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Multifunctional Roles for the PH Domain of Dbs in Regulating Rho GTPase Activation*

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Dbl family members are guanine nucleotide exchange factors specific for Rho guanosine triphosphatases (GTPases) and invariably possess tandem Dbl (DH) and pleckstrin homology (PH) domains. Dbs, a Dbl family member specific for Cdc42 and RhoA, exhibits transforming activity when overexpressed in NIH 3T3 mouse fibroblasts. In this study, the PH domain of Dbs was mutated to impair selectively either guanine nucleotide exchange or phosphoinositide binding *in vitro* and resulting physiological alterations were assessed. As anticipated, substitution of residues within the PH domain of Dbs integral to the interface with GTPases reduced nucleotide exchange and eliminated the ability of Dbs to transform NIH 3T3 cells. More interestingly, substitutions within the PH domain that prevent interaction with phosphoinositides yet do not alter *in vitro* activation of GTPases also do not transform NIH 3T3 cell and fail to activate RhoA *in vivo* despite proper subcellular localization. Therefore, the PH domain of Dbs serves multiple roles in the activation of GTPases and cannot be viewed as a simple membrane-anchoring device. In particular, the data suggest that binding of phosphoinositides to the PH domain within the context of membrane surfaces may direct orientations or conformations of the linked DH and PH domains to regulate GTPases activation.

The Rho family GTPases[1] are an essential subset of the Ras superfamily of small molecular weight GTPases. Like Ras, Rho family GTPases cycle between GDP- and GTP-bound forms. When GDP-bound, Rho proteins are inactive and do not functionally couple to their downstream effectors. However, when GTP-bound, Rho GTPases elicit profound effects on the organization of the actin cytoskeleton, in addition to tightly regulating the activation state of transcription factors such as the serum response factor, c-Jun, and NF-κB (1–3). GDP/GTP cycling within Rho proteins is primarily accomplished through the actions of two classes of regulatory proteins. GTPase activating proteins promote the inactive, GDP-bound form of Rho proteins by enhancing their intrinsic GTPase ability to convert bound GTP to GDP. In contrast, the actions of guanine nucleotide exchange factors (GEFs) upon Rho GTPases results in Rho activation by exchanging their bound GDP for GTP.

Members of the Dbl family of oncoproteins act as GEFs exclusively for Rho GTPases (RhoGEFs) and, like constitutively activated members of Rho GTPases, can exhibit potent transformation potential within various cell types upon overexpression or constitutive activation (4–6). Dbl family members invariably contain a Dbl homology (DH) domain in tandem with an adjacent, carboxyl-terminal pleckstrin homology (PH) domain. However, outside of this region, Dbl-related proteins share little sequence conservation and typically possess a variety of protein-signaling domains, presumably reflecting diversity in regulation and cellular function.

The invariant positioning of PH domains immediately carboxyl-terminal to DH domains strongly implies a unique functional coupling. As demonstrated by several complementary studies, DH-associated PH domains are essential components of Dbl family activation of Rho family GTPases. Truncation of all or part of the PH domains of Dbl (7), Dbs (8), Lfc (9), and Lsc (10) results in the complete loss of cellular transformation. Replacing the PH domains of Lfc and Dbs with a membrane-targeting sequence restores at least partial transforming activity, indicating a role in membrane targeting for these PH domains, presumably through interaction with phosphoinositides, *i.e.* low affinity (micromolar K_D), nonspecific ligands for the vast majority of PH domains (11). In addition to possibly mediating the translocation of Dbl family proteins to cellular membranes, phosphoinositides may also potentiate or suppress the exchange activity of some Dbl family proteins in solution (12–14); however, this does not appear to be a general mechanism of regulation (15). Moreover, recent evidence indicates that PH domains associated with DH domains can accelerate the catalytic exchange of nucleotides on Rho family GTPases independent of phosphoinositides. For example, a DH/PH fragment of Trio catalyzes nucleotide exchange within Rac ~200-fold better than the isolated DH domain (16).

