

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

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Background: Phosphatidylinositol transfer protein, cytoplasmic 1 (PITPNC1) (alternative name, RdgB β) promotes meta-static colonization and angiogenesis in humans.

Results: We demonstrate that RdgB β is a phosphatidic acid (PA)- and phosphatidylinositol-binding protein and binds PA derived from the phospholipase D pathway.

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

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

Phosphatidylinositol transfer proteins (PITPs)³ are conserved proteins that participate in many cellular processes

including cell signaling, membrane traffic, and lipid metabolism (1). The defining feature of the PITPs is the presence of an N-terminal PITP domain. The five members of the PITP family in the human genome are grouped as Class I and Class II based on phylogenetic analysis (Ref. 2 and see Fig. 1). Class I comprises PITP α and PITP β , which are single domain proteins consisting of a hydrophobic cavity that sequesters a single molecule of either phosphatidylinositol (PI) or phosphatidylcholine (PC) (3–5). Except for a very brief period after unloading, PITP α and PITP β are loaded with their lipid cargo; half the population contains PI, and the other half contains PC (6). PITP α , the founding member of the Class I PITP family, is not essential for life but is required for axonal outgrowth (7, 8), and mice lacking a functional PITP α or a having a PITP mutant unable to bind PI have neurodegenerative disorders (9, 10). In contrast, PITP β is essential (11) and is required for retrograde traffic from the Golgi to the endoplasmic reticulum and for maintenance of the nuclear architecture (12). The RdgB proteins, RdgB α 1 (alias PITPNM1; Nir2), RdgB α 2 (PITPNM2; Nir3), and RdgB β (PITPNC1), belong to Class II and are less well characterized with respect to their lipid binding properties (1, 2). Based on the structure of PITP α containing PI, four residues (Thr-59, Lys-61, Glu-86, and Asn-90) are essential for PI binding in PITP α (4). These residues are conserved in the entire PITP family, and mutation of any of these residues in PITP α leads to loss of PI binding. Despite the availability of the structures of PITP α and of PITP β containing PC (3, 5), residues responsible for PC binding have been difficult to identify. We have recently reported that mutation of Cys-95 to threonine causes a loss of PC transfer in PITP β (12). Cys-95 resides in the lipid binding pocket (see Fig. 2B), and in RdgB proteins, it is

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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; PI(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; GTP γ S, guanosine 5'-(γ -thio)triphosphate; PET, phosphatidylethanol; FIPI, 5-fluoro-2-indolyl deschlorohalopemide; Dm, *Drosophila melanogaster*; sp1, splice variant 1; sp2, splice variant 2.

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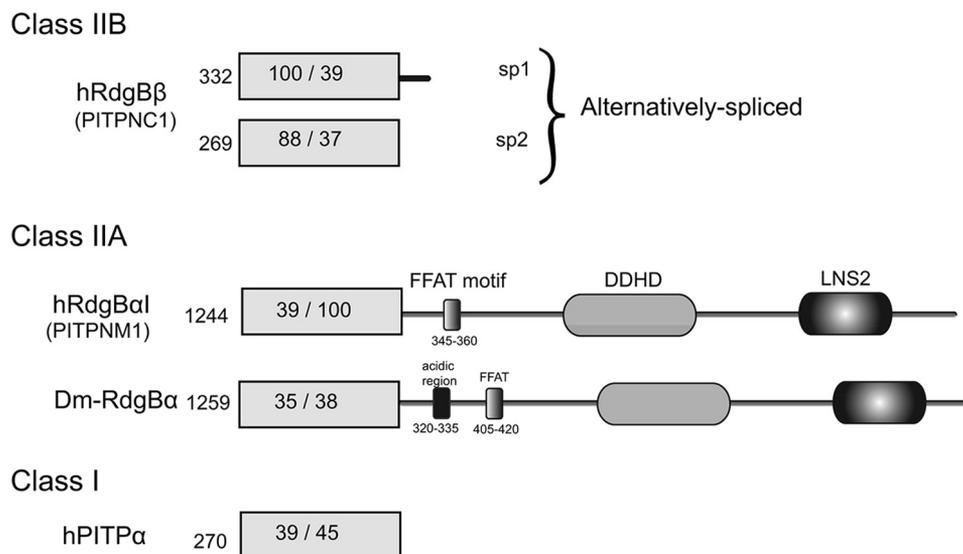


FIGURE 1. **Class I and Class II PITPs.** Shown is the domain organization of PITP family members examined here. PITPs are grouped into Classes I and II. Class II is subdivided into A and B, and RdgB β belong to Class IIB. The splice variants of RdgB β are shown. The protein sequences were analyzed by BLAST, and the percentage of primary sequence identity of each PITP domain to human RdgB β and to human RdgB α 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-RdgB α , was identified in *Drosophila*. The single Dm-RdgB α 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-RdgB α are characterized by abnormal termination of the light response and profound loss of the electroretinogram amplitude shortly after initial light exposure. In addition, the rhabdomeric 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 rhabdomere results in the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) by phospholipase C (PLC), and Dm-RdgB α could potentially facilitate the delivery of PI to the rhabdomeric membranes for the regeneration of the PLC substrate, *i.e.* PI(4,5)P₂ (17). RdgB α is also required for normal localization of rhodopsin in rhabdomeres (18), and rhodopsin levels are reduced in Dm-RdgB α mutants (19). In mammals, RdgB α 1 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 RdgB α 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 RdgB α 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 RdgB α 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 RdgB α , RdgB β is a soluble protein with two splice variants: a long form (332 amino acids), RdgB β splice variant 1 (sp1), and a short form, RdgB β splice variant 2 (sp2) (268 amino acids) (Fig. 1). RdgB β consists of a PITP domain followed by a C-terminal extension of 60 amino acids, whereas RdgB β -sp2 consists of the PITP domain only. The C-terminal extension of RdgB β -sp1 is phosphorylated at two serine residues that form a docking site for 14-3-3 proteins (23). The PITP domain of RdgB β is only 39% identical to both Class I and Class IIA PITPs and 35% identical to Dm-RdgB α (Fig. 1). RdgB β 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 RdgB β and consequently in metastatic colonization. This can be recapitulated by increased expression of RdgB β in MDA-231 breast cancer cells.

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

EXPERIMENTAL PROCEDURES

Materials—*myo*-[2-³H]inositol, [*methyl*-³H]choline chloride, and [1-¹⁴C]acetic acid were purchased from American Radiolabeled Chemicals (UK). *sn*1-Palmitoyl-*sn*2-oleoylphos-

phatidylcholine and *sn*1-palmitoyl-*sn*2-oleoyl phosphatidic acid were obtained from Avanti Polar Lipids (Alabaster, AL). *N*-Trinitrophenyl phosphatidylethanolamine (PE), *sn*1-palmitoyl-*sn*2-pyrenedecanoyl-PC, -PA, and *sn*1-octadecanoyl-*sn*2-pyrenedecanoyl-PI were synthesized as described previously (25, 26). Bacterial phospholipase D (*Streptomyces* sp. (P4912)) was obtained from Sigma.

