Phosphatidylinositol Transfer Protein, Cytoplasmic 1 (PITPNC1) Binds and Transfers Phosphatidic Acid*

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Background: Phosphatidylinositol transfer protein, cytoplasmic 1 (PITPNC1) (alternative name, RdgBβ) binds and transfers phosphatidic acid (PA) in cellular processes. The effect of PA binding and transfer has been described for many years. However, it is not clear whether this binding and transfer is a constitutive property of the protein or if it is a consequence of other cellular processes. We demonstrate that RdgBβ is a constitutive property of the protein and that it binds and transfers PA.

Results: We demonstrate that RdgBβ is a constitutive property of the protein and that it binds and transfers PA.

Conclusion: RdgBβ is the first lipid-binding protein identified that can bind and transfer PA.

Significance: PA bound to RdgBβ is a likely effector downstream of phospholipase D.

Phosphatidylinositol transfer proteins (PITPs) are versatile proteins required for signal transduction and membrane traffic. The best characterized mammalian PITPs are the Class I PITPs, PITPα (PITPNA) and PITPβ (PITPNB), which are single domain proteins with a hydrophobic cavity that binds a phosphatidylinositol (PI) or phosphatidylinositol molecule. In this study, we report the lipid binding properties of an uncharacterized soluble PITP, phosphatidylinositol transfer protein, cytoplasmic 1 (PITPNC1) (alternative name, RdgBβ), of the Class II family. We show that the lipid binding properties of this protein are distinct to Class I PITPs because, besides PI, RdgBβ binds and transfers phosphatidic acid (PA) but hardly binds phosphatidylcholine. RdgBβ when purified from Escherichia coli is preloaded with PA and phosphatidylglycerol. When RdgBβ was incubated with permeabilized HL60 cells, phosphatidylglycerol was released, and PA and PI were now incorporated into RdgBβ. After an increase in PA levels following activation of endogenous phospholipase D or after addition of bacterial phospholipase D, binding of PA to RdgBβ was greater at the expense of PI binding. We propose that RdgBβ, when containing PA, regulates an effector protein or can facilitate lipid transfer between membrane compartments.

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3 The abbreviations used are: PITP, phosphatidylinositol transfer protein; DAG, diacylglycerol; HL60, human promyelocytic leukemia cell line; PA, phosphatidic acid; PC, phosphatidylcholine; PI, phosphatidylinositol; P(4,5)P2, phosphatidylinositol 4,5-bisphosphate; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PITPNC1, phosphatidylinositol transfer protein, cytoplasmic 1; PLC, phospholipase C; PLD, phospholipase D; PMA, phorbol 12-myristate 13-acetate; RdgB, retinal degeneration type B; GTPyS, guanosine 5’-(γ-thio)triphosphate; PEt, phosphatidylethanolamine; FIPI, 5-fluoro-2-indolyl deschlorohalopemide; Dm, Drosophila melanogaster; sp1, splice variant 1; sp2, splice variant 2.
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![Diagram](https://via.placeholder.com/150)

**FIGURE 1. Class I and Class II PITPs.** Shown is the domain organization of PITP family members examined here. PITPs are grouped into Classes I and II. Class II is subdivided into A and B, and RdgBβ belong to Class II B. The splice variants of RdgBβ are shown. The protein sequences were analyzed by BLAST, and the percentage of primary sequence identity of each PITP domain to human RdgBβ and to human RdgBα is given. h, human; FFAT, two phenylalanines (FF) in an acidic tract; DDHD, heavy metal binding domain containing Asp and His residues.

replaced with threonine (see Table 1), suggesting that RdgB proteins may not bind or transfer PC.

The RdgB proteins are further subdivided into Classes IIA and IIB (Fig. 1). In addition to the N-terminal PITP domain, Class IIA proteins contain an FFAT (two phenylalanines (FF) in an acidic tract) motif followed by two other domains, DDHD (heavy metal binding domain containing Asp and His residues) and LNS2, of unknown function (Fig. 1). The first member of the Class IIA PITPs, Dm-RdgBα, was identified in *Drosophila*.

The single Dm-RdgBα isoform is localized to the submicrovillar cisternae, thought to be a subcompartment of the endoplasmic reticulum in close proximity to the microvillar plasma membrane of *Drosophila* photoreceptors cells (13). Loss of function mutants in Dm-RdgBα are characterized by abnormal termination of the light response and profound loss of the electroretinogram amplitude shortly after initial light exposure. In addition, the rhodobacteric membranes become vesiculated, leading to photoreceptor cell degeneration, giving the Class II proteins their name: retinal degeneration type B (RdgB). Notably, retinal degeneration is also seen when the gene for diacylglycerol (DAG) kinase, *rdgA*, which catalyzes conversion of DAG to PA, is mutated (14–16).

