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Quantitative Analysis of the Effect of Phosphoinositide Interactions on the Function of Dbl Family Proteins*

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Normally, Rho GTPases are activated by the removal of bound GDP and the concomitant loading of GTP catalyzed by members of the Dbl family of guanine nucleotide exchange factors (GEFs). This family of GEFs invariantly contain a Dbl homology (DH) domain adjacent to a pleckstrin homology (PH) domain, and while the DH domain usually is sufficient to catalyze nucleotide exchange, possible roles for the conserved PH domain remain ambiguous. Here we demonstrate that the conserved PH domains of three distinct Dbl family proteins, intersectin, Dbs, and Tiam1, selectively bind lipid vesicles only when phosphoinositides are present. While the PH domains of intersectin and Dbs promiscuously bind several multiphosphorylated phosphoinositides, Tiam1 selectively interacts with phosphatidylinositol 3-phosphate (K_D ~5–10 μM). In addition, and in contrast to recent reports, catalysis of nucleotide exchange on nonprenylated Rac1 provided by various extended portions of Tiam1 is not influenced by (*a*) soluble phosphoinositide head groups, (*b*) dibutyl versions of phosphoinositides, or (*c*) lipid vesicles containing phosphoinositides. Likewise, GEF activity afforded by DH/PH fragments of intersectin and Dbs are also not altered by phosphoinositide interactions. These results strongly suggest that unless all relevant components are localized to a lipid membrane surface, Dbl family GEFs generally are not intrinsically modulated by binding phosphoinositides.

Rho GTPases cycle between inactive and active states based upon conformational alterations imposed by the state of bound guanine nucleotide. Rho GTPases bound to GDP are inactive in downstream signaling, while GTP-bound versions modulate a plethora of downstream effectors typically associated with morphological alterations of the cytoskeleton and activation of stress response genes (1–5). Consistent with their central role in regulating cellular differentiation and proliferation, constitutively active Rho GTPases are sufficient to promote cellular transformation. Similarly, Ras-induced transformation is dependent on Rac1, a Rho GTPase (6–9). Since the proper control of a multitude of signaling cascades by G proteins depends critically upon the state of bound nucleotide, G proteins have evolved several, tightly controlled processes for regulating the binding and hydrolysis of guanine nucleotides. For Rho GTPases, the exchange of bound GDP for GTP is catalyzed by a large class of guanine nucleotide exchange factors (GEFs)[1] related to the gene product for Dbl (diffuse B-cell lymphoma) (10). Similarly to constitutively active forms of Rho GTPases, the unregulated activation of Dbl family members is generally associated with cellular transformation, and many Dbl family members are proto-oncogenic (7, 11).

Dbl family proteins invariantly contain an ~300-amino acid span composed of a Dbl homology (DH) domain in tandem with a pleckstrin homology (PH) domain (12, 13). DH domains are sufficient to catalyze nucleotide exchange; however, exchange activity is often enhanced by inclusion of the adjacent PH domain (14). While DH domains serve as the major docking site for Rho GTPases, roles for the adjacent, conserved PH domains remain unclear. The invariant DH/PH domain architecture in all Dbl family members strongly suggests that associated PH domains have a unique and highly conserved role in regulating nucleotide exchange. Simple structural stabilization of the DH domain by the adjacent PH domain as suggested by the Tiam1-DH/PH·Rac1 crystal structure (15) is an unsatisfactory explanation for the universal pairing of DH and PH domains. A multitude of other domains could easily be imagined to serve this purpose.

In many other proteins, PH domains bind phosphoinositides to function as regulated tethers to cellular membranes (16–20). Although PH domains typically share very low sequence identity, all possess a common β-sandwich fold capped at one end with a C-terminal helix. Numerous studies indicate that PH domains generally bind phosphoinositides with a wide degree of affinity and specificity via clusters of basic residues located within the highly variable loops between strands β_1/β_2 and β_3/β_4 (21–23). Several reports present conflicting data describ-

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[1] The abbreviations used are: GEF, guanine nucleotide exchange factor; DH, Dbl homology; PH, pleckstrin homology; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PtdIns(3,4,5)P_3, phosphatidylinositol 3,4,5-trisphosphate; PtdIns(3,4)P_2, phosphatidylinositol 3,4-bisphosphate; PtdIns(3)P, phosphatidylinositol 3-phosphate; GST, glutathione *S*-transferase; SUV, small unilamellar vesicle; Ins(1,3,4)P_3, inositol phosphate; mant-GTP, methylanthronyl-GTP; SPR, surface plasmon resonance; ITC, isothermal titration calorimetry; PDZ, PSD-95/Dlg/ZO1.

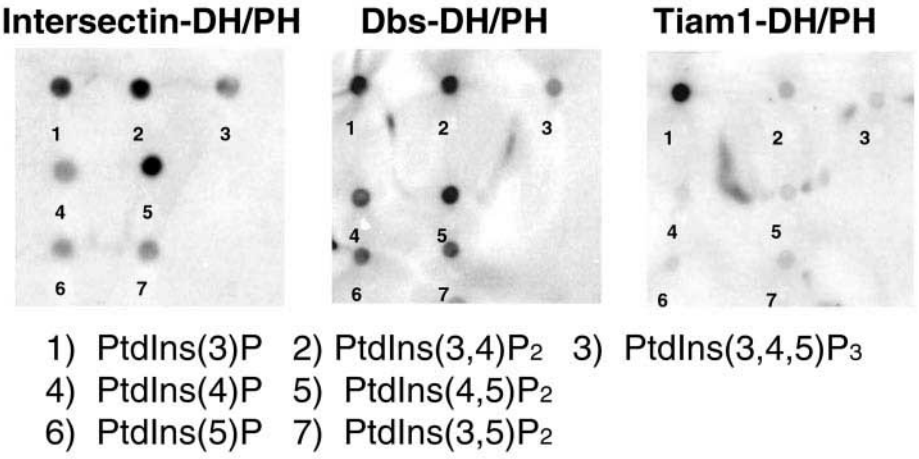

FIG. 1. **Analysis of GEF phosphoinositide binding specificity using a dot-blot screen.** Radiolabeled GST-DH/PH fusion proteins were applied to nitrocellulose filters containing various phosphoinositides. After washing, the bound protein was visualized using a PhosphorImager as described under "Experimental Procedures."

ing how phosphoinositide binding to DH-associated PH domains modulates GEF activity on Rho GTPases (24–28). Specifically, for Tiam1 acting on Rac1, one report (28) identifies phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) as an obligate activator of nucleotide exchange activity, while another report (27) describes the identical phosphoinositide as inhibitory to exchange and phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3) and phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P_2) as activators of GEF activity.

