains a lipid phosphatase catalytic domain sequence in which residues known to be critical for catalysis in related enzymes are completely conserved (30). This motif is not conserved in the related proteins PPAPDC1 and PPAPDC3/NET39 neither of which exhibit lipid phosphatase activity[3] (31) (Fig. 3*A*). Mutation of conserved residues in each of the phosphatase catalytic motif sequences abolished PDP1/PPPAPDC2 activity (Fig. 3*B*). Unlike the LPPs, hydropathy analysis of PDP1/PPPAPDC2 and the related PPAPDC1 and PPAPDC3/NET39 proteins predicts a four-transmembrane helical topology that orients the C1 and C2 catalytic domain sequences in the cytoplasmic face of the membrane (Fig. 3*D*, *model 1*) (31) (available on-line from EMBnet). However, because an experimentally validated mechanism for these enzymes based on the structure of related fungal vanadium-dependent oxidases requires that the C1, C2, and C3 sequences must cooperate during catalysis (30), this orientation is incompatible with the observation shown here and reported by others that PDP1/PPAPDC2 is an active phosphatase. We found that a C-terminal GFP tag appended to PDP1/PPPAPDC2 was readily susceptible to proteolysis in cells treated with trypsin in the presence of saponin to permeabilize the plasma membrane under conditions where HuK4, a *bona fide* type 2 ER resident transmembrane protein (32) with a similar C-terminal tag, was resistant to degradation implying a cytosolic localization of the C terminus (Fig. 3*C*). The fourth predicted transmembrane helical region in PDP1/PPPAPDC2, PPAPDC1, and PPAPDC3/

[3] A. J. Morris, unpublished results.