Detection of light at the rhodomere results in the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) by phospholipase C (PLC), and Dm-RdgBα could potentially facilitate the delivery of PI to the rhodobacteric membranes for the regeneration of the PLC substrate, *i.e.* PI(4,5)P₂ (17). RdgBα is also required for normal localization of rhodopsin in rhodobacter (18), and rhodopsin levels are reduced in Dm-RdgBα mutants (19).

In mammals, RdgBα is an essential protein (20) and is required for anterograde transport from the trans-Golgi network to the plasma membrane (21). It has been proposed that RdgBα functions by maintaining a critical pool of DAG in the Golgi by regulating its consumption via the CDP-choline pathway (21). In *Caenorhabditis elegans*, the single RdgBα is required in the sensory neurons for synaptic transmission and behavioral plasticity (22). Interestingly, the normal phenotype of *C. elegans* can also be restored by inactivating mutations of the DAG kinase, causing accumulation of DAG. The normal phenotype of RdgBβ loss of function mutants in *Drosophila* and in *C. elegans* can be restored by expression of the PITP domain alone, indicating that this domain is central to the biological activity of the protein (19, 22).

In contrast to RdgBα, RdgBβ is a soluble protein with two splice variants: a long form (332 amino acids), RdgBβ splice variant 1 (sp1), and a short form, RdgBβ splice variant 2 (sp2) (268 amino acids) (Fig. 1). RdgBβ consists of a PITP domain followed by a C-terminal extension of 60 amino acids, whereas RdgBβ-sp2 consists of the PITP domain only. The C-terminal extension of RdgBβ-sp1 is phosphorylated at two serine residues that form a docking site for 14–3–3 proteins (23). The PITP domain of RdgBβ is only 39% identical to both Class I and Class IIA PITPs and 35% identical to Dm-RdgBα (Fig. 1). RdgBβ is a short lived protein that is abundant in the heart (23). Moreover, its expression can be regulated by microRNA-126 (24). Loss of microRNA-126 occurs in many human cancers including breast cancer and results in increased levels of RdgBβ and consequently in metastatic colonization. This can be recapitulated by increased expression of RdgBβ in MDA-231 breast cancer cells.

Here we examined the lipid binding properties of RdgBβ and report that unlike Class I PITPs RdgBβ binds PA in addition to PI. When the cells were stimulated to activate phospholipase D, binding of PA to RdgBβ was significantly increased at the expense of PI. The ability to bind PA by RdgBβ is shared by the PITP domain of both the Dm-RdgBα and human RdgBα but not PITPα.

**EXPERIMENTAL PROCEDURES**

*Materials:* *myo-[2-3H]Inositol, [methyl-3H]choline chloride,* and [1-14C]acetate acid were purchased from American Radiolabeled Chemicals (UK). sn1-Palmitoyl-sn2-oleoylphos-
phatidycholine and sn1-palmitoyl-sn2-oleoyl phosphatidic acid were obtained from Avanti Polar Lipids (Alabaster, AL). N-\textsuperscript{6}Trinitrophenyl phosphatidylethanolamine (PE), sn1-palmitoyl-sn2-pyrene-decanoyl-PC, -PA, and sn1-octadecanoyl-sn2-pyrene-decanoyl-PI were synthesized as described previously (25, 26). Bacterial phospholipase D (Streptomyces sp. (P4912)) was obtained from Sigma.

Recombinant PITP\textalpha{} and RdgB\beta{} (human) were expressed from pRSET vectors and purified as described previously (4). The Dm-RdgB\alpha{}-PITP cDNA coding for the N-terminal PITP domain (amino acids 1–281) was cloned into the pRSET-C plasmid vector, and the human RdgB\alpha{} PITP domain (amino acids 1–277) was cloned into the pET21a-His plasmid (a gift from Dr. Sima Lev). For site-directed mutagenesis, primers were designed, and point mutations were introduced according to the Stratagene QuickChange protocol. The oligonucleotides were manufactured by Sigma-Aldrich. The mutated sequences were verified by DNA sequencing (Eurofins MWG Operon). The His-tagged proteins were expressed in BL21(DE3)pLysS competent Escherichia coli and purified using nitritotriacetic acid-agroase resin (HIS-Select nickel affinity gel, Sigma) as described previously (4). The purity and the yield of the proteins varied between constructs. PITP\textalpha{} and Dm-RdgB\alpha{} (1–281) proteins were expressed very well, whereas the yields of RdgB\beta{} and RdgB\beta{} (1–277) were much lower. Protein concentration was assessed using the bicinchoninic acid (BCA) assay. This was readjusted after SDS-PAGE analysis according to the amount of protein of the correct molecular weight present using AIDA software with comparison to 1 \mu{}g of PITP\textalpha{}.