To define potential functions underpinning the invariant conservation of DH domains with PH domains, we have used several techniques to measure interactions between phosphoinositides and the DH/PH fragments of Tiam1, Dbs (Dbl's big sister), and intersectin. Here we demonstrate that phosphoinositide binding localizes to the PH domains with no appreciable binding attributable to the DH domains. Also, the PH domains possess measurable affinities only for lipid vesicles containing phosphoinositides. While the DH/PH fragments of intersectin and Dbs bind various phosphoinositides, Tiam1 preferentially binds phosphatidylinositol 3-phosphate (PtdIns(3)P) with low micromolar affinity. Among PH domains, preferential binding of PtdIns(3)P is rare and may implicate Tiam1 in events that occur on the surface of endosomal components where PtdIns(3)P is localized. In addition, and in contrast with previous studies (24–28), we find no evidence that phosphoinositides modulate the ability of Dbl family proteins to catalyze *in vitro* nucleotide exchange within soluble Rho family GTPases.

EXPERIMENTAL PROCEDURES

Protein Production—The coding regions for each construct (human intersectin-DH-(1229–1445), intersectin-DH/PH-(1229–1580), murine Dbs-DH-(623–831), Dbs-DH/PH-(623–967), and human Tiam1-DH/PH-(1033–1401)) were expressed in *Escherichia coli* strain BL21(DE3) and purified to homogeneity as previously described (7, 15).[2] The coding region for Tiam1-PSD-95/Dlg/ZO1/DH/PH (residues 858–1406) was amplified by polymerase chain reaction, subcloned into pPROEX HTA (Life Technologies, Inc.), and overexpressed as a hexahistidine fusion in *E. coli* strain BL21(DE3). Cells were lysed by French press and clarified by ultracentrifugation, and the supernatant was loaded onto a nickel-chelating Sepharose column (Amersham Pharmacia Biotech) followed by elution with an imidazole gradient. Fractions rich in Tiam1-PDZ/DH/PH were pooled and loaded onto an S-200 gel filtration column to isolate highly purified Tiam1-PDZ/DH/PH. An insect cell expression vector harboring the coding region of the last 1199 residues of Tiam1 fused to a KT3 epitope of SV40 large T antigen was kindly supplied by Onyx Pharmaceuticals and expressed in 1 liter of High Five insect cells (Invitrogen) for 48 h at 27 °C. The cells containing the overexpressed Tiam1 fragment (hereafter called Tiam1-PH/PDZ/DH/PH) were harvested, lysed by sonication, and clarified by ultracentrifugation. Visualization by Coomassie Blue staining and Western blotting with monoclonal antibody directed against the KT3 tag (Babco) verified the presence of Tiam1-PH/PDZ/DH/PH in the soluble fraction of the lysate. This supernatant was subsequently applied to a HiTrap SP ion exchange column (Amersham Pharmacia Biotech) followed by elution with a linear salt gradient. Fractions rich in Tiam1-PH/PDZ/DH/PH were pooled and concentrated for analysis. For dot-blot screens, DH/PH proteins were expressed as GST fusions (see below). Human Rac1 (residues 1–188, C188S) and Cdc42 (residues 1–188, C188S) were expressed and purified bound to GDP as previously described (15). Protein concentrations were determined by A_{280} using calculated extinction coefficients.

Dot-Blot Screens—Dot-blot assays were performed exactly as described (23). Briefly, DH/PH fragments were amplified by polymerase chain reaction and subcloned into pGEX-2TK. Expressed and purified DH/PH proteins fused to GST were radiolabeled with ^{32}P using protein kinase A and were applied to nitrocellulose filters containing various phosphoinositides prior to extensive washing and visualization of bound radioactivity using a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA). One-microliter spots of phospholipids (at 2 mg/ml) were placed on the nitrocellulose in the pattern shown in Fig. 1.

Vesicle Preparation—Phospholipids were purchased from Matreya, Inc. Small unilamellar vesicles (SUVs) were prepared by bath sonication of a dispersion of lipids into aqueous 20 mM Hepes (pH 7.5) and 150 mM NaCl. SUVs contained (by molar fraction) 80% dipalmitoyl phosphatidylcholine, 17% dipalmitoyl phosphatidylserine, 3% of a phosphohyinositide (PtdIns(3)P, PtdIns(3,4)P_2, PtdIns(4,5)P_2, or PtdIns (3,4,5)P_3), and 0.1% *N*-biotinylated dipalmitoyl phosphatidylethanolamine when indicated.

Surface Plasmon Resonance—SUV binding was monitored by surface plasmon resonance using a BIAcore 2000 instrument. A streptavidin (SA5) chip (BIAcore) was mounted in the instrument, and SUV surfaces were created in each flow cell by capturing the SUVs via biotin-streptavidin interactions. An empty flow cell was maintained to control for nonspecific binding to the carboxymethylated dextran chip. Equal amounts of SUV were immobilized on each respective flow cell as judged by increases in response units displayed on the sensorgrams. Typically, SUVs were loaded onto flow cell surfaces until 2000 response units were achieved. The surfaces were stable and did not decay significantly throughout the titrations.

Experiments were performed at 25 °C with 100 μl/min as the flow rate. Proteins were dialyzed against 20 mM Hepes (pH 7.5) and 150 mM NaCl, filtered by centrifugation, and degassed. Each surface of the biosensor was then exposed to 25-μl injections of protein solution (association phase) followed by 100 μl of buffer (dissociation phase) via the kinject command. Between injections, a 5-μl pulse of 1 M NaCl and 50 mM NaOH regenerated the SUV surfaces. Each GEF was injected at concentrations ranging from 0 to 50 μM.

Raw sensorgrams from each titration were aligned, and the signal due to binding the empty flow cell was subtracted from each curve. The data were then globally fit to a 1:1 Langmuir binding isotherm using BIAevaluation 3.0 software (29). The resulting dissociation constants (K_D) were obtained from the average of several experiments. Representative titrations are plotted as the steady state binding response (R_{eq}) over the range of GEF concentrations.