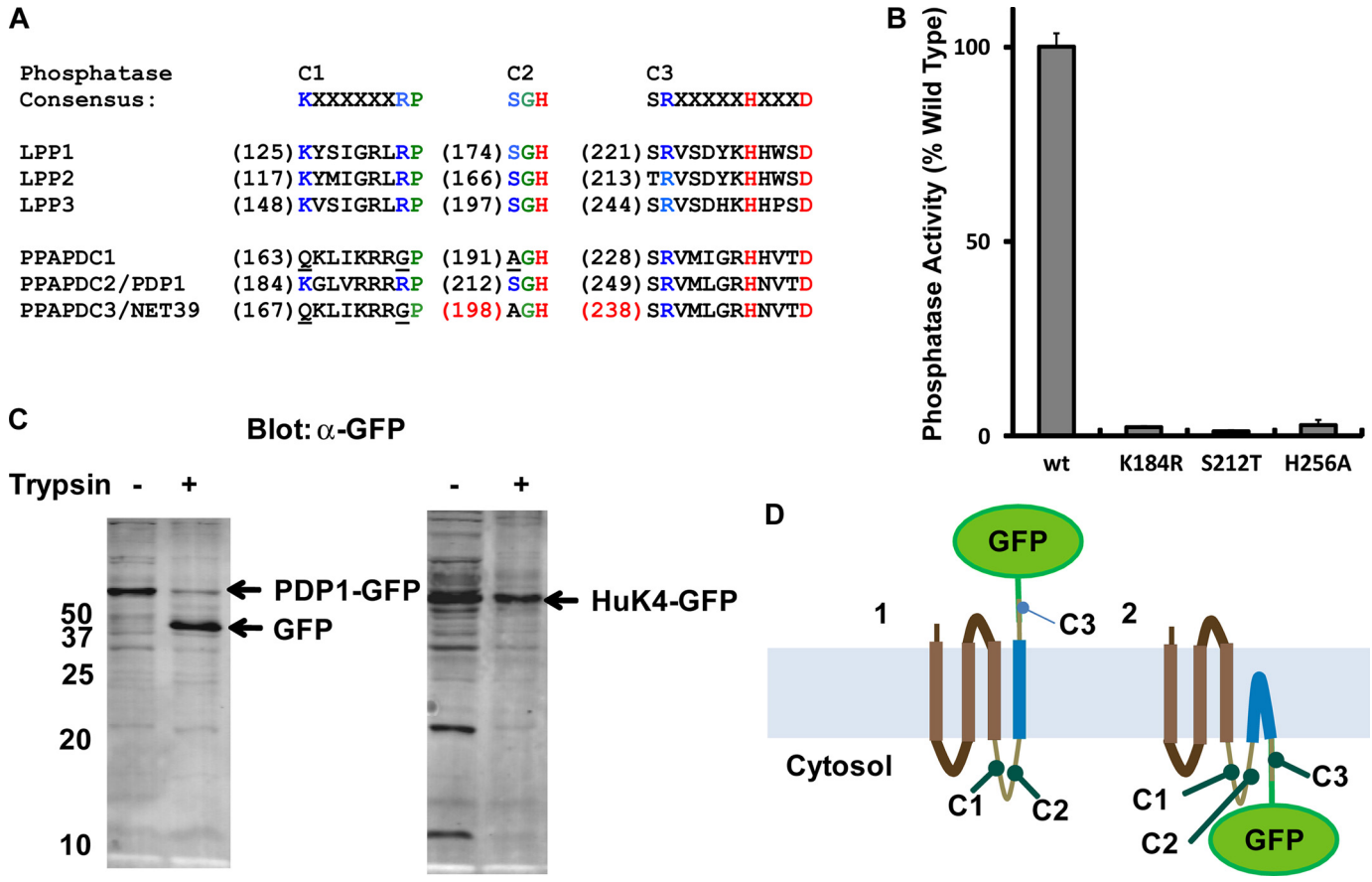

FIGURE 3. **Requirement of PDP1 catalytic domain residues for sequential dephosphorylation of isoprenoid diphosphate substrates.** *A*, catalytic motif sequence alignment of PDP1/PPPAPDC2 with LPPs1–3, PPAPDC1, and PPAPDC3/NET39. *B*, the indicated PDP1/PPPAPDC2 mutants were expressed in insect cells using baculovirus vectors and polyisoprenoid diphosphate phosphatase activity determined and normalized to expression levels determined by Western blotting using the PDP1/PPPAPDC2-selective antibody. *C*, PDP1/PPPAPDC2 or HuK4 with C-terminal GFP tags were expressed in HEK293 cells. Cells were permeabilized by treatment with saponin and incubated with vehicle or trypsin. Proteins were analyzed by Western blotting using an anti GFP antibody. *D*, transmembrane topology models of PDP1/PPPAPDC2 as predicted by hydropathy analysis (*1*) or suggested by our experimental observations (*2*).

NET39 is longer (28 residues) than transmembrane helical regions 1–3, which have 19–22 residues, and a proline residue is conserved between all three proteins in the center of this region. We speculate that this region may form a kinked helix that orients the C terminus of the protein toward the cytoplasm, which would juxtapose the C1, C2, and C3 catalytic sequences to form a complete catalytic site (Fig. 3*D*, *model 2*).

Inducible Expression of PDP1/PPPAPDC2 in Yeast Causes Growth Defects and Sterol Auxotrophy—In budding yeast the primary role of the mevalonate pathway is synthesis of the essential sterol ergosterol. Mutations in mevalonate pathway genes result in sterol auxotrophy, and growth and viability can be restored by supplementation of growth medium with ergosterol (33). Yeast do not contain a PDP1/PPPAPDC2 homolog, but we reasoned that if PDP1/PPPAPDC2 was a functional polyisoprenoid diphosphate phosphatase overexpression of this enzyme in yeast would interfere with ergosterol synthesis. We generated yeast strains harboring constructs for expression of wild-type and catalytically inactive PDP1/PPPAPDC2 S212T under control of a galactose-inducible promoter. Induction of expression of wild-type PDP1 but not PDP1/PPPAPDC2 S212T resulted in a dramatic decrease in the growth of these strains either on agar plates or in suspension cultures. Remarkably, we found that supplementation of the growth medium with ergosterol under conditions that favor uptake of this sterol largely complemented these growth defects (Fig. 4, *A–C*). Using our mass spectrometry methods, we found that levels of FPP were significantly reduced when PDP1 expression was induced in these strains (Fig. 4*D*). These results suggest that the apparent preference of PDP1/PPPAPDC2 for polyisoprenoid diphosphates observed *in vitro* using detergent-solubilized substrates is preserved in a more physiological setting.

Subcellular Localization of Recombinant PDP1/PPPAPDC2—Our PDP1-selective antibody was ineffective for indirect immunofluorescence so we examined the subcellular localization of recombinantly expressed C-terminal epitope tagged variants of PDP1 in HEK293 cells. At 12 h post transfection PDP1-GFP localization substantially overlapped with the ER marker calnexin. Similar localization patterns were observed in a variety of mammalian cells (not shown), and localization was independent of the epitope tag used to visualize the protein (Fig. 5, *A* and *B*). Interestingly, when examining the subcellular localization of PDP1in HEK293 cells by confocal microscopy, in addition to the predominant ER distribution revealed by wide-field microscopy, we often observed pronounced PDP1 staining around the rim of the nucleus. At times greater than 36 h post transfection, multinucleate cells appeared (see also Fig. 8), and localization of PDP1 to the nuclear rim was variably