The His-tagged proteins were stored in aliquots in PIPES buffer (20 mM PIPES, 137 mM NaCl, 3 mM KCl, pH 6.8) at ~80 °C. Transfer of Radiolabeled PI and PC Using Microsomes as a Donor—100 \mu{}l of [\textsuperscript{3}H]inositol-labeled or [\textsuperscript{3}H]choline-labeled microsomes (6 mg/ml) were incubated with an equal volume of liposomes (PC/PI, 98:2 molar ratio; 80 nmol of phospholipid) together with 50 \mu{}l of recombinant proteins at 25 °C for 20 min (27). [\textsuperscript{3}H]Inositol-labeled microsomes were prepared from rat liver, whereas [\textsuperscript{3}H]choline-labeled microsomes were obtained from HL60 cells. The reaction was quenched by the addition of 50 \mu{}l of ice-cold 0.2 M sodium acetate, 0.25 M sucrose, pH 5.0. The tubes were vortexed and left on ice for 10 min. Following centrifugation at 12,000 × g for 10 min at 4 °C, 150 \mu{}l of supernatant from each reaction was counted in a liquid scintillation counter. Results are expressed as a percentage of the total input count. Error bars indicate mean ± S.D. or S.E. as indicated.

Transfer of Radiolabeled PI and PC Using Permeabilized HL60 Cells as a Donor—The phospholipid transfer assays were carried out as described previously (6). In brief, HL60 cells were prelabeled with [\textsuperscript{3}H]inositol or [\textsuperscript{3}H]choline for 48 h. The cells were permeabilized with streptolysin O, centrifuged to remove the cytosolic components, and incubated with liposomes (PC/PI, 98:2, mol/mol) together with 50 \mu{}l of recombinant proteins at 37 °C for 20 min. The samples were processed as described above.

Binding of Cellular Lipids by PITPs—Association of cellular lipids with the PITPs was analyzed exactly as described (4, 6). In brief, HL60 cells were labeled with 1 \mu{}Ci/ml [\textsuperscript{14}C]acetate in RPMI 1640 medium for 48 h. The cells were permeabilized with streptolysin O, and the leaked cytosol was removed by centrifugation. Permeabilized cells (~10\textsuperscript{7} cells) were incubated with 120 \mu{}g of PITP\textalpha{} or RdgB\beta{} protein (100 \mu{}l) for 20 min at 37 °C in the presence of 2 mM Mg\textsuperscript{2+}-ATP and 100 mM Ca\textsuperscript{2+} buffered with 3 mM EGTA. A sample of the protein was retained and run on SDS-PAGE. At the end of the incubation, the cells were removed by centrifugation, and the recombinant proteins in the supernatant were captured on nickel beads. An aliquot of the recovered proteins was run on SDS-PAGE to assess their recovery, and the rest of the sample was used for lipid extraction. The lipids were resolved by thin layer chromatography using a Whatman silica gel 60 TLC plate using chloroform/methanol/acetic acid/water (75:45:3:1, v/v) as the eluent. Lipids extracted from the permeabilized HL60 cells (approximately 100,000 dpm) were analyzed alongside for comparison. The TLC plates were exposed to Fuji phosphorimaging screens and analyzed using a Fuji BAS1000 phosphorimaging system. Both the SDS-PAGE and TLC images were analyzed using AIDA software.

Mass Spectrometric Analysis of Phospholipids—For the mass spectrometric analyses, the binding assay was scaled up 5-fold; i.e. 5 × 10\textsuperscript{7} HL60 cells (unlabeled) were incubated with 600 \mu{}g of a purified transfer protein. Lipids were also directly extracted from the E. coli-expressed PITPs (600 \mu{}g/sample). Disposable borosilicate glassware was used for all lipid extractions. Phospholipids were analyzed using a Quatro Ultima triple quadrupole mass spectrometer (Micromass, Wythenshawe, UK) equipped with direct infusion or nanoflow interfaces, depending upon sample size, as described previously (28). An initial full screening for glycerophospholipid species was undertaken under both positive and negative ionization for m/z values up to 1300. More detailed selective precursor ion scanning of all phospholipids of potential interest was then pursued including for PA, PC, PE, PI, phosphatidylserine, and related lysophospholipids. Bacterial lipids including phosphatidylglycerol (PG) were absent from all samples. The PA species were identified by scanning for the precursors of the glycerophospholipid fragment (m/z ~153), and their identities and dominant acyl compositions were confirmed by specific peak fragmentations, whereas the PI species were detected by scanning for the precursor of inositol phosphate (m/z ~241) as described previously (28).