Isothermal Titration Calorimetry—Inositol phosphates were pur-

[2] W. Pruitt, M. Baumeister, K, Rossman, M. Lemmon, J. Sondek, B. Kay, and C. Der, submitted for publication.

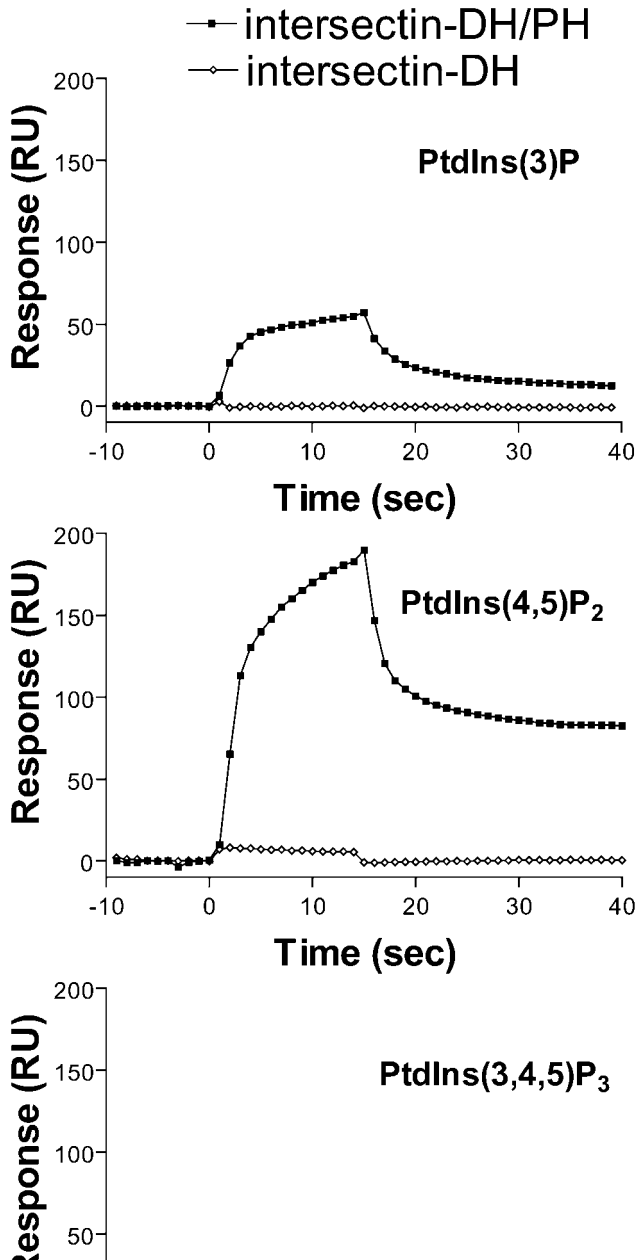

FIG. 2. **Binding of intersectin-DH/PH protein to phosphoinositide-containing vesicles by surface plasmon resonance.** GEF binding to phosphoinositides was analyzed using SPR in a BIAcore 2000. Intersectin-DH and intersectin-DH/PH solutions (50 μM) were injected over immobilized SUV surfaces (3% of indicated phosphoinositide, 17% dipalmitoyl phosphatidylserine, 80% dipalmitoyl phosphatidylcholine, 0.1% *N*-biotinylated dipalmitoyl phosphatidylethanolamine by molar fraction) for 15 s (association phase), and then flow was switched to buffer alone (dissociation phase). Binding was monitored by an increase in response units (*RU*) as a function of time. Raw data were normalized to the signal achieved from nonspecific binding to an empty flow cell surface.

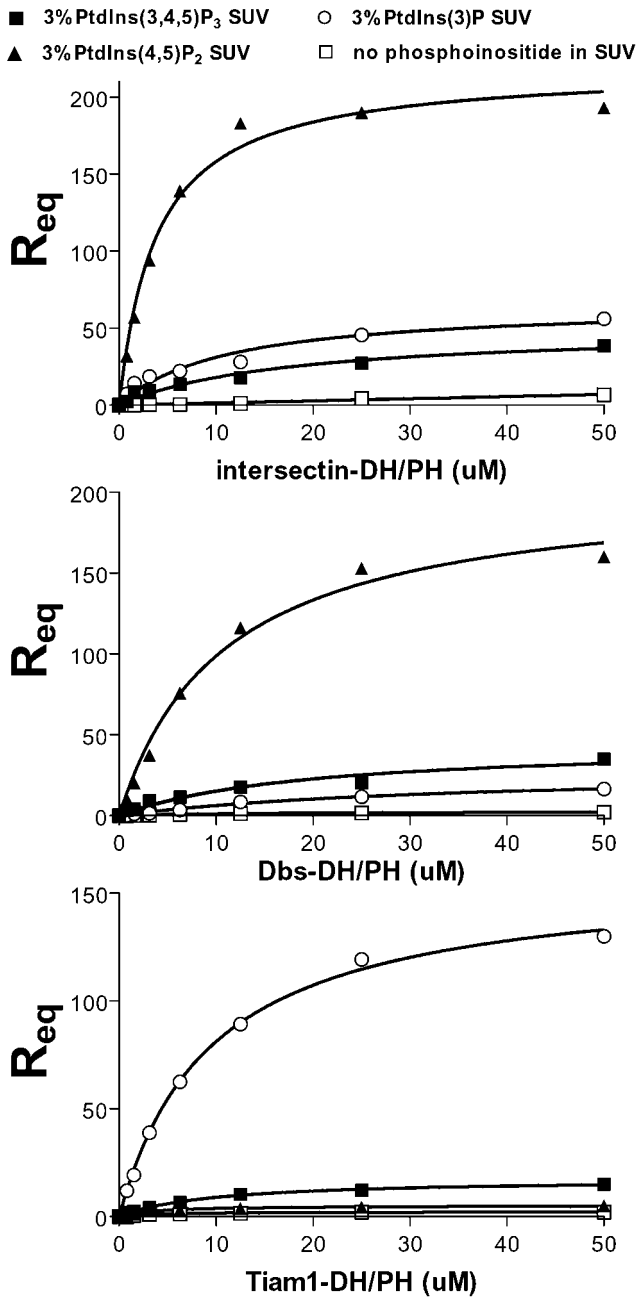

FIG. 3. **Differential specificity of intersectin, Dbs, and Tiam1 DH/PH fragments for phosphoinositide-containing SUVs.** GEF solutions (0, 0.8, 1.5, 3.1, 6.2, 12.5, 25, and 50 μM) were injected over various SUV sensor surfaces in the same manner as depicted in Fig. 2. The sensorgrams were aligned and globally fit to a 1:1 binding model using BIAevaluation software as described under "Experimental Procedures." The resulting binding curves are presented as the steady state binding response (R_{eq}) as a function of GEF concentration. Estimated K_D values from the best fit curves are presented in Table I.