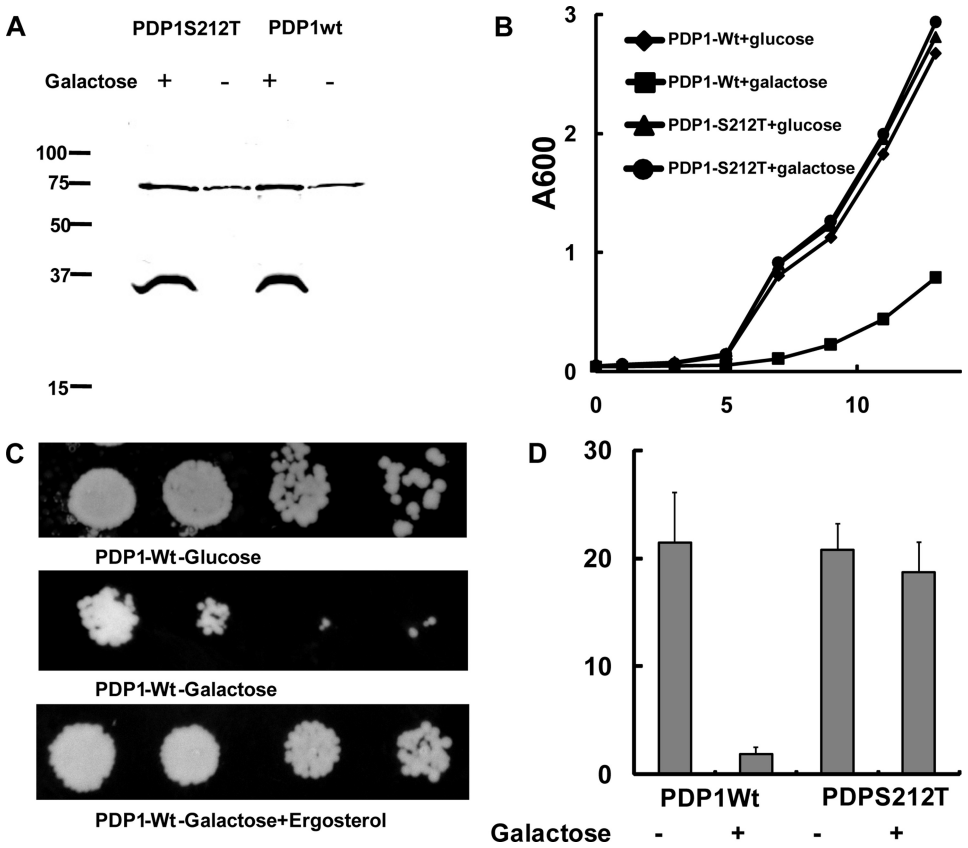

FIGURE 4. **Inducible expression of PDP1/PPPAPDC2 in yeast causes growth defects and sterol auxotrophy.** *A*, galactose inducible expression of HA-tagged wild-type and catalytically inactive S212T PDP1/PPPAPDC2 in yeast was analyzed by Western blotting. A nonspecific immunoreactive protein of ~75-kDa molecular mass is present in all samples. *B*, effect of induction of wild-type PDP1/PPPAPDC2 and catalytically inactive PDP1/PPPAPDC2 S212T on growth of yeast in liquid cultures. *C*, effects of overexpression of wild-type PDP1/PPPAPDC2 on growth of yeast on agar plates and complementation of the growth defect by inclusion of ergosterol in the growth medium. *D*, levels of FPP were determined in extracts from yeast expressing wild-type or S212T PDP1/PPPAPDC2. Data are means ± S.D. ($n = 3$) expressed as picomoles/10 nmol of phospholipid phosphorous.

associated with distortion of the nuclear envelope, which could be prominently observed by confocal microscopy examination of cells counterstained for the inner nuclear envelope protein lamin-associated protein 1-C (*LAP1C*, Fig. 5*C*). These nuclear envelope "herniations," are often observed in cells overexpressing proteins that localize to the inner nuclear membrane (34). To investigate the basis for this apparent nuclear association of PDP1, nuclei were purified from PDP1-expressing cells and membrane proteins remaining associated with these purified nuclei were extracted with 1% Triton X-100, which effectively removed the ER markers calnexin and calreticulin. PDP1-GFP was prominently retained in these detergent-extracted nuclei (not shown). Resistance to detergent extraction is a well established characteristic of nuclear envelope-localized integral membrane proteins and suggests that as recently shown for the related protein NET39/PPAPDC3 (31), PDP1 localizes in part to the inner nuclear membrane.

A Chemical Reporter Strategy for Monitoring Cellular Isoprenoid Phosphate Metabolism—A direct evaluation of the physiological role of PDP1 in isoprenoid phosphate metabolism requires measurements of PDP1 substrates and products in mammalian cells. Although the mass spectrometry-based assays described above were highly effective when used to monitor hydrolysis of isoprenoid diphosphate substrates *in vitro* and could be used to quantitate FPP in extracts from yeast, to date we have been unable to reliably measure FPP or GGPP in extracts from several cultured mammalian cells, although exogenously added FPP and GGPP are efficiently recovered by our extraction method. This is likely a consequence of the low levels of FPP and GGPP in mammalian cells and the poor specificity offered by monitoring the phosphate or diphosphate product ions in selective reaction monitoring mode mass spectrometry. Incorporation of the anilinogeranyl moiety of the protein isoprenylation reporter AGOH into proteins implies conversion to the diphosphate derivative, which is a good farnesyl transferase substrate (18, 35). Because the anilino moiety ionizes strongly in positive mode ESI, we reasoned that AGOH could be used as a selective chemical probe to monitor cellular isoprenoid phosphate metabolism. To test this idea, we incubated HeLa cells with 100 μM AGOH for various times and prepared extracts from these cells, including d5-AGPP, as a recovery standard. We observed time-dependent conversion of AGOH into AGP and AGPP (Fig. 6*A*). Definitive identification of these species was accomplished by positive mode MS/MS analysis (Fig. 6, *B* and *C*). These results provide a direct demonstration of a mammalian cell pathway for conversion of an exogenously provided isoprenol to its mono- and diphosphate derivatives and suggest that, in addition to its demonstrated value as a probe for monitoring protein isoprenylation, AGOH can also be used as a reporter for studies of polyisoprenoid phosphate metabolism in live cells.