Assay of Pyrene-Lipid Binding by PITPs—To study the binding of pyrene-labeled phospholipids by the transfer proteins, we used a previously described dequenching assay (25). Briefly, donor vesicles in which the pyrene fluorescence is effectively quenched by N-trinitrophenyl PE were prepared by injecting 10 \mu{}l of an ethanol solution of pyrenyl phospholipid/sn1-palmitoyl-sn2-oleoylphosphatidylcholine/N-trinitrophenyl PE (2:88: 10, mol/mol) mixture (4 nmol of total phospholipid) into 2 ml of a buffer (20 mM Tris-Cl, 100 mM NaCl, 5 m MDTA) in a cuvette. After a 1-min equilibration period, aliquots of a transfer protein were added, and the fluorescence intensity (with excitation at 348 nm and emission at 395 nm) was recorded after each addition. If the pyrenyl phospholipid incorporates into the transfer protein, pyrene fluorescence increases because the labeled lipid is removed from the quenching environment, i.e. the donor vesicle. Fluorescence intensity versus protein con-
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centrations plots were constructed to assess the relative affinity of a pyrene phospholipid for the transfer protein. When transfer was monitored, acceptor vesicles (80 nmol of phospholipid) consisting of sn1-palmitoyl-sn2-oleylphosphatidylcholine/sn1-palmitoyl-sn2-oleoyl phosphatidic acid (98:2, mol/mol) and prepared by the ethanol injection method were also added.

Lipid Binding following Incubation with PMA, GTPγS, or Bacterial Phospholipase D—HL60 cells were labeled with 1Ci/ml [14C]acetate in RPMI 1640 medium for 48 h. After washing twice in PIPES buffer, the cells were added to a mixture of GTPγS (100 μM final), PMA (100 nM final), 0.2 unit of bacterial phospholipase D, MgATP (2 mM final), Ca2+ buffered with 3 mM EGTA at 100 mM (pCa 7) or 10 μM (pCa 5), streptolysin O (0.4 IU/ml final), and a recombinant protein (120 μg) as indicated in a final incubation volume of 600 μl. The inhibitors FIP1 (750 nM) and U73122 (10 μM) were also added where indicated. After incubation for 20 min at 37 °C, the cells were centrifuged, and 500 μl of the supernatant was used to recapture the proteins as described above (6). The residual cells were quenched with 375 μl of chloroform/methanol (1:2, v/v), and the lipids were extracted and analyzed by TLC as described (6).

RESULTS

Comparison of the PI and PC Transfer Properties of the Two Splice Variants of RdgBβ with PITPα—We have recently reported that the PI transfer activity of RdgBβ-sp1 is low when compared with PITPα (23). RdgBβ-sp1 requires 100-fold higher concentrations and furthermore does not achieve the
same level of transfer as PITPα. Two splice variants of RdgBβ have been identified that differ at their C termini. The canonical RdgBβ (332 amino acids) has a 60-amino acid C-terminal extension compared with RdgBβ-sp2 (268 amino acids), which has 9 amino acids. The C-terminal extension precedes the G-helix, which in PITPα swings outward, dislodging the C-terminal extension; this exposes the hydrophobic cavity required for lipid exchange to take place at the membrane (29). Thus, it is possible that the C-terminal extension of RdgBβ-sp1 is the main cause for the difference in activity between PITPα and RdgBβ-sp1. Alternatively, the difference could lie in the PITP domain itself as RdgBβ is only ~40% identical to the Class I PITPs. To distinguish between these possibilities, we examined the lipid transfer activity of RdgBβ-sp2, which is of similar length to PITPα (270 amino acids).

To determine PI transfer activity, we used two different assays: a microsome assay (30) and a cytosol-depleted permeabilized HL60 cell assay (6), both containing [3H]inositol-labeled

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PI. Vesicles composed of PC/PI (98:2) were used as the acceptors. The His-tagged proteins were expressed in E. coli, and the concentration of the purified proteins was adjusted according to the degree of purity of the recombinant proteins (Fig. 2A). The two splice variants of RdgBβ showed very similar PI transfer activity regardless of the assay used (Fig. 2, C and E). Compared with PITPα, both RdgBβ proteins required a much higher concentration and did not achieve the same degree of activity. Furthermore, the RdgBβ proteins showed greater activity in the microsome-liposome assay compared with the permeabilized HL60 cell assay, whereas PITPα was equally effective in either assay.