chased from Echelon or Calbiochem. Each D-*myo*-inositol phosphate ligand (inositol 1,3-bisphosphate ($Ins(1,3)P_2$), D-*myo*-inositol 1,3,4-trisphosphate, D-*myo*-inositol 1,3,5-trisphosphate, D-*myo*-inositol 1,4,5-trisphosphate, D-*myo*-inositol 1,3,4,5-tetrakisphosphate, and D-*myo*-inositol 1,2,3,4,5,6-hexakisphosphate) was dissolved in isothermal titration calorimetry (ITC) buffer (20 mM phosphate, pH 7.5, 150 mM NaCl), and their concentrations were based on the amount stated by the supplier. Protein solutions were dialyzed exhaustively against ITC buffer, filtered, and degassed before each injection. Titrations were performed at 20 °C using an Omega MCS calorimeter (MicroCal, Inc.). Twenty 15-μl injections or 30 10-μl injections of each inositol phosphate (260–360 μM) were titrated into a GEF solution (26–36 μM) in the calorimeter cell (1.39 ml). Data were analyzed by integrating the peaks with Origin 5.0 software. Heats of dilution were subtracted from heats of binding, and the data were fit to a one-binding site model (30).

Nucleotide Exchange Assay—Guanine nucleotide exchange assays were performed as previously described in 2-ml reactions (15). Briefly,

nucleotide exchange was monitored as the increase in relative fluorescence of the GTP analog mant-GTP upon binding G protein in a reaction buffer containing 20 mM Tris (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 50 μg/ml bovine serum albumin, and 10% glycerol. Prior to the addition of GEF, a 1 μM concentration of the appropriate G protein was incubated with 200 nM mant-GTP at 20 °C in a thermostatted cuvette, and fluorescence was measured using a PerkinElmer Life Sciences LS-50B (λ_{ex} = 360 nm; λ_{em} = 440 nm; slits = 5/5 nm) (31, 32). After equilibration, 10 nM GEF or buffer (uncatalyzed trace) was added.

RESULTS

Dot-Blot Screens—In order to rapidly screen Dbl PH domains for phosphoinositide affinity, we utilized an established dot-blot assay (23). This qualitative method has been used to identify PH domain-phosphoinositide interactions, and results typically correlate well with other modes of binding analysis. The DH/PH regions from intersectin, Dbs, and Tiam1 fused to GST were expressed, purified, and radiolabeled with protein kinase A as previously described (23). These ^{32}P-labeled GST-DH/PH proteins were next applied to nitrocellulose filters that were spotted previously with various phosphoinositides (see "Experimental Procedures"). As shown in Fig. 1, intersectin and Dbs recognized several phosphoinositides, while Tiam1 specifically interacted with only PtdIns(3)P of the panel of phosphoinositides screened. The GST protein alone did not display significant signal for any lipid (data not shown). This differential phosphoinositide selectivity observed for these GEFs using the dot-blot assay prompted us to further evaluate these interactions using more quantitative measurements.

TABLE I
SPR results for DH/PH fragments binding to phosphoinositide-containing SUVs

K_D values (μM) were estimated from best fits to the SPR data presented in Fig. 3. Values reported with a greater than sign reflect lack of saturable binding for the highest concentration of GEF (50 μM) examined. Each dissociation constant is the mean of several experiments with the errors cited as S.D.

SUV	Intersectin-DH/PH	Dbs-DH/PH	Tiam1-DH/PH
3% PtdIns(3,4,5)P_3	> 50	> 50	> 50
3% PtdIns(4,5)P_2	4.2 ± 2	11 ± 6	> 50
3% PtdIns(3)P	> 50	> 50	10 ± 7

Surface Plasmon Resonance—Given the ability of the DH/PH fragments of Tiam1, Dbs, and intersectin to bind phosphoinositides on a dot-blot screen, we sought to assess these protein-lipid interactions in a more physiological situation. SUVs composed of 3% molar fraction phosphoinositide were immobilized on a streptavidin SA chip (BIAcore) in a BIAcore 2000 instrument via biotinylated phosphatidylethanolamine (33). Solutions of highly purified, untagged, catalytically active DH and DH/PH fragments (see "Experimental Procedures") were injected over SUV surfaces followed by buffer to promote dissociation. Binding was monitored in real time using surface plasmon resonance (SPR). Fig. 2 displays representative sensorgrams of intersectin-DH and intersectin-DH/PH applied to flow cells coated with PtdIns(3)P-, PtdIns(4,5)P_2-, and PtdIns(3,4,5)P_3-containing vesicles respectively. The DH domain of intersectin or that of Dbs (data not shown) displayed no measurable affinity for any lipid surface tested, while extended fragments of intersectin or Dbs containing the tandem DH and PH domains possessed significant affinity for SUVs doped with phosphoinositides. This differential binding between DH and DH/PH domains implicates the PH domain of