Recombinant PITP α and RdgB β (human) were expressed from pRSET vectors and purified as described previously (4). The Dm-RdgB α -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 α 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 QuikChange 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 nitrilotriacetic acid-agarose resin (HIS-Select nickel affinity gel, Sigma) as described previously (4). The purity and the yield of the proteins varied between constructs. PITP α and Dm-RdgB α (1–281) proteins were expressed very well, whereas the yields of RdgB β and RdgB α (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 μ g of PITP α . 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 μ l of [^3H]inositol-labeled or [^3H]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 μ l of recombinant proteins at 25°C for 20 min (27). [^3H]inositol-labeled microsomes were prepared from rat liver, whereas [^3H]choline-labeled microsomes were obtained from HL60 cells. The reaction was quenched by the addition of 50 μ 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 $12,000 \times g$ for 10 min at 4°C , 150 μ 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 \pm 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 [^3H]inositol or [^3H]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 μ 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 $\mu\text{Ci/ml}$ [^{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 ($\sim 10^7$ cells) were incubated with 120 μg of PITP α or RdgB β protein (100 μl) for 20 min at 37°C in the presence of 2 mM Mg^{2+} -ATP and 100 nM Ca^{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.* 5×10^7 HL60 cells (unlabeled) were incubated with 600 μg of a purified transfer protein. Lipids were also directly extracted from the *E. coli*-expressed PITPs (600 μg /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 μl of an ethanol solution of pyrenyl phospholipid/*sn*1-palmitoyl-*sn*2-oleylphosphatidylcholine/*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 mM EDTA) 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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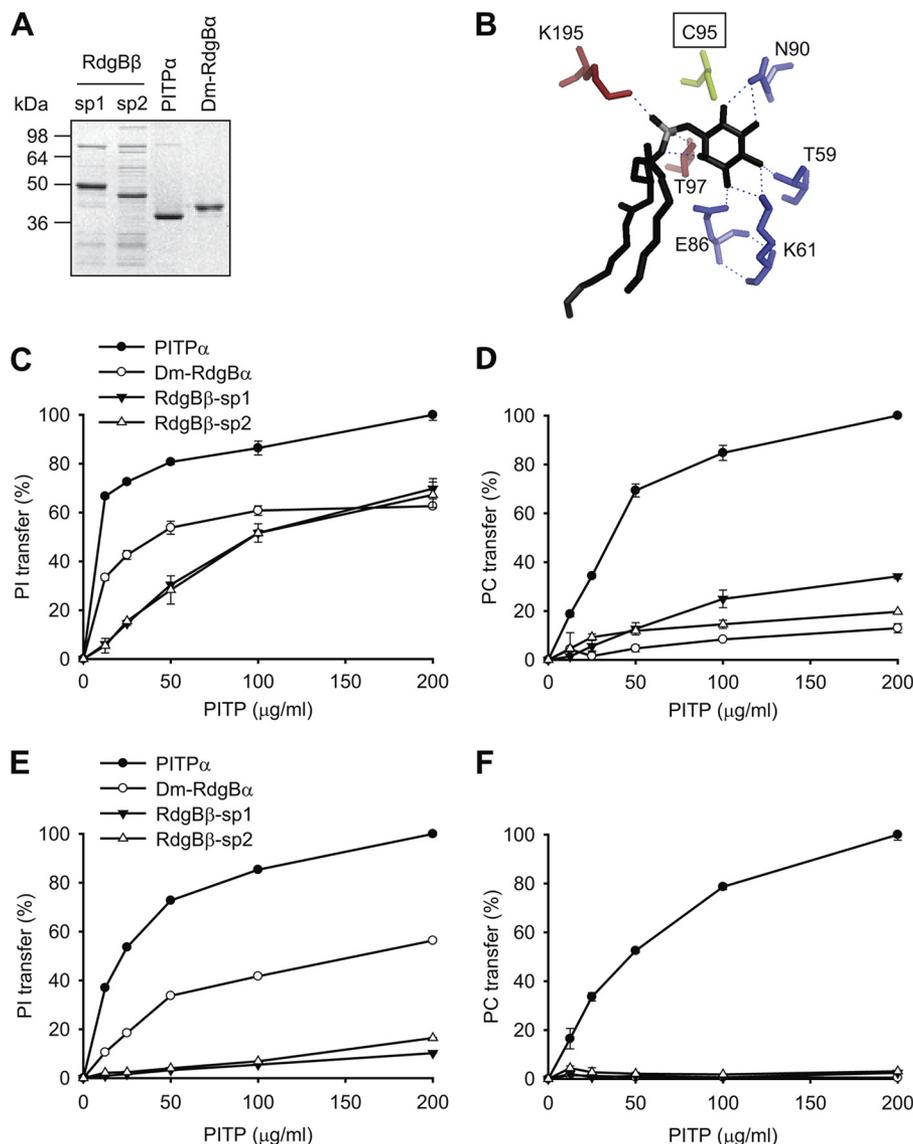


FIGURE 2. Comparison of PI and PC transfer by Class I and II PITPs. *A*, recombinant human PITP α , human RdgB β -sp1 (long form) and RdgB β -sp2 (short form), and the PITP domain of Dm-RdgB α (1 μ g each) were separated by SDS-PAGE and stained with Coomassie Blue to demonstrate their degree of purity. *B*, residues that coordinate the inositol ring of PI in the lipid binding pocket of PITP α (Lys-61, Asn-90, Thr-59, and Glu-86) are conserved in RdgB β . The location of residue Cys-95 in PITP α is indicated. This residue is replaced by a threonine in RdgB proteins. *C*, PI transfer activity by PITP α and RdgB proteins using [3 H]inositol-labeled microsomes as donor and liposomes as acceptors. *D*, PC transfer using [3 H]choline-labeled microsomes as donor and liposomes as acceptors. *E*, PI transfer using permeabilized HL60 cells prelabeled with [3 H]inositol with liposomes as acceptors. *F*, PC transfer using permeabilized HL60 cells prelabeled with [3 H]choline with liposomes as acceptors. The results are representative of a minimum of three experiments, and two or more protein preparations were assessed. The activity of PITP α was set as 100%. Results are shown from individual experiments performed in duplicate (\pm range indicated by error bars).

centration 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 *sn*1-palmitoyl-*sn*2-oleylphosphatidylcholine/*sn*1-palmitoyl-*sn*2-oleoyl phosphatidic acid (98:2, mol/mol) and prepared by the ethanol injection method were also added.

Lipid Binding following Incubation with PMA, GTP γ S, or Bacterial Phospholipase D—HL60 cells were labeled with 1 μ Ci/ml [14 C]acetate in RPMI 1640 medium for 48 h. After washing twice in PIPES buffer, the cells were added to a mixture of GTP γ S (100 μ M final), PMA (100 nM final), 0.2 unit of bacterial phospholipase D, MgATP (2 mM final), Ca $^{2+}$ buffered with 3 mM EGTA at 100 nM (*p*Ca 7) or 10 μ M (*p*Ca 5), streptolysin O (0.4 IU/ml final), and a recombinant protein (120 μ g) as

indicated in a final incubation volume of 600 μ l. The inhibitors FIPI (750 nM) and U73122 (10 μ M) were also added where indicated. After incubation for 20 min at 37 $^{\circ}$ C, the cells were centrifuged, and 500 μ l of the supernatant was used to recapture the proteins as described above (6). The residual cells were quenched with 375 μ l 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 RdgB β with PITP α —We have recently reported that the PI transfer activity of RdgB β -sp1 is low when compared with PITP α (23). RdgB β -sp1 requires 100-fold higher concentrations and furthermore does not achieve the

TABLE 1

Inositol headgroup binding

Gray, residues Thr-59, Lys-61, Glu-86, and Asn-90 are conserved in all sequences. Residue 95 is a threonine in the class II proteins (green) but a cysteine residue in the class I sequences (yellow).