In addition to transferring PI, Class I PITPs can also transfer PC. We therefore compared the PC transfer activity of RdgBβ splice variants with PITPα using both assays. For PI transfer, four residues (Thr-59, Lys-61, Glu-86, and Asn-90; mouse PITPα numbering) that coordinate the inositol ring are essential (Fig. 2B); these residues are conserved in RdgBβ. Regarding PC transfer, we have recently shown that mutation of cysteine 95 to threonine in PITPβ reduces the transfer of this lipid (12). This residue is replaced with threonine in the Class II PITPs (Table 1). Cys-95 resides in the head group binding pocket (Fig. 2B) and is not essential for PI transfer activity (12). Unlike PITPα, RdgB proteins showed very low PC transfer in either assay (Fig. 2, D and F). The PC transfer activity was 20% of that of PITPα in the microsome-liposome assay (Fig. 2D), whereas no activity was detected when permeabilized cells were used as donors (Fig. 2F). The low level of PC transfer activity exhibited by RdgBβ was also observed for another Class II RdgBβ protein, the PITP domain of Dm-RdgBa. The Dm-RdgBa PITP domain was active for PI transfer in either assay, but the maximal activity attained was ~50% of PITPα (Fig. 2, C and E).

Lipid Binding Properties of the Soluble Class II PITP, RdgBβ—The low level of PC transfer exhibited by RdgBβ prompted us to examine its lipid binding properties. This was measured by exposing the His-tagged recombinant proteins to pre-permeabilized HL60 cells prelabeled with [14C]acetate (6). The recombinant proteins were recaptured using nickel beads, and the lipids were extracted from the eluted proteins and analyzed by TLC and mass spectrometry. Compared with PITPα, which binds PI and PC, RdgBβ mainly bound PI and PA (Fig. 3A). The lipid binding of the individual species is expressed as a percentage of total lipid bound to a protein (Fig. 3C). PITPα bound near equal proportions of PI and PC. No PA binding was observed. In comparison, RdgBβ bound PI and PA in near equal proportions, whereas PC binding was low (~5%) (Fig. 3C).

It is well documented that isolated PITPα contains one molecule of PI or PC, which undergoes constant exchange with membrane lipids within minutes; the apo form is present only transiently at a membrane after the release of bound lipid (29). Thus, the amount of radioactivity found in RdgBβ after incubation with the labeled HL60 cells provides a measure of lipid exchange (Fig. 3D). Relative to PITPα, RdgBβ contained less radioactivity per pmol of protein, implying that a large fraction of the RdgBβ molecules may not have undergone lipid exchange.

Recombinant PITPα when purified from E. coli comes preloaded with PG, which is immediately exchanged when exposed to PI or to PC (28). To identify which lipids were associated with recombinant RdgBβ when purified from E. coli, the extracted lipids were analyzed by mass spectrometry (electrospray ionization-MS/MS). Precursor scans of the m/z — 153 fragment revealed that...
recombinant RdgBβ contained not only PG but also PA (Fig. 4A). When RdgBβ was incubated with the HL60 cells, the PG was deposited to the HL60 cells (Fig. 4), and RdgBβ now bound PA and PI (Figs. 5 and 6). Precursor scans of the m/z -153 fragment revealed that the PA bound to RdgBβ was enriched in C16:0/16:1 PA (m/z 645; -27%) and C16:1/18:1 PA (m/z 671; -23%) species as compared with the cellular PA pool of which these species comprised 16 and 11%, respectively (Fig. 5, A and B).

Analysis of the molecular species composition of PI of HL60 cells revealed a mixture of species containing a saturated or monounsaturated fatty acid or a polyunsaturated acyl residue in different combinations (Fig. 6C and Ref. 28). Compared with the cells, PI bound by RdgBβ was enriched in arachidonyl (C20:4)-containing species at the expense of C18:0/18:1 species (Fig. 6A), whereas PITPα preferred PI species containing shorter saturated or monosaturated acyl chains as reported previously (28) (Fig. 6B).

To study PA binding and transfer properties of RdgBβ using purified proteins and liposomes of defined composition, we used a previously devised fluorescence dequenching assay (25).  