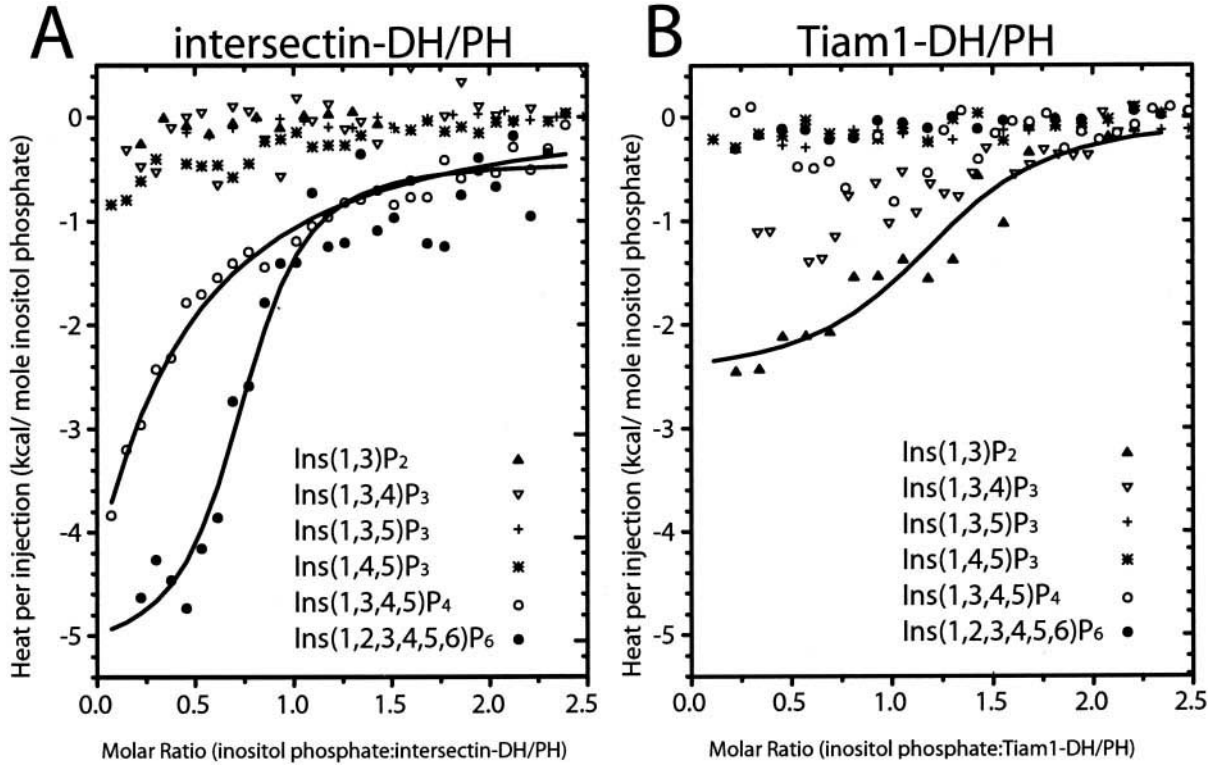

FIG. 4. **Titration calorimetry of inositol phosphates binding to intersectin-DH/PH and Tiam1-DH/PH.** Various inositol phosphates were titrated into solutions of intersectin-DH/PH (*A*) and Tiam1-DH/PH (*B*) in a calorimeter as described under "Experimental Procedures." The heat evolved per injection is displayed as a function of molar ratio (inositol phosphate/GEF). Curves of best fit are shown for titrations displaying detectable binding. For intersectin-DH/PH (*A*), the dissociation constants from the D-*myo*-inositol 1,2,3,4,5,6-hexakisphosphate (*Ins(1,2,3,4,5,6)P_6*) and D-*myo*-inositol 1,3,4,5-tetrakisphosphate (*Ins(1,3,4,5)P_4*) titrations displayed are 0.8 and 10 μM, respectively. For Tiam1-DH/PH (*B*), the estimated K_D value for the Ins(1,3)P_2 titration displayed is 5 μM. Due to limitations in protein quantity, Dbs-DH/PH was not evaluated by calorimetry.