PDP1/PPPAPDC2 Regulates Isoprenoid Diphosphate Levels and Protein Isoprenylation in Mammalian Cells—As observed in yeast (Fig. 4) prolonged overexpression of PDP1/PPPAPDC2 is very toxic to mammalian cells (see below). To study acute effects of PDP1 overexpression we therefore derived HeLa cell lines expressing PDP1/PPPAPDC2 under control of a tetracycline-inducible promoter. Addition of the inducer resulted in rapid induction of PDP1/PPPAPDC2 expression in these cells (Fig. 7*A*). Cells were incubated with AGOH overnight, PDP1/PPPAPDC2 expression was induced for 24 h, and extracts were prepared. In comparison to uninduced cells, levels of AGPP were dramatically reduced in the PDP1-expressing cells. Moreover, induction of PDP1/PPPAPDC2 expression almost completely abolished the incorporation of AGOH into proteins 24 h

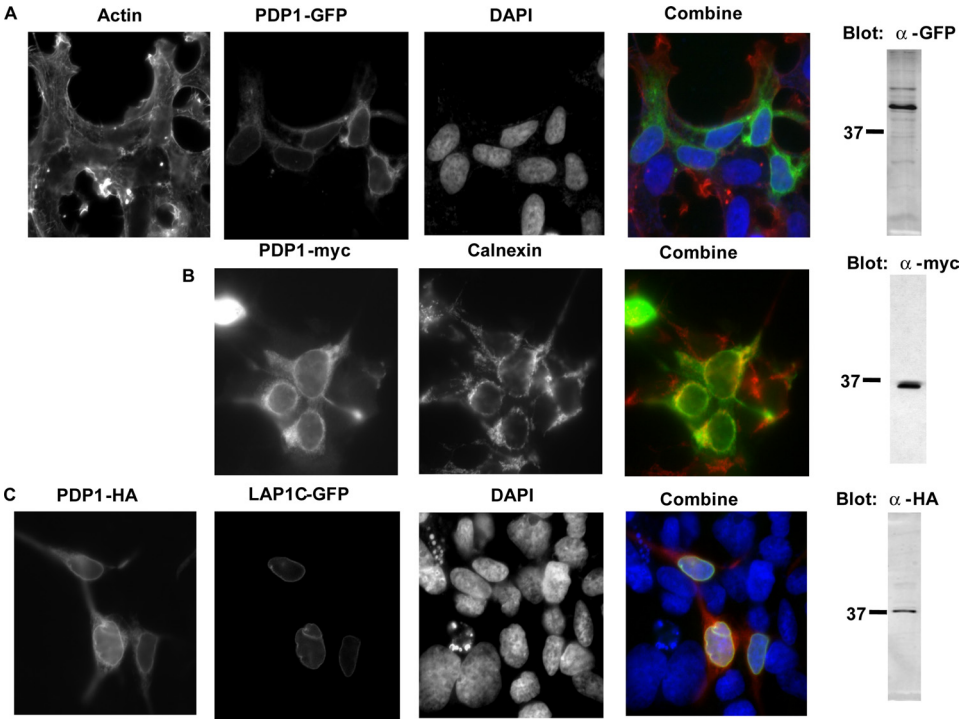

FIGURE 5. **Subcellular localization of PDP1/PPPAPDC2.** GFP (*A*), Myc (*B*), or HA (*C*)-tagged variants of PDP1/PPPAPDC2 were transiently expressed in HEK293 cells and visualized ~12 h post transfection (*A* and *B*) or ~48 h post transfection (*C*) by indirect immunofluorescence and wide-field (*A* and *B*) or confocal (*C*) microscopy. Cells were stained with rhodamine phalloidin, DAPI, or antibodies to visualize the ER and nuclear envelope markers calnexin and lamin-associated protein 1-C (*LAP1C*). Western blots of total membrane fractions isolated from these cells using antibodies that selectively recognize the recombinantly expressed epitope-tagged proteins are also shown.

post induction without significantly altering total protein levels (Fig. 7, *B* and *C*). Taken together, these results show that manipulations of PDP1 expression can regulate isoprenoid phosphate metabolism and protein isoprenylation in mammalian cells.