Inositol headgroup binding	
Class II	
hRdgBα1-O00562	DGPGGSGQYTHKVIYHVGSHIPGWFRALLPKAALQVEEESWNAYPYTTRTRY
hRdgBα2-Q9BZ72	DGPGGSGQYTHKVIYHVGSMHIPSWFERSILPKAALRVVEESWNAYPYTTRTRF
DmRdgBα-NP_511149.2	DGPGGNGQYTKKIYHVGNHLPGWIKSLLPKSALTVEEEAWNAYPYTTRTRY
hRdgBβ-Q9UKF7	DPHHGNGQFTEKRVYLNSKLPSPWARAVVVK-IFVYVTEKAWNYPYTITEY
Class I	
hPITPα-P48739	K-DGEKGQYTHKVIYHLKSKVPAFVRMIAPEGSLVFHEKAWNAYPYC RTIV
hPITPβ-Q00169	K-DGEKGQYTHKVIYHLQSKVPTFVRMLAPEGALNIHEKAWNAYPYC RTVI
 * * * * * * *

* , identical, fully conserved residues; ; , conservative substitution between residues with strongly similar properties; . , conservative substitution between residues with weakly similar properties.

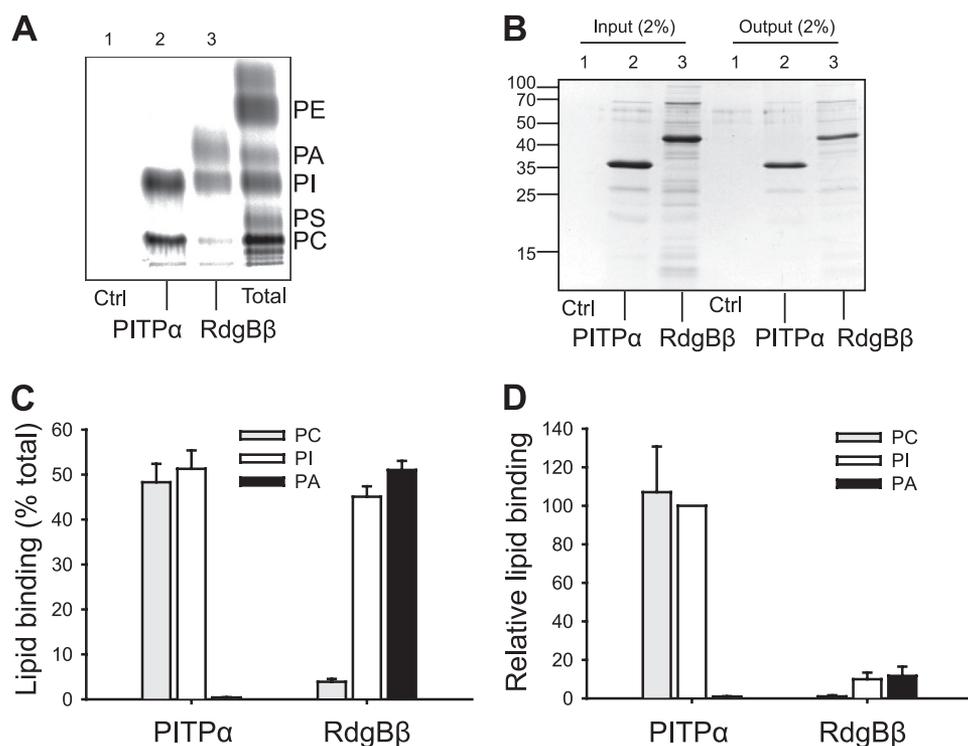


FIGURE 3. Analysis of the lipid binding specificity of RdgBβ. A, His-tagged RdgBβ and PITPα (120 μg) were incubated for 20 min with prepermeabilized HL60 cells (~10⁷ cells) prelabeled with [¹⁴C]acetate for 48 h. The cells were then removed by centrifugation, and RdgBβ and PITPα in the supernatant was captured using nickel beads. The lipids bound to the protein were extracted and separated by TLC. B, to monitor capture by the nickel beads, a sample of the protein (~2 μg) was analyzed by SDS-PAGE and stained with Coomassie Blue. C, quantification of the lipid bound to PITPα (n = 5) and RdgBβ (n = 6) expressed as a percentage of total lipid binding (PC + PI + PA) to each PITP. Error bars are S.E. D, the lipid bound adjusted for the amount of recombinant protein recovered. PI bound by PITPα was set at 100%. PITPα, n = 5; RdgBβ, n = 3. Error bars are S.E. Ctrl, control.

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 RdgB β showed very similar PI transfer activity regardless of the assay used (Fig. 2, C and E). Compared with PITP α , both RdgB β proteins required a much higher concentration and did not achieve the same degree of activity. Furthermore, the RdgB β proteins showed greater activity in the microsome-liposome assay compared with the permeabilized HL60 cell assay, whereas PITP α 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 RdgB β splice variants with PITP α using both assays. For PI transfer, four residues (Thr-59, Lys-61, Glu-86, and Asn-90; mouse PITP α numbering) that coordinate the inositol ring are essential (Fig. 2B); these residues are conserved in RdgB β . Regarding PC transfer, we have recently shown that mutation of cysteine 95 to threonine in PITP β 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 PITP α , RdgB proteins showed very low PC transfer in either assay (Fig. 2, D and F). The PC transfer activity was 20% of that of PITP α 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 RdgB β was also observed for another Class II RdgB protein, the PITP domain of Dm-RdgB α . The Dm-RdgB α PITP domain was active for PI transfer in either assay, but the maximal activity attained was ~50% of PITP α (Fig. 2, C and E).

Lipid Binding Properties of the Soluble Class II PITP, RdgB β —The low level of PC transfer exhibited by RdgB β 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 PITP α , which binds PI and PC, RdgB β 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). PITP α bound near equal proportions of PI and PC. No PA binding was observed. In comparison, RdgB β bound PI and PA in near equal proportions, whereas PC binding was low (~5%) (Fig. 3C).

It is well documented that isolated PITP α 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 RdgB β after incubation with the labeled HL60 cells provides a measure of lipid exchange (Fig. 3D). Relative to PITP α , RdgB β contained less radioactivity per pmol of protein, implying that a large fraction of the RdgB β molecules may not have undergone lipid exchange.

Recombinant PITP α when purified from *E. coli* comes pre-loaded with PG, which is immediately exchanged when exposed to PI or to PC (28). To identify which lipids were associated with