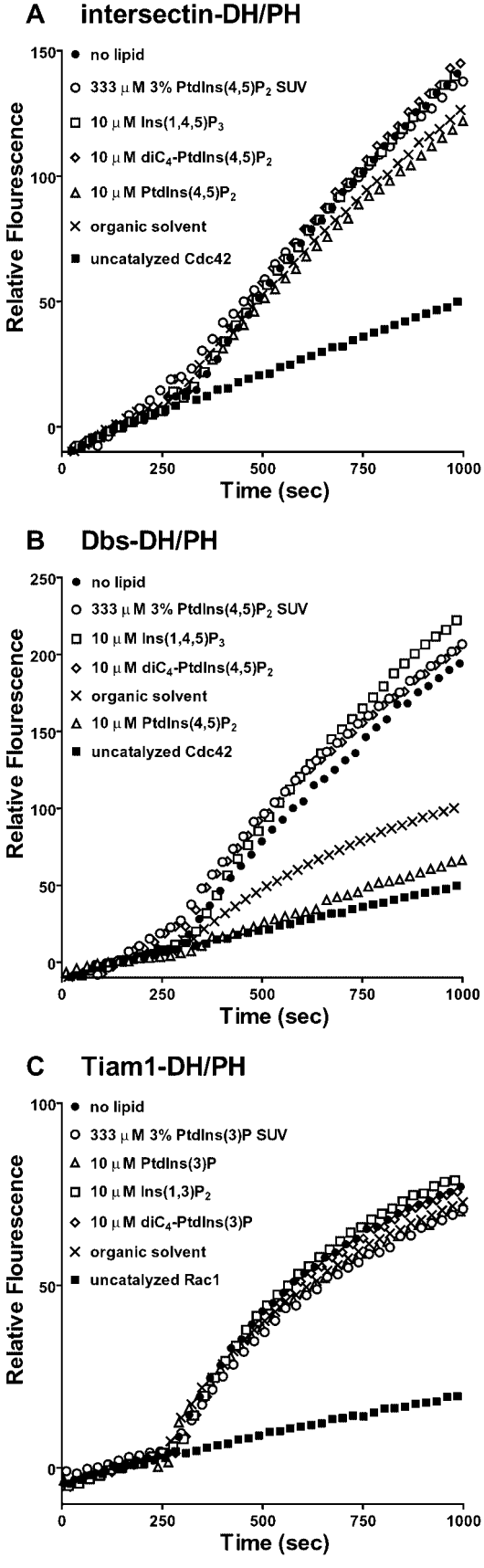

FIG. 5. **Effect of phosphoinositides on DH/PH-catalyzed nucleotide exchange on Cdc42 and Rac1.** Guanine nucleotide exchange of GDP was monitored *in vitro* by fluorescence of mant-GTP as described under "Experimental Procedures." One micromolar of the appropriate G protein substrate (for intersectin-Cdc42 (*A*), for Dbs-Cdc42 (*B*), and for Tiam1-Rac1 (*C*)) was incubated with lipid additives prior to the addition of 10 nM GEF-DH/PH or buffer for the uncatalyzed trace (■). For intersectin-DH/PH (*A*) and Dbs-DH/PH (*B*), reactions contained 10 μM D-*myo*-inositol 1,4,5-trisphosphate (*Ins(1,4,5)P3*) (□),10 μM dibutyl-PtdIns(4,5)P_2 (◇), 333 μM 3% PtdIns(4,5)P_2 SUVs (○), no lipids (●), 22 μl of 1:1 $CHCl_3/CH_3OH$ solvent (×), or 10 μM PtdIns(4,5)P_2 in 22 μl of 1:1 $CHCl_3/CH_3OH$ solvent (△). For Tiam1-DH/PH (C), reactions contained similar additives with the exception of 10 μM Ins(1,3)P_2 (○),10 μM dibutyl-PtdIns(3)P (◇), 333 μM of 3% PtdIns(3)P SUVs (○), and 10 μM PtdIns(3)P in 20 μl of 1:1 $CHCl_3/CH_3OH$ solvent (△).

Dbl family GEFs in mediating phosphoinositide-dependent membrane localization.

We measured GEF binding to SUVs over a range of DH/PH concentrations to obtain quantitative binding constants. Dissociation constants for each GEF-SUV binding curve were estimated by globally fitting the sensorgrams to a 1:1 Langmuir binding model, and resulting binding isotherms are presented as the steady state binding signal as a function of GEF concentration in Fig. 3. All GEFs displayed little affinity for vesicles that lack phosphoinositides (80% dipalmitoyl phosphatidylcholine, 20% dipalmitoyl phosphatidylserine, and trace amounts of *N*-biotinylated dipalmitoyl phosphatidylethanolamine (0.1% molar fraction)). In contrast, intersectin-DH/PH and Dbs-DH/PH bound with micromolar range K_D values to vesicles containing phosphoinositides, with higher affinity for PtdIns(4,5)P_2 relative to PtdIns(3,4,5)P_3 and PtdIns(3)P. Tiam1-DH/PH showed significant specificity for SUVs containing PtdIns(3)P, consistent with the dot-blot results, but did not bind significantly to PtdIns(4,5)P_2 or PtdIns(3,4,5)P_3. The estimated dissociation constants for these titrations are summarized in Table I and correlate very well with the pattern of binding observed using the dot-blot assay.

Titration Calorimetry—To investigate the selectivity of these PH domains for the head groups of phosphoinositides in solution, ITC was used to measure heats of binding upon titration of individual inositol phosphates into solutions of GEFs (see Fig. 4). The small amount of heat released during the titrations strongly limits our ability to discern specificities between PH domains and phosphoinositides. However, consistent with the other methods of analysis, Tiam1-DH/PH displayed the greatest affinity for Ins(1,3)P_2, the head group of PtdIns(3)P, and consistently bound with an approximate K_D of 5 μM, which is similar to the K_D of 10 μM measured by SPR for the interaction of Tiam1-DH/PH and SUVs doped with PtdIns(3)P. The only other inositol phosphate releasing any measurable amount of heat upon binding Tiam1-DH/PH was Ins(1,3,4)P_3. Intersectin-DH/PH exhibited increasing affinity for inositol phosphates with an increasing number of phosphate groups (negative charge). This trend for intersectin argues that, at least in bulk solution, in the absence of negative phospholipid membranes, intersectin favors phosphoinositide head groups possessing a large degree of localized negative charge, with little stereospecificity.

Nucleotide Exchange Assay—The combination of our detection of selective phosphoinositide affinity by DH-associated PH domains, coupled with previous reports of phosphoinositide-induced modulation of GEF catalysis in solution, prompted us to evaluate the effects of phosphoinositides on guanine nucleotide exchange catalyzed by the DH/PH portions of intersectin, Dbs, and Tiam1 (see Fig. 5) (34). Consistent with previous work (14, 15),[2] the DH/PH portions of all three Dbl family GEFs efficiently catalyze the exchange of bound GDP for GTP within the appropriate G protein substrates as compared with reactions lacking GEF (uncatalyzed reactions). However, the addition of 333 μM SUVs containing 3% PtdIns(4,5)P_2, 10 μM PtdIns(4,5)P_2, water-soluble 10 μM dibutyl-PtdIns(4,5)P_2, or 10 μM Ins(1,4,5)P_3, the corresponding soluble head group, did not