Overexpression of PDP1/PPPAPDC2 Causes Cell Growth Arrest, Defective Cytokinesis, and Actin Disorganization and Inhibits Rho GTPase Activation and Membrane Association—Although overexpression of PDP1 was toxic to yeast and mammalian cells, acute overexpression of the related broad specificity LPP enzymes is generally well tolerated (36–38). To investigate the effects of PDP1/PPPAPDC2 overexpression on mammalian cell viability we compared overexpression of PDP1/PPPAPDC2 and a representative LPP enzyme, LPP3, on the growth of HEK293 cells. Control or LPP3-expressing cells grew at approximately the same rate as their untransfected counterparts. By contrast, cells expressing PDP1/PPPAPDC2 stopped growing, and their numbers modestly declined (Fig. 8*A*). 36 h post transfection the number of PDP1/PPPAPDC2-expressing cells containing two or more nuclei was significantly increased in comparison to control cells, and this difference was even greater at 72 h (Fig. 8, *B* and *C*). Expression of the inactive PDP1/PPPAPDC2 S212T mutant did not induce either growth arrest or accumulation of multinucleate cells (not shown) indicating that these effects require PDP1 catalytic activity. Rho family GTPases play critical roles in regulation of the cell cycle, and inactivation of certain members of this family such as cdc42 results in defects in cytokinesis leading to an accumulation of multinucleate cells (39). Because the PDP1 substrates GGPP and its precursor FPP are required for isoprenylation of Rho family GTPases we considered the possibility that phenotypes observed in cells overexpressing PDP1 arise through inhibition of Rho GTPase function. Rho GTPases are well established regulators of the actin cytoskeleton. The HEK293 cells used for examinations of the effects of PDP1/PPPAPDC2 overexpression on cell growth do not contain a well defined actin cytoskeleton, but, by contrast, vascular smooth muscle cells exhibit pronounced and well characterized actin-based morphologies and inhibition of Rho function in these cells leads to characteristic changes in organization of polymerized actin and actin-rich structures at the plasma membrane. In comparison to cells infected with a GFP-expressing control virus, lentivirus-mediated overexpression of PDP1/PPPAPDC2 in mouse aortic vascular smooth muscle cells resulted in retraction, partial rounding, a "spindle-like" morphology, and dramatic depletion of filamentous actin leading to a complete loss of actin stress fibers (Fig. 6*D*). Overexpression of PDP1/PPPAPDC2 but not GFP in these cells led to a decrease in both total and membrane-associated levels of RhoA comparable to those observed in cells treated with the mevalonate pathway inhibitor mevastatin (Fig. 6*E*). Lentivirus-mediated overexpression of PDP1/PPPAPDC2 in these cells decreased RhoA activation to 31 ± 5% of control (mean ± S.E., from three independent experiments) measured using a rhotekin binding assay. These results suggest that PDP1/PPPAPDC2 regulates pools of isoprenoid diphosphate substrates required for Rho family GTPase function and maintenance of the morphology of vascular smooth muscle cells.

DISCUSSION

Membrane-associated activities that dephosphorylate polyisoprenoid diphosphates have been reported previously, but the identity of the enzyme(s) responsible has been unclear (10). Here we show that the atypical integral membrane lipid phosphatase enzyme PDP1/PPPAPDC2 can dephosphorylate polyisoprenoid diphosphates, including FPP and GGPP *in vitro*. PDP1/PPPAPDC2 also dephosphorylated several glycero- and sphingo-phospholipid substrates with lower activity. Although the physiological relevance of the activity of this enzyme against phospholipid substrates remains to be determined, we obtained experimental evidence indicating that PDP1/PPPAPDC2 is a functional polyisoprenoid diphosphate phosphatase that can deplete pools of FPP and GGPP required for sterol synthesis and protein isoprenylation in yeast and mammalian cells. Evi-