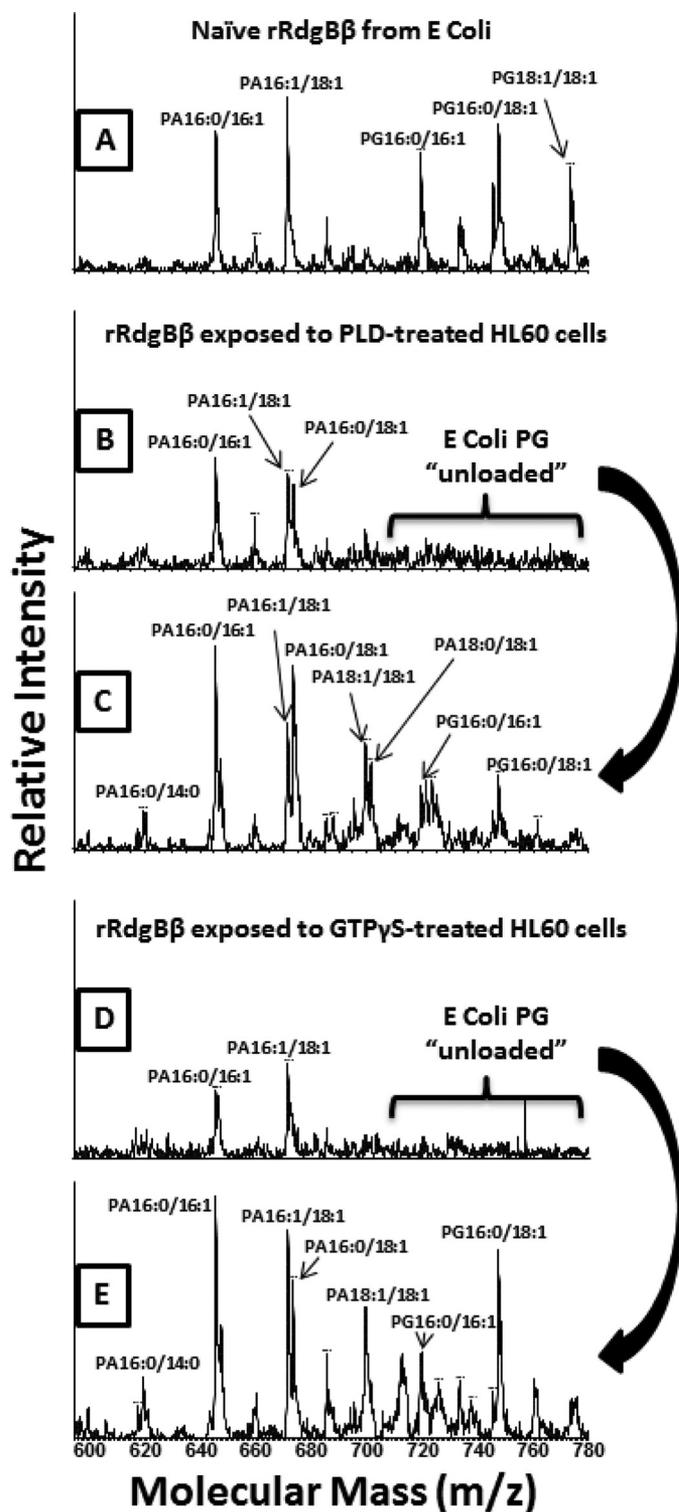
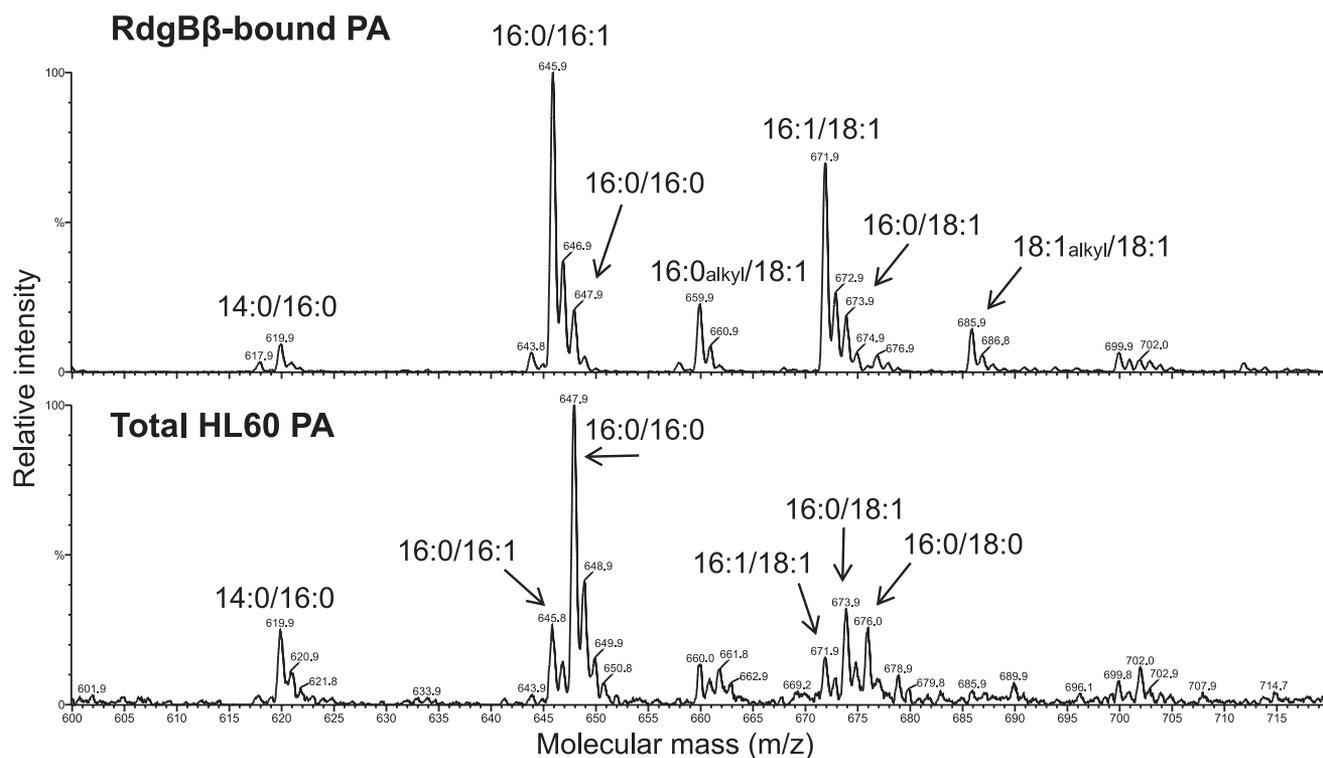


FIGURE 4. Mass spectrometric analysis of lipids associated with RdgB β before and after exposure to HL60 cells. A, lipids extracted directly from recombinant RdgB β expressed in *E. coli*. B, RdgB β exposed to HL60 cells in the presence of bacterial phospholipase D. C, HL60 cells after exposure to RdgB β and bacterial PLD (0.2 unit). D, RdgB β exposed to HL60 cells in the presence of GTP γ S and pCa 5 to activate endogenous PLD. E, HL60 cells after exposure to RdgB β , GTP γ S, and pCa 5.

recombinant RdgB β 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

A

Precursors of m/z -153

B

Mass (m/z)		Dominant Molecular species	Whole HL60 PA (% mean \pm S.E.)	RdgBβ-bound PA (% mean \pm S.E.)	RdgBβ-bound PA after GTPγS (% mean \pm S.E.)
619	C30:0	14:0/16:0	11.06 \pm 1.73	5.02 \pm 0.63**	4.01 \pm 1.56*
643	C32:2	16:1/16:1	5.58 \pm 0.51	6.14 \pm 0.93	7.21 \pm 2.05
645	C32:1	16:0/16:1	16.77 \pm 0.71	26.90 \pm 5.27	23.09 \pm 0.81**
647	C32:0	16:0/16:0	11.50 \pm 0.50	7.09 \pm 0.58**	6.34 \pm 0.69**
671	C34:2	16:1/18:1	11.30 \pm 0.28	23.09 \pm 2.80**	24.65 \pm 2.40**
673	C34:1	16:0/18:1	10.55 \pm 0.73	7.32 \pm 0.59**	11.90 \pm 1.30
699	C36:2	18:1/18:1	11.53 \pm 0.63	6.09 \pm 1.14**	9.65 \pm 2.74
701	C36:1	18:0/18:1	7.54 \pm 0.54	6.02 \pm 1.61	7.26 \pm 2.32
			(n = 5)	(n = 6)	(n = 4)

FIGURE 5. RdgBβ binds cellular PA species selectively. A, HL60 cells (5×10^7 cells) were permeabilized with streptolysin O and incubated with His-tagged RdgBβ (600 μ g) 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 \pm 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 \pm 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 ** ($p < 0.005$).