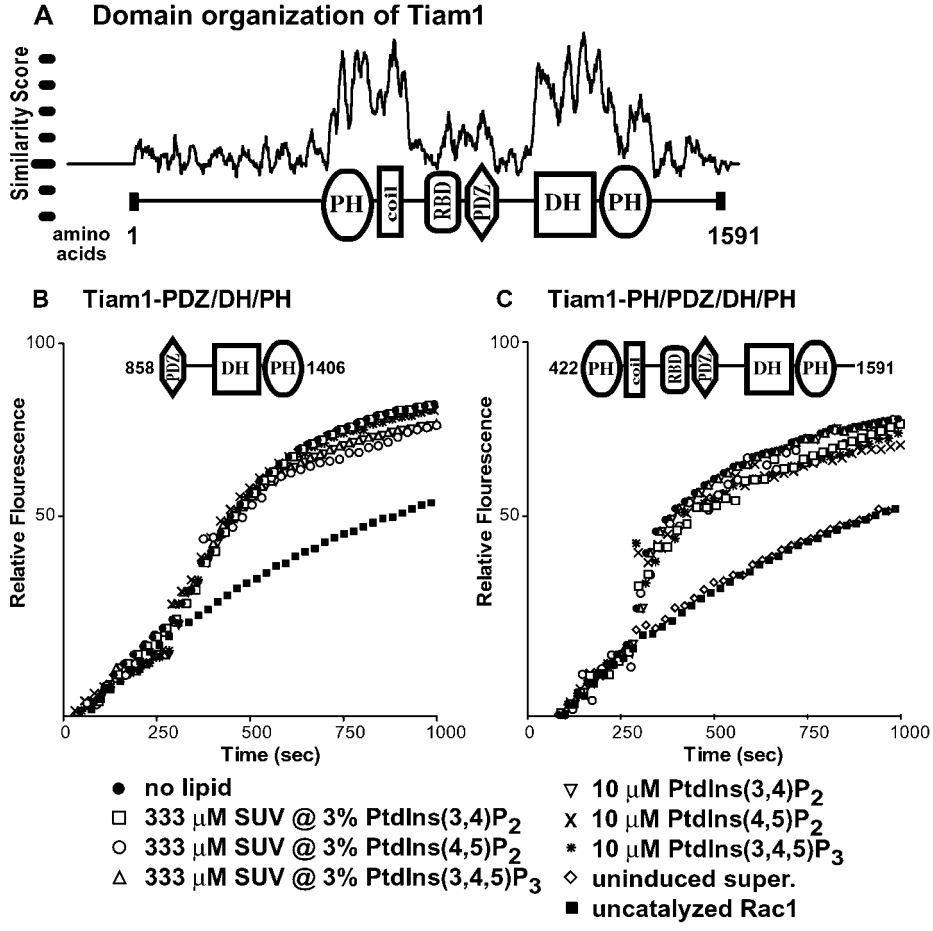

FIG. 6. **Effect of phosphoinositides on Tiam1-catalyzed nucleotide exchange on Rac1.** Full-length sequences of Tiam1, Stef, and Still-life were aligned using ClustalX and analyzed for regions of high similarity using Plotsimilarity in GCG (53, 54). The domain organization of Tiam1 is displayed under corresponding regions of a similarity plot *A*, labeled domains include a region predicted to form a coiled-coil (*coil*) and a putative Ras binding domain (*RBD*). Guanine nucleotide exchange assays were performed exactly as described in the legend to Fig. 5. For Tiam1-PDZ/DH/PH (*B*), and Tiam1-PH/PDZ/DH/PH (*C*), reactions contained 333 μM 3% PtdIns(3,4)P_2 SUVs (□), 333 μM 3% PtdIns(4,5)P_2 SUVs (○), 333 μM 3% PtdIns(3,4,5)P_3 SUVs (△), 10 μM PtdIns(3,4)P_2 (×), 10 μM PtdIns(4,5)P_2 (*), or 10 μM PtdIns(3,4,5)P_3 (*inverted triangle*). A reaction containing the equivalent amount of soluble lysate from uninduced cells in place of purified Tiam1 is also displayed (◇).

significantly affect catalysis by intersectin-DH/PH (Fig. 5*A*) on Cdc42. Similar results were obtained using SUVs containing 3% PtdIns(3,4,5)P_3, (data not shown). In contrast, the addition of 10 μM PtdIns(4,5)P_2 dramatically decreased the rate of Dbs-catalyzed nucleotide exchange on Cdc42 (Fig. 5*B*). However, this inhibition was also observed upon the addition of equivalent amounts of the neat organic solvent required to solubilize PtdIns(4,5)P_2 in the absence of SUVs. In a similar fashion, SUVs with added PtdIns(3)P, 10 μM dibutyl-PtdIns(3)P, or 10 μM Ins(1,3)P_2 had no effect on nucleotide exchange catalyzed by Tiam1-DH/PH operating on Rac1. Therefore, under various conditions, phosphoinositides fail to alter guanine nucleotide exchange within nonprenylated forms of Rac1 or Cdc42 catalyzed by three distinct Dbl family GEFs.

Like most Dbl family GEFs, Tiam1 contains multiple domains aside from the signature DH/PH region that may influence exchange activity (Fig. 6*A*). Consequently, two larger fragments of Tiam1 were analyzed for exchange activity potentially regulated by phosphoinositides. A 73-kDa segment of Tiam1 spanning the PDZ domain through the DH/PH region catalyzes guanine nucleotide exchange within Rac1 similar to the smaller DH/PH fragment (Fig. 6*B*). Furthermore, similar to our previous observations, the exchange activity of Tiam1-PDZ/DH/PH is not modulated by phosphoinositides under a variety of conditions (Fig. 6*B*). Similarly, a 135-kDa fragment of Tiam1 produced in baculovirus and spanning the C-terminal PH domain through the N-terminal PH domain (28) also efficiently catalyzes nucleotide exchange within Rac1 but not Cdc42 that cannot be altered by the additions of various phosphoinositides (Fig. 6*C* and data not shown). The catalyzed exchange cannot be attributed to exogenous lipids or other contaminants, since soluble lysate derived from uninfected Sf9 cells imparts no exchange activity to Rac1 above spontaneous rates (Fig. 6*C*). Apparently in solution, the minimal DH/PH fragment of Tiam1 accurately recapitulates the exchange activity of extended portions of Tiam1, and potential *in vitro* regulation of exchange activity by phosphoinositides is not imparted by other domains of Tiam1 or the original source of heterologous expression.

DISCUSSION

The strict conservation of PH domains adjacent to DH domains in Dbl family members strongly suggests that these PH domains share a highly conserved function essential to the intrinsic mechanism of guanine nucleotide exchange catalyzed by Dbl family proteins. In order to elucidate potential functions for these PH domains, the DH/PH portions of intersectin, Dbs, and Tiam1 were functionally characterized for selectivity and affinity toward a series of phosphoinositides under a variety of experimental conditions. PH domains generally bind phosphoinositides, and consistent with this observation, the DH/PH fragments of intersectin, Dbs, and Tiam1 preferentially bound phosphoinositides relative to other anionic membrane lipids (*i.e.* dipalmitoyl phosphatidylserine). The DH/PH fragment of Dbs promiscuously binds phosphoinositides, showing little selectivity between various phosphoinositides blotted on nitrocellulose membranes. The intersectin DH/PH portion appears a little more selective, preferring PtdIns(4,5)P_2. When phosphoinositides are presented within a background of anionic SUVs and affinities are measured by SPR, the DH/PH fragments of both Dbs and intersectin bind PtdIns(4,5)P_2, with K_D values of 11 and 4 μM, respectively, and both were able to discriminate PtdIns(4,5)P_2 from PtdIns(3,4,5)P_3 and PtdIns(3)P. These results emphasize the importance of accounting for the physical format used to present phosphoinositides to PH domains.