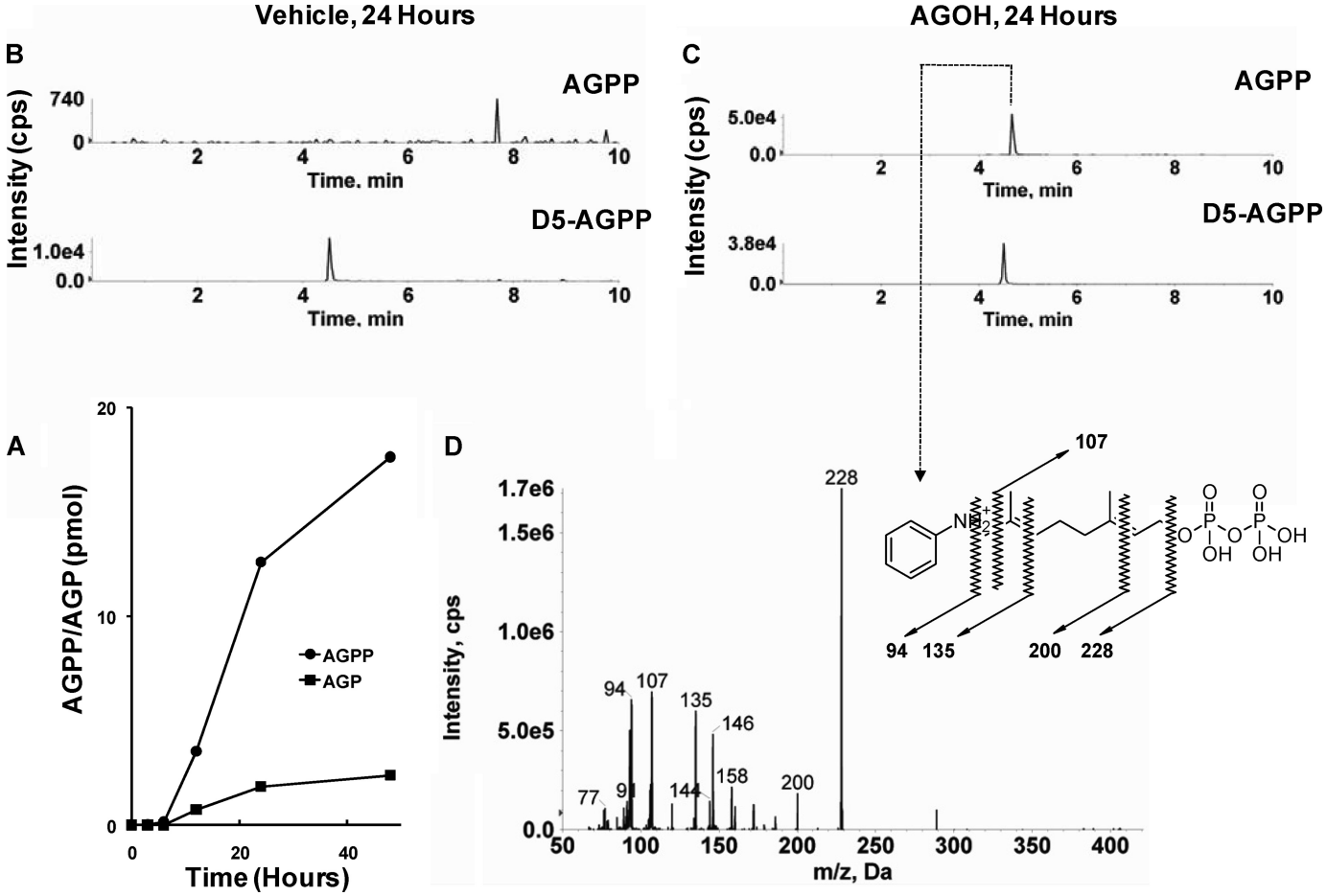

FIGURE 6. **Use of AGOH as a reporter for monitoring isoprenoid diphosphate metabolism in mammalian cells.** *A*, HeLa cells were incubated with 100 μM AGOH for various times, and extracts were prepared for analysis of AGP and AGPP levels by tandem mass spectrometry. *B* and *C*, extracted ion chromatograms showing quantitation of AGPP and the internal standard d5-AGPP in extracts from HeLa cells incubated with vehicle or AGOH for 24 h. *D*, positive mode MS/MS analysis of AGPP detected by selective reaction monitoring in the sample from cells incubated with AGOH for 24 h.

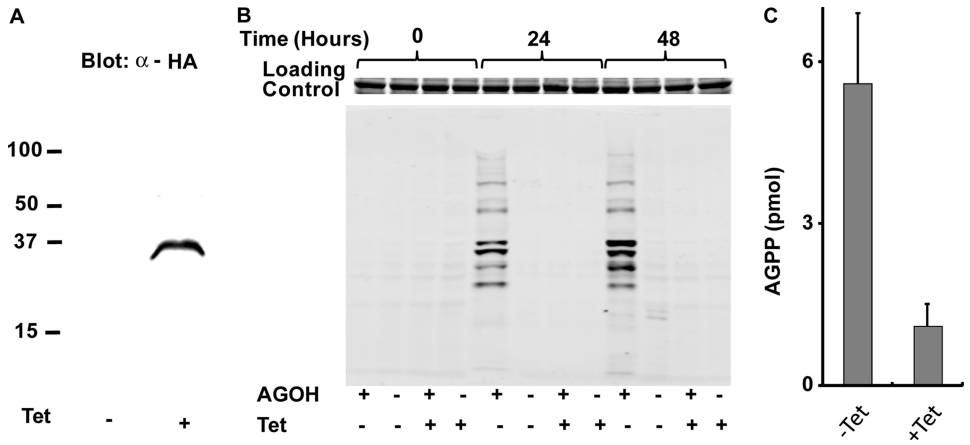

FIGURE 7. **Inducible expression of PDP1/PPPAPDC2 in HeLa cells decreases AGPP levels and incorporation of AGOH into proteins.** *A*, detection of PDP1/PPPAPDC2-HA in equal quantities of membranes from control and tetracycline-treated PDP1/PPPAPDC2 HeLa cells by Western blotting. *B*, time-dependent incorporation of AGOH into proteins in HeLa cells is decreased by tetracycline-induced expression of PDP1/PPPAPDC2. *C*, levels of AGPP were measured in HeLa cells incubated with AGOH for 24 h with and without tetracycline induction of PDP1/PPPAPDC2 expression. Data shown are means ± S.D. ($n = 3$) and are picomoles/10 nmol of phospholipid phosphorous.