**Figure 5.** RdgBβ binds cellular PA species selectively. A, HL60 cells (5 x 10^6 cells) were permeabilized with streptolysin O and incubated with His-tagged RdgBβ (600 μg) for 20 min at 37 °C. The cells were removed by centrifugation, and the RdgBβ present in the supernatant was recaptured with nickel beads. The lipids were extracted from the protein and analyzed by mass spectrometry. A representative electrospray ionization-MS/MS precursor scan of m/z -153 fragment in negative ionization showing the PA composition of RdgBβ-bound lipid and whole HL60 cell lipid extract is shown. B, comparison of the percent distribution of the whole cell PA molecular species with RdgBβ-bound PA (mean ± S.E. (n = 6)). RdgBβ-bound PA was also determined after HL60 cells were stimulated with GTPγS and pCa 5 to activate endogenous PLD (mean ± S.E. (n = 6)). Species identified represent the dominant species from MS/MS fragmentation analysis. A significant difference between whole cell and RdgBβ-bound PA for each molecular species is indicated by either * (p < 0.05) or ** (p < 0.005).
used as a control, bound pyrenyl-PI efficiently, whereas binding of pyrenyl-PC was less efficient, and that of pyrenyl-PA was negligible (Fig. 7A). In contrast, RdgBβ bound PA best, whereas PI binding was low, and that of PC was negligible (Fig. 7B). Replotting of these data shows clearly that RdgBβ bound PA far more efficiently than PI, whereas the reverse was true for PITPα (Fig. 7, C and D).

When acceptor vesicles were included in the assay, PA transfer by RdgBβ was most efficient (data not shown).

**FIGURE 6. RdgBβ and PITPα prefer different molecular species of PI.** HL60 cells (5 × 10⁷ cells) were permeabilized with streptolysin O, washed, and incubated with His-tagged RdgBβ or PITPα (600 μg) for 20 min at 37 °C. The cells were removed by centrifugation, and the proteins in the supernatant were captured with nickel beads. The lipids were extracted from the protein and analyzed by mass spectrometry. A representative electrospray ionization-MS/MS precursor scan of m/z – 241 precursor in negative ionization showing the PI molecular species present in RdgBβ (A) and PITPα (B) is shown. C, comparison of the fractional representation of the whole cell PI molecular species with RdgBβ-bound and PITPα-bound PI. Species identified represent the dominant species from MS/MS fragmentation analysis. A significant difference between whole cell and PITPα-bound PI for each molecular species is indicated by either * (p < 0.05) or ** (p < 0.005).
Increased Binding of Cellular PA to RdgB When HL60 Cells Are Stimulated with GTP-S—As the amount of PA in resting HL60 cells is low (<1%), exchange of the preloaded PA may not occur. RdgBβ when produced in E. coli is already preloaded with PA (and PG) (see Fig. 4A). Addition of GTP-S to acutely permeabilized HL60 cells leads to a pronounced activation of both PLC and phospholipase D (PLD) (31, 32). PLC catalyzes the hydrolysis of PI(4,5)P2 to generate DAG and inositol triphosphate. In the presence of MgATP, DAG is phosphorylated to PA. PLD catalyzes the hydrolysis of PC, leading to the direct formation of PA. Thus, two separate pathways can produce PA upon stimulation of the cells with GTP-S (see Fig. 13).

To examine whether an increase in cellular PA levels following the activation of PLC or PLD increases PA binding by RdgBβ, the lipid binding assay was modified as follows. Recombinant RdgBβ or PITPα was added together with the permeabilizing agent, streptolysin O, Ca2+ buffered with 3 mM EGTA at 100 nm (pCa 7) or 10 μM (pCa 5), and the stimulus, GTP-S. It has been shown that 10 μM Ca2+ alone stimulates PLC and PLD somewhat, but in the presence of GTPγS, it causes a robust activation of both types of phospholipases (31, 33). In the presence of 10 μM Ca2+ alone, a small increase in PA binding to RdgBβ was observed, whereas in the presence of Ca2+ plus GTPγS, substantially increased PA binding at the expense of PI was observed (Fig. 8, A and B). As expected, lipid binding to PITPα was insensitive to the manipulation of the cellular PA levels (Fig. 8, A and B).

To identify whether the increased PA bound by RdgBβ was derived from the PLD or PLC pathway, we also stimulated the permeabilized cells with PMA, which activates PLD but not PLC in intact cells. In contrast to GTPγS, PMA did not increase binding of PA to RdgBβ (Fig. 8). To explore this further, we monitored the production of PA in the permeabilized HL60 cells after stimulation with PMA in the absence or presence of ethanol. (Ethanol causes PLD to produce the stable metabolite phosphatidylethanol (PEt) instead of PA.) PMA failed to increase the level of PA, and in the presence of ethanol, no PET was produced. Thus, in the permeabilized cells, PMA could not be used to assess the source of the PA.