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 monounsaturated 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). PITPα,

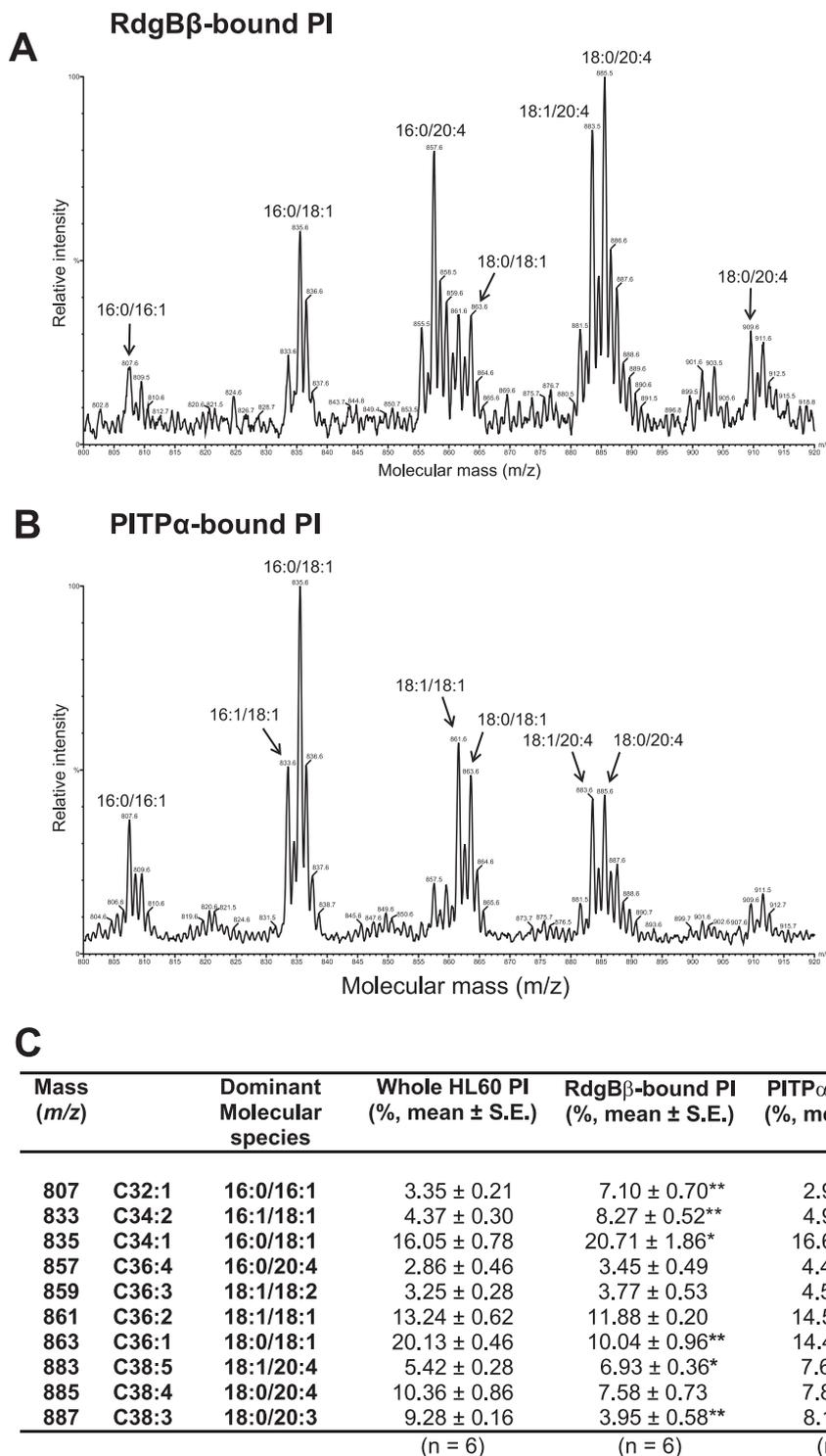


FIGURE 6. **RdgBβ** and **PITPα** prefer different molecular species of PI. HL60 cells (5×10^7 cells) were permeabilized with streptolysin O, washed, and incubated with His-tagged RdgBβ or PITPα (600 μg) for 20 min at 37 °C. The cells were removed by centrifugation, and the proteins in the supernatant 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 PITP-bound PI 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).

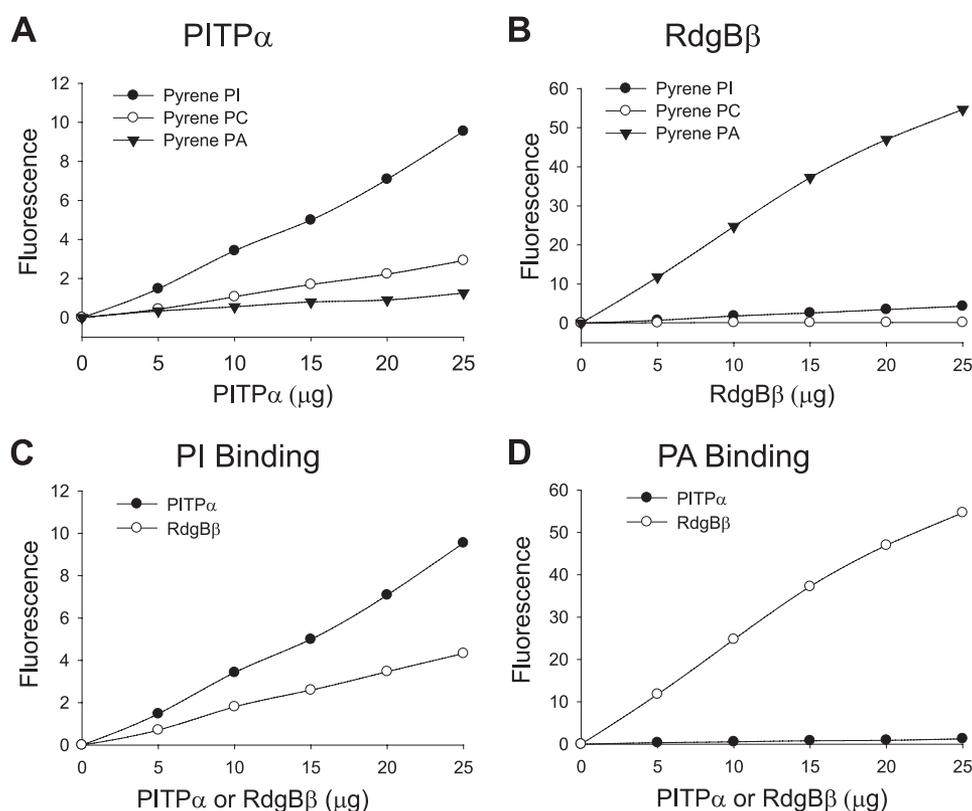


FIGURE 7. **RdgB β but not P1TPa binds pyrene-labeled PA.** Quenched donor vesicles consisting of a pyrene-labeled phospholipid/*sn*1-palmitoyl-*sn*2-oleylphosphatidylcholine/*N*-trinitrophenyl PE (2:88:10, mol/mol) were titrated with P1TPa or RdgB β . The increase in fluorescence intensity of the different pyrenyl lipid as a function of P1TPa or RdgB β 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 P1TPa versus RdgB β . A representative experiment of three is shown.

Increased Binding of Cellular PA to RdgB β When HL60 Cells Are Stimulated with GTP γ S—As the amount of PA in resting HL60 cells is low (<1%), exchange of the preloaded PA may not occur. RdgB β when produced in *E. coli* is already preloaded with PA (and PG) (see Fig. 4A). Addition of GTP γ 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 γ S (see Fig. 13).

To examine whether an increase in cellular PA levels following the activation of PLC or PLD increases PA binding by RdgB β , the lipid binding assay was modified as follows. Recombinant RdgB β or P1TPa was added together with the permeabilizing agent, streptolysin O, Ca²⁺ buffered with 3 mM EGTA at 100 nM (*p*Ca 7) or 10 μ M (*p*Ca 5), and the stimulus, GTP γ S. It has been shown that 10 μ M Ca²⁺ alone stimulates PLC and PLD somewhat, but in the presence of GTP γ S, it causes a robust activation of both types of phospholipases (31, 33). In the presence of 10 μ M Ca²⁺ alone, a small increase in PA binding to RdgB β was observed, whereas in the presence of Ca²⁺ plus GTP γ S, substantially increased PA binding at the expense of PI was observed (Fig. 8, *A* and *B*). As expected, lipid binding to P1TPa was insensitive to the manipulation of the cellular PA levels (Fig. 8, *A* and *B*).