The PH domain of Dbs makes direct contacts to Cdc42, and these interactions are necessary for efficient guanine nucleotide exchange *in vitro* (17). In this study, we define functional

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[1] The abbreviations used are: GTPase, guanosine triphosphatase; Dbl, diffuse B-cell lymphoma; Dbs, the big sister of Dbl; DH, Dbl homology; ELISA, enzyme-linked immunosorbent assay; GEF, guanine nucleotide exchange factor; GST, glutathione *S*-transferase; HA, hemagglutinin; $Ins(4,5)P_2$, inositol 4,5-bisphosphate; Lfc, the first cousin of LBC; Lsc, the second cousin of LBC; mant, *N*-methylanthraniloyl; PBS, phosphate-buffered saline; PH, pleckstrin homology; $PtdIns(4,5)P_2$, phosphatidylinositol 4,5-bisphosphate; SUVs, small unilamellar vesicles; Tiam1, T-cell lymphoma invasion and metastasis 1; Tris, tris(hydroxymethyl)aminomethane; wt, wild-type; GFP, green fluorescent protein; EGFP, enhanced GFP; BSA, bovine serum albumin.

roles for the PH domain of Dbs in directly mediating cellular transformation associated with downstream signaling events, especially the *in vivo* activation of GTPases. We found that mutations within the PH domain of Dbs, previously shown to be necessary for exchange *in vitro*, completely eliminated the ability of Dbs to transform NIH 3T3 cells. More interestingly, mutations within the PH domain that prevent binding of phosphoinositides, but not *in vitro* nucleotide exchange by Dbs, also prevent Dbs from activating RhoA *in vivo* and do not allow the transformation of NIH 3T3 cells by a normally highly oncogenic form of Dbs. These effects were not caused by the mislocalization of Dbs, because none of the mutations affected subcellular targeting as assessed by cellular fractionations and indirect immunofluorescence. Therefore, the PH domain of Dbs must be functioning in some capacity other than a simple membrane anchor dependent upon binding phosphoinositides to alter the gross subcellular distribution of Dbs leading to GTPase activation. Indeed, the data are more consistent with phosphoinositide binding being required to direct orientation or conformations of the invariantly associated DH and PH domains with respect to cellular membranes and membrane-resident GTPases for regulated guanine nucleotide exchange.

EXPERIMENTAL PROCEDURES

Molecular Constructs—pAX142-*dbs*-HA6 and pCTV3H-*dbs*-HA6 contain cDNAs that encode fragments of murine Dbs fused to an HA epitope tag and include the Dbs DH/PH domain along with amino- and carboxyl-terminal flanking regions (residues 525–1097) (18). pCTV3H-*dbs*-HA8 encodes an HA epitope-tagged Dbs DH domain (residues 525–833), whereas pCTV3P-dbs-HA7 encodes the Dbs DH domain fused to the plasma membrane-targeting sequence (GCMSCKCVLS) of H-Ras (Dbs DH plus C*AAX*).

GST-C21 contains the Rho·GTP binding domain of Rhotekin (19). pEGFP (Clontech) used for immunofluorescence studies contains GFP under the control of a cytomegalovirus promoter.

The Dbs DH/PH domain (murine, residues 623–967), fused to a carboxyl-terminal hexa-histidine tag, was expressed in *Escherichia coli* from pET-28a (Novagen) (17). A pET-21a (Novagen) expression construct was used to bacterially express human placental Cdc42 (residues 1–188, C188S) (17). Human RhoA (residues 1–190, C190S), fused to an amino-terminal hexa-histidine tag, was expressed from pProEX-HT (Invitrogen). The PH domain of PLC-δ_1 (residues 11–140), fused to an amino-terminal hexa-histidine tag, was also expressed from the pProEX-HT vector for bacterial expression. Site-directed substitutions placed within all Dbs DH/PH domains and Cdc42 expression constructs were prepared using the QuikChange site-directed mutagenesis kit (Stratagene) as per the manufacturer's instructions and were verified by automated sequencing.