In contrast, using three highly complementary approaches, Tiam1-DH/PH specifically recognized PtdIns(3)P but did not bind significantly to either PtdIns(4,5)P_2 or PtdIns(3,4,5)P_3. PtdIns(3)P is required for proper localization of regulators of endocytic membrane fusion through specific interactions with FYVE domains (35–40) and is not normally associated with PH domain function. To date, very few PH domains have been implicated in selectively binding PtdIns(3)P, and these past studies have relied mainly on qualitative dot-blot analyses (41, 42). Unfortunately, the C-terminal PH domain of Tiam1 is lacking traditionally defined consensus sequences normally associated with binding the 3′-phosphate of inositol phosphates. Also, within the Tiam1-DH/PH·Rac1 crystal structure, the β_1/β_2 and β_3/β_4 loops of the PH domain are unordered, making it difficult to assign residues potentially involved in ligating PtdIns(3)P. Aside from the DH-associated PH domain, Tiam1 contains a second N-terminal PH domain that has been reported to interact specifically with PtdIns(3,4,5)P_3, and both N- and C-terminal PH domains have been implicated in recruiting Tiam1 to the plasma membrane *in vivo* (43–45). Therefore, Tiam1 may use its two PH domains to sense distinct pools of intracellular phosphoinositides, resulting in pleiotropic responses at different cellular locations, depending on the levels of PtdIns(3,4,5)P_3 and PtdIns(3)P.

The low micromolar affinities of the PH domains of intersectin and Dbs for PtdIns(4,5)P_2, as well as Tiam1 for PtdIns(3)P, are consistent with the dissociation constants commonly reported for the majority of PH domains interacting with phosphoinositides (19, 46). These weak interactions appear to be insufficient to drive directly the binding of proteins to membranes, and it is thought that secondary sites of membrane attachment are required to effectively localize proteins to membrane surfaces (19). Consistent with this notion, the DH-associated PH domains of Tiam1, Dbl, Vav, and Sos1 lack critical structural determinants required for high affinity phosphoinositide binding as highlighted by several crystal structures of PH domains in complex with inositol phosphates (47, 48) and complementary functional analyses (49). For Dbl family GEFs, interactions between membrane-resident GTPases and DH domains, coupled with interactions between specific phosphoinositides and PH domains, mostly likely serves to enhance membrane affinity of Dbl family proteins while simultaneously ensuring the proper subcellular localization and fidelity of Dbl family proteins for specific G proteins.

While we have quantitatively defined the specific phosphoinositides that bind the DH-associated PH domains of intersectin, Dbs, and Tiam1, we have been unable to observe any allosteric regulation of exchange activity mediated by concentrations of phosphoinositides sufficient to occupy fully the PH domains adjacent to DH domains. Recent reports (27, 28) present conflicting data describing the *in vitro* regulation of Tiam1-catalyzed exchange on nonprenylated Rac1 mediated by phosphoinositides. In one instance (27), both PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 modestly enhanced (~2-fold) guanine nucleotide exchange mediated by Tiam1, while PtdIns(4,5)P_2 reduced GDP release, and phosphoinositide regulation was attributed to the N-terminal PH domain. Conversely, a second report (28) finds Tiam1-catalyzed exchange on Rac1 robustly activated (~5–6-fold) by PtdIns(4,5)P_2 or PtdIns(4)P interacting through the C-terminal PH domain. To address these discrepancies, we demonstrated that similar to the DH/PH and PDZ/DH/PH elements of Tiam1, the identical Tiam1 construct (28) used in previous studies (Tiam1-PH/PDZ/DH/PH) effectively catalyzes release of GDP on Rac1 under a variety of experimental conditions yet is not modulated by phosphoinositides.

There are several experimental aspects that must be considered when attempting to measure guanine nucleotide exchange potentially modified by phosphoinositides. For instance, guanine nucleotide exchange is traditionally evaluated using filtration-based assays requiring separation of bound *versus* free nucleotides, and previous relevant reports have used solid-phase separation techniques to monitor exchange at single, arbitrary time points (24, 25, 27, 28). Unfortunately, physical separation of solution components introduces the potential to perturb equilibrium conditions (50, 51). In contrast, the studies described here rely upon the continuous, real time analysis of exchange activity using fluorescence spectroscopy and do not require altering the solution conditions in order to measure exchange. Furthermore, small amounts of contaminating organic solvents normally required to solubilize phosphoinositides can dramatically affect guanine nucleotide exchange, presumably by destabilizing GEFs (see Fig. 5*B*). Reduced exchange activity would then be attributed inappropriately to the added phosphoinositides as opposed to the vehicle solvent.

Nevertheless, the invariant association of DH domains with C-terminal PH domains strongly suggests a strictly conserved functional requirement that requires a reasonable explanation. Previously determined crystal structures of PH domains bound to inositol phosphates reveal that conformational alterations induced upon complex formation are localized strictly to the β_1/β_2 and β_3/β_4 loops of the PH domains (21, 47, 48). In the recent crystal structure of a DH/PH fragment of Tiam1 bound to nucleotide-depleted Rac1 (15), the PH domain fails to engage any part of Rac1, and it is difficult to imagine how phosphoinositide binding to the PH domain allosterically influences DH domain-mediated exchange in the absence of a membrane surface. However, the physical nature of a two-dimensional surface may introduce steric constraints on the ability of DH domains linked to PH domains to engage membrane-resident GTPases. The structural consequences of these membrane-induced steric constraints may mediate a conserved functional linkage between DH and PH domains. For example, the structures of the DH/PH element of Sos1 (52) as well as the complex of Tiam1-DH/PH bound to Rac1 (15) and Dbs-DH/PH bound to Cdc42[3] indicate a large degree of conformational heterogeneity arising within the loops of the PH domain as wells as between the PH domains with respect to the DH domains. It seems plausible that the simultaneous engagement of GTPases and phosphoinositides by the DH and PH domains, respectively, may serve not only to regulate the intracellular localization of the GEFs but may also dictate (*a*) the proper orientation of the PH domains relative to the DH domains and (*b*) specific loop conformations within PH domains that affect regulation of exchange activity by as yet unclear molecular mechanisms. Undoubtedly, it seems clear that future experiments using prenylated G proteins resident in lipid vesicles and containing specific phosphoinositides will be necessary to dissect the molecular and functional details dictating the invariant conservation of PH domains adjacent to DH domains.

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