To identify whether the increased PA bound by RdgB β 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 GTP γ S, PMA did not increase binding of PA to RdgB β (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 GTP γ S stimulation (34). GTP γ S stimulation enhanced PA binding, and in the additional presence of either alcohol, PA binding to RdgB β was unaffected despite the reduction in cellular PA to resting levels (Fig. 9). In addition, RdgB β was now found to bind a small amount of PEt or phosphatidylbutanol (Fig. 9A). We also monitored cellular PA and the phosphatidylalcohol after GTP γ S stimulation. GTP γ S 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 RdgB β binds PA preferentially to the

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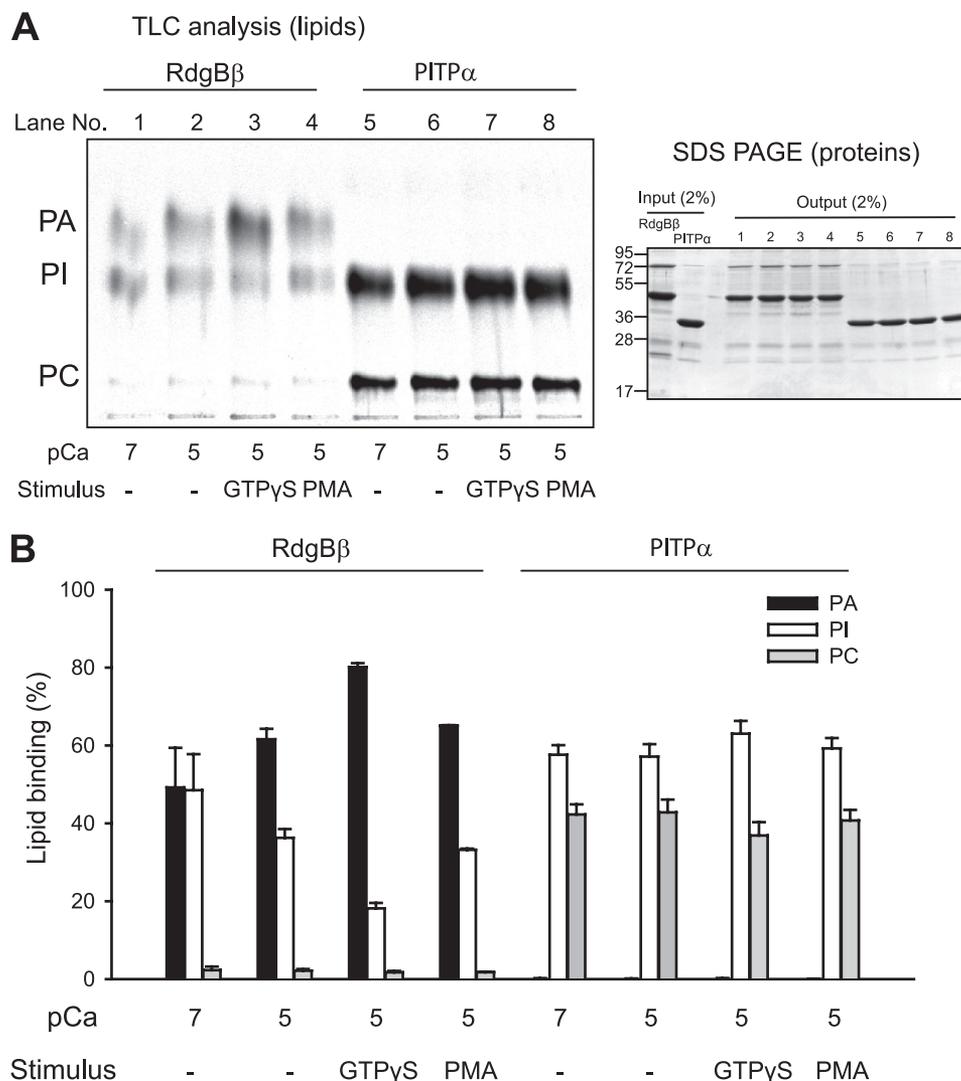


FIGURE 8. **Increased PA binding by RdgB β after stimulation of HL60 cells with GTP γ S.** [14 C]acetate-labeled HL60 cells were incubated with RdgB β or PITP α in the presence of GTP γ S (100 μ M) or PMA (100 nM) and calcium-buffered at pCa 5 (10 μ M) or pCa 7 (100 nM) together with the permeabilizing agent streptolysin O. After 20 min, the cells were removed by centrifugation, and the PITPs were captured from the supernatant using nickel beads. A, the lipids were extracted and separated by TLC, and the plate was imaged using a phosphorimaging screen. A sample of the captured proteins was also analyzed by SDS-PAGE. B, lipid binding was quantitated from two independent experiments. Error bars are S.D. ($n = 2$).

phosphatidylalcohols, and moreover, PA binding to RdgB β 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 β , we used inhibitors of PLC (U73122) and PLD (FIPI) (36). We first confirmed that FIPI is able to inhibit GTP γ S-stimulated PLD activity by monitoring the production of PEt in permeabilized cells (data not shown). Next we studied PA binding to RdgB β by incubating HL60 cells with a phospholipase inhibitor and GTP γ S. FIPI but not U73122 inhibited the GTP γ S-stimulated increase in RdgB β -bound PA (Fig. 10, A and B). We conclude that RdgB β mainly binds PA derived from the PLD pathway. This conclusion is also supported by the analysis of the molecular species of PA bound by RdgB β after GTP γ S 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 GTP γ S, 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 RdgB β .

To confirm that increased PA binding to RdgB β 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 RdgB β (Fig. 10C). The dominant molecular species bound to RdgB β were C16:0/16:1 and C16:1/18:1 PA (Fig. 4D). When compared with GTP γ S, radiolabeled PA bound to RdgB β was much greater, suggesting that the amount of PA present is the limiting factor (Fig. 10).

RdgB α Proteins Are Also PA-binding Proteins—Above we showed RdgB β as a PI/PA- rather than a PI/PC-binding and transfer protein. Similar to RdgB β , the PITP domain of Dm-