Protein Expression and Purification—Protein expression in stably transfected NIH 3T3 cells was determined by Western blot analysis as described previously (9). Protein was visualized with Enhanced Chemiluminescence reagents (Amersham Biosciences). Protein expression and purification of Dbs DH/PH domains, Cdc42(C188S) and RhoA(C190S) from *E. coli*, were performed as described previously (17, 20).

Guanine Nucleotide Exchange Assays—Fluorescence spectroscopic analysis of *N*-methylanthraniloyl (mant)-GTP incorporation into bacterially purified Cdc42 and RhoA was carried out using a PerkinElmer Life Sciences LS 50B spectrometer at 25 °C. Exchange reaction assay mixtures, containing 20 mM Tris, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, and 100 μM mant-GTP (BIOMOL), and either 1 or 2 μM (as indicated) of either Cdc42 or RhoA protein, were prepared and allowed to equilibrate with continuous stirring. After equilibration, Dbs DH/PH domain proteins were added at 200 nM, and the rates of nucleotide loading (k_{obs}) of Rho GTPases were determined by monitoring the decrease in Cdc42 or RhoA tryptophan fluorescence (λ_{ex} = 295 nm, λ_{em} = 335 nm) in response to binding mant-GTP. The rates (k_{obs}) of guanine nucleotide exchange were determined by fitting the data as single-exponential decays utilizing the program GraphPad Prism. Data were normalized to wild-type curves to yield the percent GDP released.

Cell Culture, Transfection, and Transformation Assays—NIH 3T3 cells were maintained in Dulbecco's modified Eagle medium (high glucose) supplemented with 10% bovine calf serum (NIH 3T3, JRH, Lenexa, KS). Primary focus formation assays were performed in NIH 3T3 cells exactly as described previously (21). Briefly, NIH 3T3 cells were transfected by calcium phosphate coprecipitation in conjunction with a glycerol shock. Focus formation was scored at 14 days. NIH 3T3 cells that stably express pCTV3H, pCTV3H-*dbs*-HA6, or the pCTV3H versions of Dbs mutants were generated by calcium phosphate coprecipitation followed by selection for 14 days in growth medium supplemented with hygromycin B (200 μg/ml). Multiple drug-resistant colonies (>100) were pooled together to establish stable cell lines. All assays for transformation were performed in triplicate.

Docking of Ins(4,5)P_2 onto the PH Domain of Dbs—Potential lipid binding pockets on the surface of the Dbs PH domain were predicted using the SiteID option of SYBYL (Tripos Inc.). A coordinate file for the phosphoinositol ring of phosphatidylinositol 4,5-bisphosphate (Ins(4,5)P_2) was created using SYBYL. All docking procedures utilized the DOCK suite of programs (version 4.0, I. D Kuntz, University of California at San Francisco) and were carried out in a manner previously reported (22). Briefly, a molecular surface for each potential binding pocket was generated using the surface calculation algorithm MS (23) and used as an input for generating space-filling spheres using Sphgen. A scoring grid encompassing each pocket plus an additional radius of ~8 Å was calculated using the grid. Parameters used for docking Ins(4,5)P_2 into the Dbs PH domain were standard DOCK parameters for the single anchor search method using torsion drive and torsion minimization. Residues 846–852 in the disordered β1/β2 loop region were modeled in and minimized for some docking procedures. To validate our docking procedure, inositol 1,4,5-trisphosphate was successfully docked into the previously determined structure of the PH domain of PLC-δ bound to inositol 1,4,5-trisphosphate (RCSB accession number 1MAI).