dence that yeast homologs of the broad specificity LPP enzymes are isoprenoid phosphatases has been presented (40), and we cannot discount the possibility that the mammalian LPPs are also relevant polyisoprenoid diphosphate phosphatases. Using the assays developed here, we found that a recombinantly expressed representative LPP, LPP3, could also dephosphorylate FPP and GGPP *in vitro*, but this enzyme did not display the preference for these substrates exhibited by PDP1/PPPAPDC2 in these *in vitro* assays (not shown). Overexpression of the LPP enzymes is generally well tolerated in mammalian cells but results in effects on growth and cell cycle progression in part through interference with responses to bioactive lipid growth factors (36, 38, 41). By contrast, we found that overexpression of PDP1/PPPAPDC2 has acutely deleterious effects on cell growth, morphology, and viability pointing to a fundamental difference in the physiological substrate preferences between these enzymes. The approaches developed here for studies of PDP1/PPPAPDC2 can now be

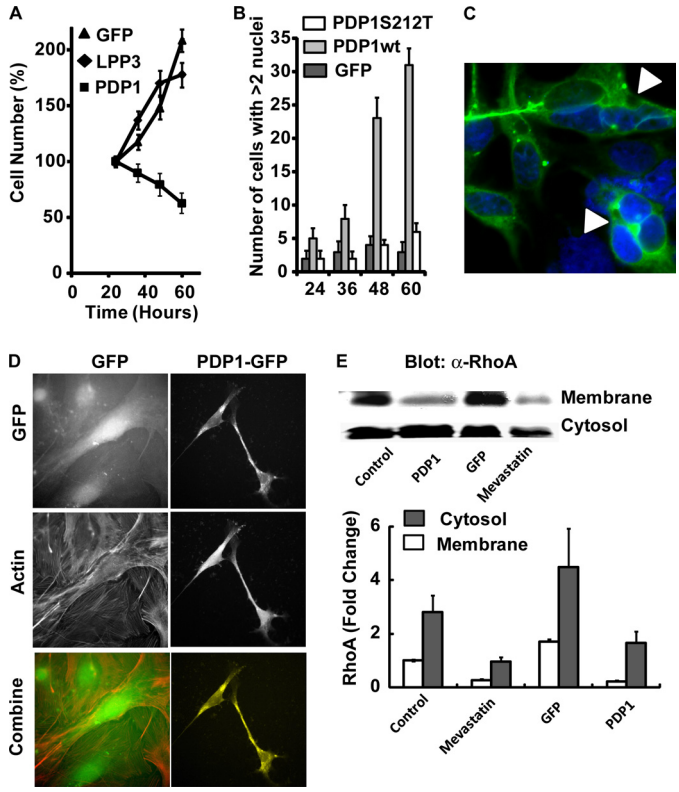

FIGURE 8. **Effect of overexpression of PDP1/PPPAPDC2 on cell morphology, actin organization, growth, cytokinesis, and Rho GTPase activity.** *A*, HEK293 cells at ~20% confluence were transfected with vectors for expression of PDP1/PPPAPDC2-GFP (■) or LPP3-GFP (◆) or GFP (▲) alone and examined by fluorescence microscopy at the indicated times. Transfected cells were identified by visualization of GFP fluorescence and counted. The data shown are mean numbers of cells/field ± S.D. for 3–5 fields of cells from the same slide. *B*, HEK93 cells were transfected with vectors for expression of GFP (*black bars*), PDP1/PPPAPDC2-GFP (*gray bars*), and catalytically inactive PDP1/PPPAPDC2 S212T-GFP (*white bars*), and the number of cells expressing the GFP marker with greater than two nuclei was quantitated in equivalent fields of cells. Data shown are means ± S.D. ($n = 3$). *C*, cells expressing PDP1/PPPAPDC2 GFP were stained with DAPI and DAPI or GFP visualized by fluorescence microscopy 60 h post transfection. Multinucleate cells are identified by *arrows*. *D*, primary cultures of mouse aortic vascular smooth muscle cells were infected with lentivirus vectors for expression of GFP or PDP1/PPPAPDC2 GFP and stained to visualize GFP or F-actin. *E*, effect of lentivirus-mediated overexpression of PDP1/PPPAPDC2-GFP or GFP or treatment with 20 μM mevastatin on membrane association of RhoA in vascular smooth muscle cells was monitored by Western blotting. The *lower panel* shows summarized data for RhoA distribution between the membrane and cytosol fractions normalized to control from three separate experiments (means ± S.E., $n = 3$).

used to probe the role of LPPs as regulators of polyisoprenoid diphosphate metabolism. RNA interference approaches identify a role for PDP1/PPAPDC2 in dephosphorylation of PSDP in cell cultures models (29). Further loss of function studies will now be needed to establish the physiological role of these enzymes in polyisoprenoid diphosphate metabolism.