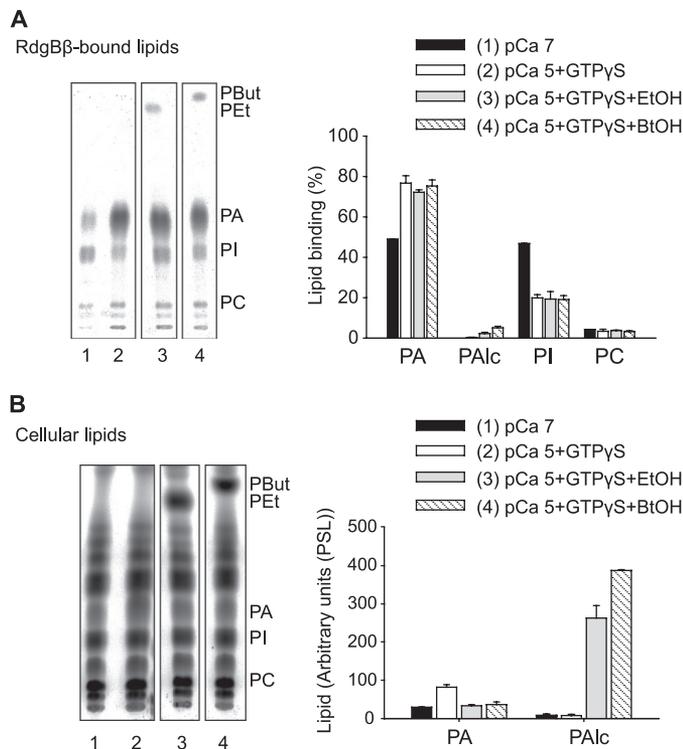


FIGURE 9. RdgBβ binds phosphatidylalcohols poorly compared with PA. HL60 cells prelabeled with [¹⁴C]acetate were incubated with RdgBβ in the presence of pCa 7 (100 nM) (control), GTPγS (100 μM) and pCa 5 (10 μM), and ethanol (2%) or butanol (BtOH) (0.5%) as indicated in the presence of streptolysin O for 20 min. *A*, TLC of radiolabeled lipids bound to RdgBβ and their quantitation. *B*, TLC of the cellular lipids and their quantitation. PBuT, phosphatidylbutanol; PSL, photostimulated luminescence; PAIc, phosphatidylalcohol. The results are quantitated from four independent experiments. Error bars are S.E. (*n* = 4).

RdgBα 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 P1TP 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 P1TP 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 P1TPα are conserved in all the Class II P1TPs (Table 1), and we demonstrate above that the P1TP domain of RdgBα proteins can transfer PI. The P1TP domains of human RdgBβ and Dm-RdgBα hardly bound and transferred PC (Figs. 12 and 2). However, the P1TP 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 P1TP domain, it did not transfer PC (Fig. 12).

Cysteine 95 is important for PC transfer by P1TPβ 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

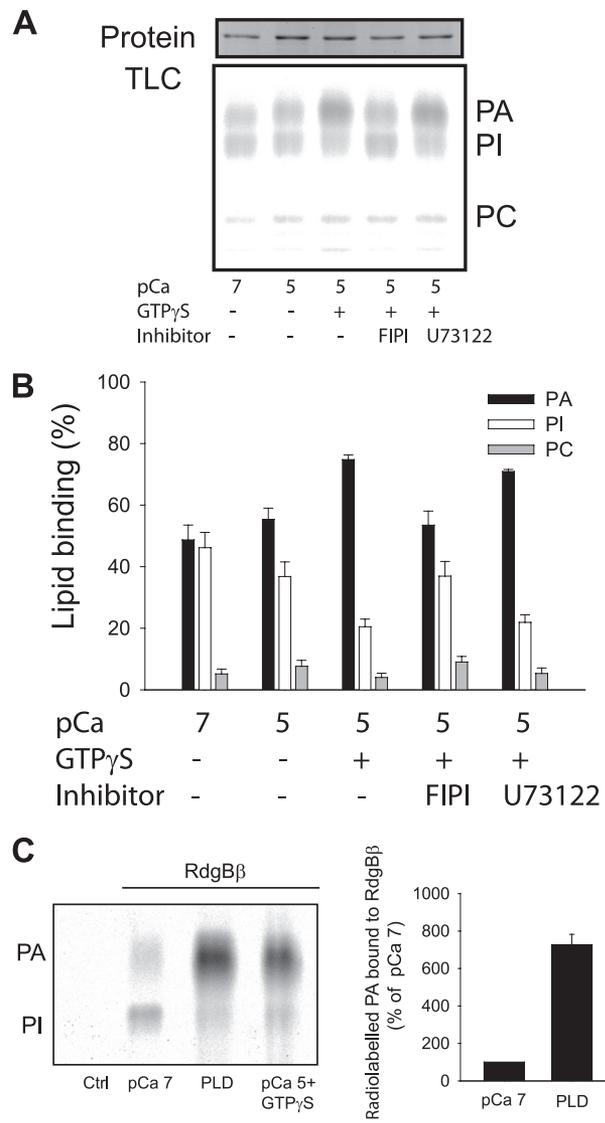


FIGURE 10. Increased PA binding to RdgBβ is dependent on endogenous phospholipase D activation and is mimicked by exogenous bacterial phospholipase D. [¹⁴C]Acetate-labeled HL60 cells were incubated with RdgBβ (120 μg) in the presence of GTPγS (100 μM), FIPI (750 nM), or U73122 (10 μM) and calcium-buffered at pCa 5 (10 μM) or pCa 7 (100 nM) as indicated together with the permeabilizing agent streptolysin O. After 20 min, the cells were removed by centrifugation and retained, and the P1TPs were captured from the supernatant using nickel beads. *A*, the lipids were extracted and analyzed by TLC, and a sample of the captured proteins was also analyzed by SDS-PAGE. *B*, lipid binding to RdgBβ was quantitated from three independent experiments. *C*, [¹⁴C]acetate-labeled HL60 cells were incubated with RdgBβ (120 μg) in the presence of streptolysin *sp.* PLD (0.2 unit) or GTPγS (100 μM) plus pCa 5 (10 μM) or pCa 7 (100 nM) as indicated together with the permeabilizing agent streptolysin O. After 20 min, the cells were removed by centrifugation and retained, and the P1TPs were captured from the supernatant using nickel beads. Radiolabeled PA binding to RdgBβ was quantitated from three independent experiments. Error bars are S.E. (*n* = 3). *Ctrl*, control.

Dm-RdgBα P1TP 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).

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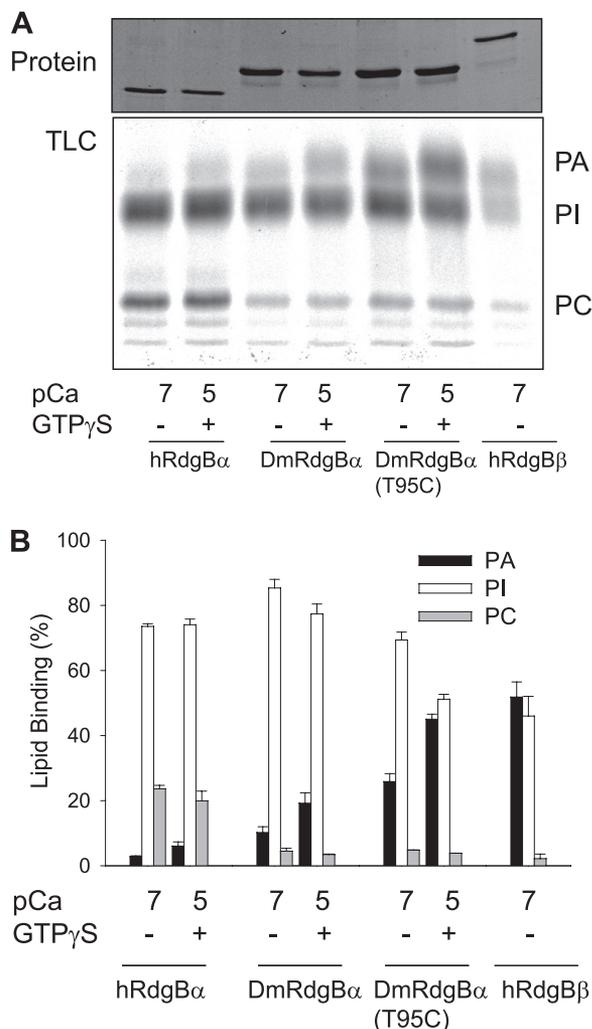


FIGURE 11. The P1TP domain of Class II RdgB α proteins also binds PA. [14 C]Acetate-labeled HL60 cells were incubated with the P1TP domain of human (*h*) RdgB α , Dm-RdgB α , Dm-RdgB α (T95C), or human RdgB β (120 μ g) in the presence of GTP γ S (100 μ M) and calcium-buffered at pCa 5 (10 μ M) or pCa 7 (100 nM) as indicated together with the permeabilizing agent streptolysin O. After 20 min, the cells were removed by centrifugation and retained, and the P1TPs were captured from the supernatant using nickel beads. *A*, the radiolabeled lipids were extracted, separated by TLC, and imaged using a phosphorimaging screen. A sample of the captured proteins was also analyzed by SDS-PAGE. *B*, lipid binding was quantitated using AIDA software. The data are means of three independent experiments \pm S.E. (error bars).