Phosphoinositide Binding Assays—The ability of His_6-tagged Dbs proteins to bind to phosphatidylinositol 4,5-bisphosphate was assessed by an enzyme linked immunosorbent assay (ELISA) utilizing a 96-well microtiter plate containing a various amounts of diC_6-modified phosphatidylinositol 4,5-bisphosphate (Echelon Biosciences Inc.). Protein stock solutions were diluted to 10 μM in buffer C (20 mM Tris, pH 7.5, 100 mM NaCl, and 5% glycerol) before adding 50 μl of each protein to the wells. 100 μl of an anti-His antibody (Santa Cruz Biotechnology) diluted 1:500 in buffer C was then incubated with each well, followed by 100 μl of an horseradish peroxidase-conjugated sheep anti-mouse IgG (Amersham Biosciences) diluted 1:1000 in buffer C. All incubations were performed for 1 h at 18 °C, and the wells were extensively washed with buffer C after each incubation step. Protein-lipid interactions were detected with the horseradish peroxidase substrate *o*-phenylenediamine (Sigma), and absorbances were measured at 490 nm.

Dbs binding to small unilamellar vesicles (SUVs) containing phosphatidylinositol 4,5-bisphosphate was measured by surface plasmon resonance using a BIAcore 2000 instrument. SUVs (containing by molar fraction 80% dipalmitoyl phosphatidylcholine, 17% dipalmitoyl phosphatidylserine, 3% phosphatidylinositol 4,5-bisphosphate, and 0.1% *N*-biotinylated dipalmitoyl phosphatidylethanolamine) were prepared by sonicating lipids into 20 mM Hepes (pH 7.5) and 150 mM NaCl. SUVs were then captured on an SA5 chip (BIAcore) via a biotin-streptavidin interaction. Equal amounts of SUVs were immobilized on each respective flow cell, whereas an empty flow cell was maintained to control for nonspecific binding. 25 μl of various Dbs proteins in 20 mM Hepes (pH 7.5) and 150 mM NaCl were injected onto the flow cells at 50 μM and followed by 100 μl of buffer. Experiments were performed at 25 °C with a flow rate of 100 μl/min. Raw sensorgrams from each experiment were aligned, and the signal due to binding the empty flow cell was subtracted from each curve using the software BIAevaluation 3.0 (24).

RhoA Activation Assays—The Rho binding domain of Rhotekin (GST-C21) (19) was expressed as a GST fusion in BL21(DE3) cells and immobilized by binding to glutathione-coupled Sepharose 4B beads (Amersham Biosciences). The immobilized GST-C21 was then used to precipitate GTP-bound RhoA from NIH 3T3 cell lysates. Cells were washed in cold phosphate-buffered saline and then lysed in 50 mM Tris-HCl, pH 7.4, 2 mM $MgCl_2$, 100 mM NaCl, 10% glycerol, 1% Nonidet P-40, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin, and 1 μg/ml phenylmethylsulfonyl fluoride. Cell lysates were then cleared by centrifugation at 10,000 × *g* for 5 min at 4 °C. Lysates used for affinity purification were normalized for endogenous Rho levels. Affinity purifications were carried out at 4 °C for 1 h, washed three times in an excess of lysis buffer, and then analyzed by Western blot. RhoA was detected by a monoclonal antibody (sc-418, Santa Cruz Biotechnology).

Immunofluorescence—NIH 3T3 cells were plated on coverslips and then transiently cotransfected with 1 μg of pEGFP (Clontech) and 3 μg of either pAX142 or pAX142-*dbs*-HA6 harboring the PH domains mu-