Theoretical and experimental evidence indicates that PDP1/PPPAPDC2 and the LPPs have different transmembrane topologies, which might account for different functions in cellular lipid metabolism. The transmembrane organization of the LPPs places the three conserved catalytic motif (C1, C2, and C3) sequences in loop regions of the proteins that link the transmembrane helices. Previously recognized homologies between these enzymes and the catalytic core of structurally characterized fungal vanadium-dependent oxidases supported by mutational studies identify roles for residues within each of the C1, C2, and C3 motifs in catalysis (30). This transmembrane organization has been verified for LPP3 and is consistent with widely reported observations that LPPs can localize to the plasma membrane where they are responsible for an "ecto" phosphatase activity that dephosphorylates extracellular substrates, including bioactive lipids such as LPA and S1P (42). When localized to intracellular sites, most notably the endoplasmic reticulum and Golgi apparatus, the active site of the LPPs would face the lumen of these organelles. By contrast, the predicted transmembrane topology of PDP1/PPPAPDC2 orients the protein with the C1 and C2 catalytic domain sequences facing the cytoplasmic face, and our observation, that a C-terminal GFP tag appended to the protein is readily susceptible to proteolysis in saponin-permeabilized cells, suggests that the fourth predicted transmembrane helix may be "kinked" to orient the C3 catalytic domain sequences adjacent to the C1 and C2 sequences. This forms a complete active site accounting for the observed catalytic activity of the enzyme. PDP1/PPPAPDC2 localized predominantly to the endoplasmic reticulum. The closely related protein PPAPDC3/NET39 was recently identified as a nuclear envelope-localized protein (31). As reported for PAPDC3/NET39 we found that a subfraction of PDP1/PPPAPDC2 retained in detergent-extracted nuclei and overexpression of PDP1/PPPAPDC2 led to alterations in nuclear envelope morphology, suggesting that PDP1/PPPAPDC2 can also localize in part to the nuclear envelope, although the significance of this remains to be determined.[4]

To conduct the work reported here we developed tandem mass spectrometry assays for polyisoprenoid diphosphates. Unlike assays for these enzymes that monitor phosphate release through chemical or radiochemical methods, these assays have high sensitivity and specificity and can be used to directly evaluate enzyme activity against mixtures of competing substrates. Although detectable using coupled enzymatic assays (43), measurements of FPP and GGPP in cells and tissues using tandem mass spectrometry have not been reported. Unfortunately, the assays we developed for *in vitro* characterization of enzyme activities lacked the specificity and sensitivity to identify FPP and GGPP in extracts from cultured mammalian cells. To investigate how changes in PDP1/PPPAPDC2 expression might impact on cellular polyisoprenoid diphosphate metabolism, we developed a chemical reporter strategy using the unnatural isoprenol, AGOH. Incubation of cells with exogenous AGOH led to time-dependent accumulation of intracellular AGP and AGPP. The widely reported use of AGOH as a probe to monitor protein isoprenylation by immunological detection of the protein-conjugated anilinogeranyl moiety implies that conversion to AGPP is a common property of many cells (18, 22, 44). The identities of the enzyme(s) responsible for conversion of AGOH to AGPP and the significance of this pathway are presently unknown. Using this approach, we found that regulated overexpression of PDP1 decreased cellular levels of

[4] M. I. McDermott and A. J. Morris, unpublished results.

AGPP, and the incorporation of the anilinogeranyl moiety of the AGOH reporter into proteins indicating that the isoprenoid diphosphate phosphatase activity of PDP1/PPPAPDC2 observed *in vitro* is likely relevant in cells. Several other lines of evidence support this idea. Sustained overexpression of PDP1/PPPAPDC2 in diverse mammalian cells led to dysregulation of cell growth and division and disorganization of the actin cytoskeleton. Rho GTPases are isoprenylated proteins with a pivotal role in these processes, and we also observed that overexpression of PDP1/PPPAPDC2 was associated with a decrease in activation and membrane association of RhoA. Finally, our studies revealed that inducible overexpression of PDP1/PPPAPDC2 in budding yeast led to growth defects and sterol auxotrophy, which would also be consistent with PDP1/PPPAPDC2-catalyzed depletion of pools of isoprenoid diphosphates required for ergosterol synthesis. Taken together, our results identify a pathway for interconversion of isoprenols and isoprenoid diphosphates in mammalian cells and indicate that PDP1/PPAPDC2 can serve as a functional component of this pathway.

There are several obvious implications of our findings. PDP1/PPAPDC2 may catalyze a previously unappreciated regulatory step in the mevalonate pathway, and tight control of PDP1/PPPAPDC2 activity could contribute to the mechanisms by which cholesterol synthesis is attenuated in response to increases in dietary cholesterol or to selective regulation of pools of isoprenoid diphosphate precursors for sterol synthesis or protein isoprenylation (1). PSDP has effects on lipid signaling enzymes that suggest a role in termination of neutrophil responses to inflammatory stimuli so dephosphorylation of this substrate by PDP1/PPPAPDC2 could impact on this process (29, 45). PDP1/PPPAPDC2 may also be a determinant of the efficacy and toxicity of clinically important mevalonate pathway inhibitors. In particular, squalene synthase inhibitors are a novel class of cholesterol-lowering agents, but their usefulness is limited by toxicity associated with the accumulation of isoprenols that are formed by the dephosphorylation of isoprenoid diphosphates (46). Finally, direct quantitation of polyisoprenoid diphosphates in cells and tissues has been challenging. The development of a chemical reporter strategy to monitor polyisoprenoid phosphate metabolism in cells with high specificity and sensitivity may facilitate new studies to identify unappreciated aspects of the regulation of this vitally important class of metabolic intermediates.

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