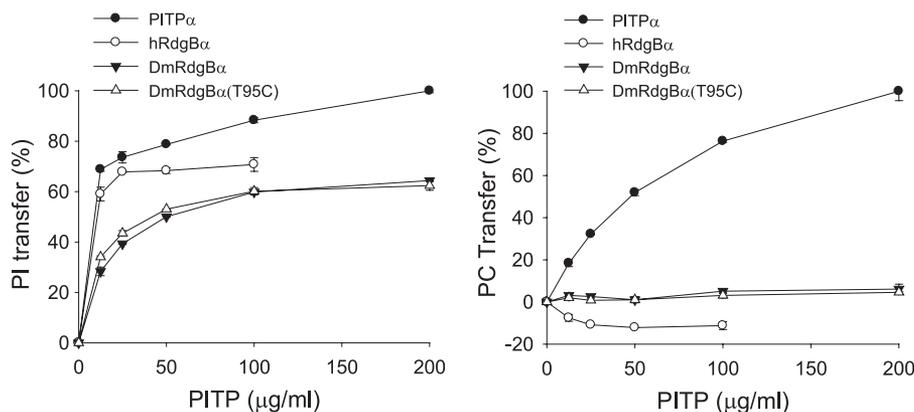


FIGURE 12. Comparison of the PI and PC transfer activity of P1TP domains of RdgB α and P1TP α . PI and PC transfer activity of P1TP α and the P1TP domains of Dm-RdgB α wild type and mutant proteins was measured using appropriately radiolabeled microsomes as donor and liposomes as acceptors. The results are representative of four independent experiments, and two or more protein preparations were assessed. The activity of P1TP α was set as 100%. Error bars are S.E. *h*RdgB α , human RdgB α .

DISCUSSION

In this study, we provide strong evidence that the P1TP domain of Class II P1TPs can bind PA in addition to binding PI and PC. Thus, the lipid binding properties of Class II P1TPs differ from those of Class I P1TPs. This in itself is not surprising given that these P1TP domains only share \sim 40% identity in their amino acid sequence. Residues that are essential for binding the inositol headgroup of PI are conserved in all P1TPs, and therefore PI binding and transfer are shared by all P1TPs. As residues important for PC binding and transfer are less obvious despite the availability of the structures of both P1TP α and P1TP β loaded with PC (3, 5), it has not been possible to predict whether RdgB β will also be competent for PC binding and transfer. In this study, we examined the lipid binding and transfer properties of RdgB β . Our results show that although all members of the P1TP family can bind and transfer PI the Class IIB P1TP, RdgB β , has a drastically reduced PC binding and transfer activity. Using HL60 cells pre-labeled with [14 C]acetate as lipid donors, RdgB β was found to bind mainly PI and PA. In contrast, P1TP α bound only PI and PC. PA binding by RdgB β was confirmed by mass spectrometry, which also showed that among the cellular PA species RdgB β 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 RdgB β is mainly derived from the PLD pathway. The ability to bind PA was conserved in all members of the Class II P1TPs. However, RdgB β bound the highest level of PA.

A striking feature of human RdgB β and the *Drosophila* RdgB α 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 P1TP α and P1TP β 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 P1TP β , PC is transferred less

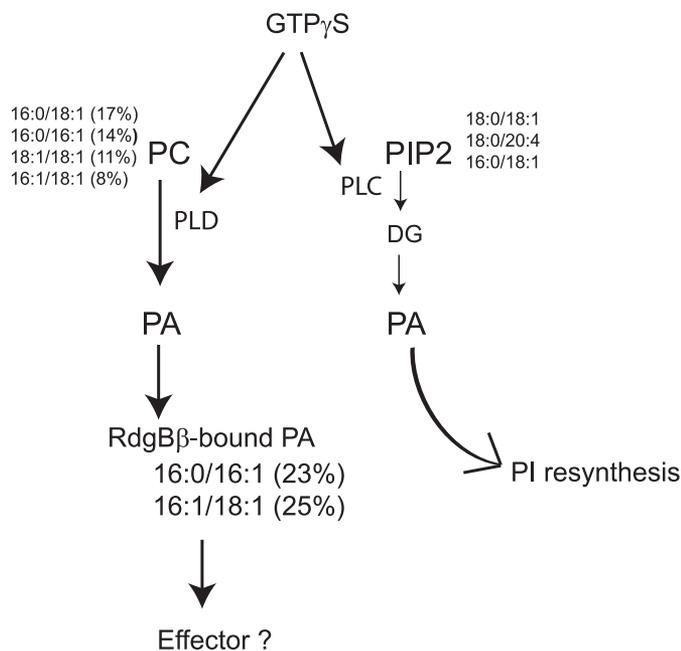


FIGURE 13. **RdgB β binds PA derived from the phospholipase D signaling pathway.** The molecular species of PA bound by RdgB β is similar to that present in PC. Other evidence to support this is the ability of the PLD inhibitor FIPI to inhibit GTP γ S-stimulated PA binding to RdgB β .

efficiently (5). Except for rodent PITP β , this residue is a phenylalanine in all Class I PITPs examined to date. In contrast, this residue is alanine in all RdgB α sequences and glycine in all RdgB β sequences. Both alanine and glycine have a small aliphatic side chain similar to that of leucine. Thus, the low transfer activity found in RdgB proteins could be influenced by the replacement of a large bulky aromatic residue (Phe-225) present in Class I PITPs with a small aliphatic residue and by replacement of Cys-95 with Thr-95.

What is the functional significance of PA binding or transfer by RdgB β ? One possibility is that PA bound to RdgB β may regulate endocytosis of the angiotensin II receptor. RdgB β is highly enriched in the heart and interacts with the angiotensin II receptor-associated protein, which itself interacts with the angiotensin II receptor (23, 39). Angiotensin II receptor-associated protein is a small (17-kDa) integral membrane protein with three transmembrane domains and a C terminus projecting into the cytoplasm (40). Angiotensin II receptor-associated protein and PLD2 can regulate the endocytosis of the angiotensin II receptor, and single nucleotide polymorphisms in either gene is associated with increased blood pressure and hypertension (41, 42). We are currently examining the inter-relationship among angiotensin II receptor endocytosis, angiotensin II receptor-associated protein, and RdgB β to decipher the significance of PA binding by RdgB β (Fig. 13). Other possible roles of RdgB β include the translocation of PA from the plasma membrane to the endoplasmic reticulum for the resynthesis of PI. However, previous studies have concluded that the PA derived from the PLD pathway may not be converted into PI, and cytosolic components are not required for the process (43).

Because RdgB α proteins also bind PA, it is possible that they are responsible for PA transfer at intermembrane contact sites.

Because PA can be metabolized into DAG, RdgB α proteins could possibly regulate the availability of DAG. In *C. elegans*, mutations in DAG kinase can rescue the phenotypes of RdgB α loss of function mutants (22). In *Drosophila* photoreceptors, the Dm-RdgB α proteins are thought to be localized at the subrhabdomeric region as is the single phospholipase D (44), whereas the hydrolysis of PI(4,5)P₂, 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)P₂ cycle running, and the possibility that RdgB α proteins could facilitate PA removal from the rhabdomere while supplying PI requires further exploration.

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