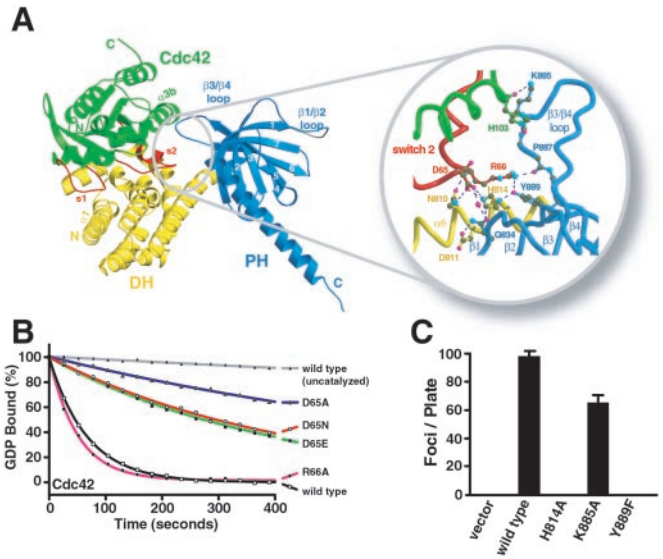

FIG. 1. **Interactions between the PH domain of Dbs and Rho GTPases are critical for transformation.** *A*, as previously reported (17), the PH domain (*blue*, disorder region in *gray*) of Dbs is an integral part of the interface with Cdc42 (*green*, switch regions are *red*) and the DH domain (*yellow*) necessary for robust GEF activity *in vitro*. The *inset* highlights specific interactions with *dashed lines* indicating potential hydrogen bonds. *B*, within Cdc42, Asp-65, but not Arg-66, is critical for exchange supported by the PH domain. Asp-65 of Cdc42 interacts primarily with His-814 of the DH domain buttressed by Gln-834 and Tyr-889 of the PH domain. Guanine nucleotide exchange reactions were performed by stimulating 2 μM of wild-type or mutant Cdc42 by 0.2 μM of the Dbs DH/PH domain. The intrinsic exchange data for the D65N, D65E, and D65A Cdc42 mutants are not shown. *C*, residues within the PH domain and integral to the interface with Cdc42 are necessary for transformation of NIH 3T3 cells mediated by Dbs (3 μg of plasmid). Data shown are representative of three assays performed on triplicate plates. The *error bars* indicate standard deviations.

tations by calcium phosphate precipitation. Cells were fixed with 3.7% formaldehyde (in PBS) for 10 min, permeabilized, and blocked in 0.1% Triton X-100, 3% BSA in PBS for 30 min. Coverslips were then incubated in a humidity chamber with an anti-HA mouse monoclonal antibody (BAbCO) for 1 h in 0.1% Triton X-100 with 0.1% BSA, washed in PBS, and then incubated with red-fluorescent Alexa Fluor 488-conjugated goat anti-mouse IgG (0.1% Triton X-100, 0.1% BSA, Molecular Probes) for 30 min in the dark. Coverslips were washed in PBS and mounted on glass slides using FluorSave reagent (Calbiochem). Cells were viewed with an Olympus IX50 inverted confocal microscope, and images were captured using the optronics digital charge-coupled device camera system.

Membrane Fractionation Analyses—Mass populations of NIH 3T3 cells stably expressing Dbs-HA6 or Dbs-HA6 harboring a PH domain mutation were washed with ice-cold PBS and resuspended in cold HYPO buffer (10 mM Tris, pH 7.4, 1 mM $MgCl_2$, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 5 μg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride). Lysates were then homogenized, harvested with HYPO buffer supplemented with 0.15 M NaCl, and then centrifuged at 38,000 rpm for 30 min at 4 °C. The supernatant (cytosolic fraction) was removed, and the pellet (particulate fraction) was resuspended in HYPO buffer supplemented with 0.15 M NaCl. The protein concentrations of the total, cytosolic, and membrane fractions were determined with a bicinchoninic acid protein assay kit (Pierce). 30 μg of protein for each fraction was resolved by SDS-PAGE, transferred to Immobilon-P membranes (Millipore), and probed with anti-HA epitope antibody (BAbCO).