We next used ethanol or butanol to divert PA to form the corresponding phosphatidylalcohol to reduce PA production following GTPγS stimulation (34). GTPγS stimulation enhanced PA binding, and in the additional presence of either alcohol, PA binding to RdgBβ was unaffected despite the reduction in cellular PA to resting levels (Fig. 9). In addition, RdgBβ was now found to bind a small amount of PET or phosphatidylbutanol (Fig. 9A). We also monitored cellular PA and the phosphatidylalcohol after GTPγS stimulation. GTPγS caused an increase in cellular PA, which was greatly reduced when ethanol or butanol was present (Fig. 9B). In their presence, an increase in the corresponding phosphatidylalcohol was observed. It was apparent that the amount of PET/phosphatidylbutanol produced greatly exceeded that of PA presumably because PA is rapidly metabolized unlike the phosphatidylalcohols (35). We conclude from this experiment that RdgBβ binds PA preferentially to the

**FIGURE 7.** RdgBβ but not PITPα binds pyrene-labeled PA. Quenched donor vesicles consisting of a pyrene-labeled phospholipid/sn1-palmitoyl-sn2-oleylphosphatidylcholine/N-trinitrophenyl PE (2:88:10, mol/mol) were titrated with PITPα or RdgBβ. The increase in fluorescence intensity of the different pyrenyl lipid as a function of PITPα or RdgBβ added is shown in A and B, respectively. In C and D, the data are replotted to show the relative binding of PI (C) and PA (D) by PITPα versus RdgBβ. A representative experiment of three is shown.
phosphatidylalcohols, and moreover, PA binding to RdgB/H9252 was still evident even when alcohols were used to divert the cellular production of PA to phosphatidylalcohols. Because PA is still produced from the phospholipase C pathway, these results do not clarify whether PA from the phospholipase C pathway makes a contribution.

As an alternative approach to establish the source of the PA bound to RdgB/H9252, we used inhibitors of PLC (U73122) and PLD (FIPI) (36). We first confirmed that FIPI is able to inhibit GTPγS-stimulated PLD activity by monitoring the production of PEt in permeabilized cells (data not shown). Next we studied PA binding to RdgB/H9252 by incubating HL60 cells with a phospholipase inhibitor and GTPγS. FIPI but not U73122 inhibited the GTPγS-stimulated increase in RdgB/H9252-bound PA (Fig. 10, A and B). We conclude that RdgBβ mainly binds PA derived from the PLD pathway. This conclusion is also supported by the analysis of the molecular species of PA bound by RdgBβ after GTPγS stimulation (Figs. 4B and 5B). The dominant PA species present are C16:0/16:1 and C16:1/18:1, which are characteristic of the PC lipids but not PI lipids (Fig. 6). During stimulation of HL60 cells with GTPγS, increases in the molecular species of C18:1/18:1 PA and C18:0/18:1 PA were observed, but these species were not found in RdgBβ.

To confirm that increased PA binding to RdgBβ was entirely due to the increased levels of PA and not some secondary effect of G-protein activation, we used the bacterial Streptomyces sp. phospholipase D to produce PA in the permeabilized cells (37). Bacterial phospholipase D hydrolyzed PC and produced PA in the permeabilized cells (Fig. 4E), and this was accompanied by an increase in PA binding to RdgBβ (Fig. 10C). The dominant molecular species bound to RdgBβ were C16:0/16:1 and C16:1/18:1 PA (Fig. 4D). When compared with GTPγS, radiolabeled PA bound to RdgBβ was much greater, suggesting that the amount of PA present is the limiting factor (Fig. 10).

RdgBα Proteins Are Also PA-binding Proteins—Above we showed RdgBβ as a PI/PA- rather than a PI/PC-binding and transfer protein. Similar to RdgBβ, the PITP domain of Dm-
Rdgbβ exhibited low PC transfer activity (Fig. 2, D and F), implying that it might also bind PA. We therefore examined the lipid binding properties of the PITP domains of human Rdgbα1 and Dm-Rdgbα before and after stimulation of HL60 cells with GTPγS. Both proteins were found to bind PA, and the binding increased when the cells were stimulated with GTPγS (Fig. 11, A and B). However, PA binding was not as prominent as seen for Rdgbβ, and nearly 70% of the lipid bound was PI. In addition, the human Rdgbα1 PITP domain bound more PC than PA.

Transfer of PI and PC by the Rdgbα Proteins—Residues that are important in binding the inositol ring in PITPα are conserved in all the Class II PITPs (Table 1), and we demonstrate above that the PITP domain of Rdgbα proteins can transfer PI. The PITP domains of human Rdgbβ and Dm-Rdgbα hardly bound and transferred PC (Figs. 12 and 2). However, the PITP domain of human Rdgbα did bind substantially more PC than human Rdgbβ (Fig. 11). We therefore analyzed the PC transfer activity of human Rdgbα(1–277) and found that, like the Dm-Rdgbα PITP domain, it did not transfer PC (Fig. 12).

Cysteine 95 is important for PC transfer by PITPβ because its replacement by threonine decreases PC transfer significantly (12). Cysteine 95 is not conserved in the Class II family of Rdgb proteins but is replaced with threonine (Table 1). To test the importance of this threonine residue for PC transfer by the Dm-Rdgbα PITP domain, it was replaced by cysteine by site-directed mutagenesis. Neither PC nor PI transfer activities were affected by the replacement (Fig. 12). Binding of PC was also unchanged in Dm-Rdgbα(T95C) (Fig. 11). However, the replacement increased binding of PA to the protein at the expense of PI, particularly upon stimulation of the cells with GTPγS (Fig. 11).
DISCUSSION

In this study, we provide strong evidence that the PITP domain of Class II PITPs can bind PA in addition to binding PI and PC. Thus, the lipid binding properties of Class II PITPs differ from those of Class I PITPs. This in itself is not surprising given that these PITP domains only share ~40% identity in their amino acid sequence. Residues that are essential for binding the inositol headgroup of PI are conserved in all PITPs, and therefore PI binding and transfer are shared by all PITPs. As residues important for PC binding and transfer are less obvious despite the availability of the structures of both PITPa and PITPβ loaded with PC (3, 5), it has not been possible to predict whether RdgBβ will also be competent for PC binding and transfer. In this study, we examined the lipid binding and transfer properties of RdgBβ. Our results show that although all members of the PITP family can bind and transfer PI the Class IIB PITP, RdgBβ, has a drastically reduced PC binding and transfer activity. Using HL60 cells prelabeled with [14C]acetate as lipid donors, RdgBβ was found to bind mainly PI and PA. In contrast, PITPa bound only PI and PC. PA binding by RdgBβ was confirmed by mass spectrometry, which also showed that among the cellular PA species RdgBβ preferably binds the C16:0/16:1 and C16:1/18:1 PA species (Fig. 13). These PA species are typically produced from PC via the PLD pathway (38). Using inhibitors specific for PLC or PLD, we confirmed that the PA bound by RdgBβ is mainly derived from the PLD pathway. The ability to bind PA was conserved in all members of the Class II PITPs. However, RdgBβ bound the highest level of PA.

A striking feature of human RdgBβ and the Drosophila RdgBα is their inability to bind substantial amounts of PC (Fig. 11). Residues binding the PC headgroup have been difficult to determine even from the crystal structures of PITPa and PITPβ loaded with PC (3, 5). Cysteine 95 lies in the lipid-binding cavity and forms a hydrogen bond with the headgroup of PC indirectly via a water molecule, and mutation of this residue to either threonine or alanine eliminates PC transfer, leaving PI transfer unaffected (12). In RdgB proteins, cysteine 95 is replaced with a threonine (Table 1). Another residue important for PC transfer is phenylalanine 225. When it is replaced with a leucine residue as is the case with mouse or rat PITPβ, PC is transferred less
Because PA can be metabolized into DAG, RdgBα proteins could possibly regulate the availability of DAG. In *C. elegans*, mutations in DAG kinase can rescue the phenotypes of RdgBα loss of function mutants (22). In *Drosophila* photoreceptors, the Dm-RdgBα proteins are thought to be localized at the subrhabdomeric region as is the single phospholipase D (44), whereas the hydrolysis of PI(4,5)P$_2$, generating DAG, occurs at the nearby but distinct microvillar plasma membrane. In this context, the movement of lipids between the microvillar plasma membrane and the subrhabdomeric region needs to occur to keep the PI(4,5)P$_2$ cycle running, and the possibility that RdgBα proteins could facilitate PA removal from the rhabdomere while supplying PI requires further exploration.

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**FIGURE 13. RdgBβ binds PA derived from the phospholipase D signaling pathway.** The molecular species of PA bound by RdgBβ is similar to that present in PC. Other evidence to support this is the ability of the PLD inhibitor FIP1 to inhibit GTPγS-stimulated PA binding to RdgBβ.
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