RESULTS

The PH Domain of Dbs Is Integral to the Interface with Rho GTPases Necessary for Guanine Nucleotide Exchange and Cellular Transformation—The recently reported structure of a fragment of Dbs in complex with Cdc42 highlights a crucial role for the PH domain in promoting an extensive set of interactions between Dbs and Cdc42 necessary for efficient guanine nucleotide exchange *in vitro* (Fig. 1*A*) (17). In particular, Tyr-889 within β4 of the PH domain is critical for exchange because its substitution to phenylalanine dramatically decreases Dbs-catalyzed exchange of both Cdc42 and RhoA without affecting the overall arrangement of domains within the Dbs·Cdc42 complex (17). To clarify the structural role of the PH domain in contributing productively to the interface between Dbs and GTPases, a series of substitutions within the interface were assessed for functional importance. In particular, Arg-66 of Cdc42 interacts with multiple residues within the PH domain of Dbs, including Tyr-889. Yet substitution of Arg-66 to alanine does not affect nucleotide exchange (Fig. 1*B*, Table I, and see Ref. 17), suggesting that Tyr-889 likely stabilizes the electronic configuration of His-814 to promote interaction with Asp-65 of Cdc42. In support of the importance of Asp-65 to nucleotide exchange, amino acid substitutions placed at Asp-65 (D65A, D65E, and D65N) severely limit the ability of Dbs to activate Cdc42 (Fig. 1*B* and Table I). Of particular note, the isosteric substitution D65N in Cdc42 should not disrupt interactions with Asn-810 of the DH domain but will disrupt hydrogen bonding with His-814 of Dbs. Consistent with an "uncoupling" of Asp-65 from Tyr-889, Cdc42 (D65N) suffers a reduction of ~7-fold in the activation by Dbs, on par with the effect of Dbs(Y889F) on wild-type Cdc42 (Table I).

TABLE I
Rate constants of Dbs catalyzed guanine nucleotide exchange of mutant Cdc42 proteins

The rates (k_{obs}) for GEF reactions were determined by fitting the data from Fig. 1*B* as single exponential decays. The -fold stimulation for each Dbs protein reflects the ratio of the k_{obs} measured for the GEF-containing reaction to the unstimulated reaction containing no GEF.

2 μM Cdc42	Intrinsic rate	Plus 0.2 μM Dbs	Stimulation
	$k_{obs}\ s^{-1} \times 10^{-3}$		*-fold*
Wild type	0.24 ± 0.01	14.89 ± 0.02	61
D65A	0.40 ± 0.00	1.05 ± 0.08	3
D65E	0.19 ± 0.00	2.34 ± 0.00	12
D65N	0.28 ± 0.03	2.60 ± 0.14	9
R66A	0.34 ± 0.02	19.56 ± 0.26	57

The *in vitro* exchange assays utilizing mutants of Dbs and Cdc42 strongly suggest that the PH domain of Dbs, acting in concert with the DH domain, is necessary to fully activate Rho family GTPases within cells. To further assess the physiological function of the PH domain of Dbs in activating Rho GTPases, we introduced a subset of the previously studied mutations into a transforming version of Dbs (Dbs-HA6) (8) and measured the capacities of the mutated proteins to transform NIH 3T3 cells (Fig. 1*C*). Dramatically, whereas Dbs-HA6 possesses potent transformation activity, either H814A or Y889F completely eliminates the focus-forming activity of Dbs-HA6 in NIH 3T3 cells. Similarly, K885A, which is also within the PH domain, significantly reduces transformation by Dbs. Because transformation of Dbs depends upon activation of RhoA in NIH 3T3 cells (25), these data suggest that the PH domain of Dbs is required for direct activation of Rho family proteins, irrespective of membrane targeting. These effects are not due to loss of affinity for phosphoinositides by Dbs, because Dbs(H814A), Dbs(Y889F), and Dbs(K885A) bind to phosphatidylinositol 4,5-bisphosphate similar to wild-type Dbs (Fig. 2, *B* and *C*).

Mapping the Interface within the PH Domain Necessary for Binding Phosphoinositides—Typically, the majority of pleckstrin homology domains bind phosphoinositide headgroups with low affinity and specificity. For instance, the PH domain of Dbs promiscuously binds several multiphosphorylated phosphoinositides with low affinity, including phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 4,5-bisphosphate (15). Primary sequence analysis and structural homology comparisons suggest Dbs binds phosphoinositides similarly to

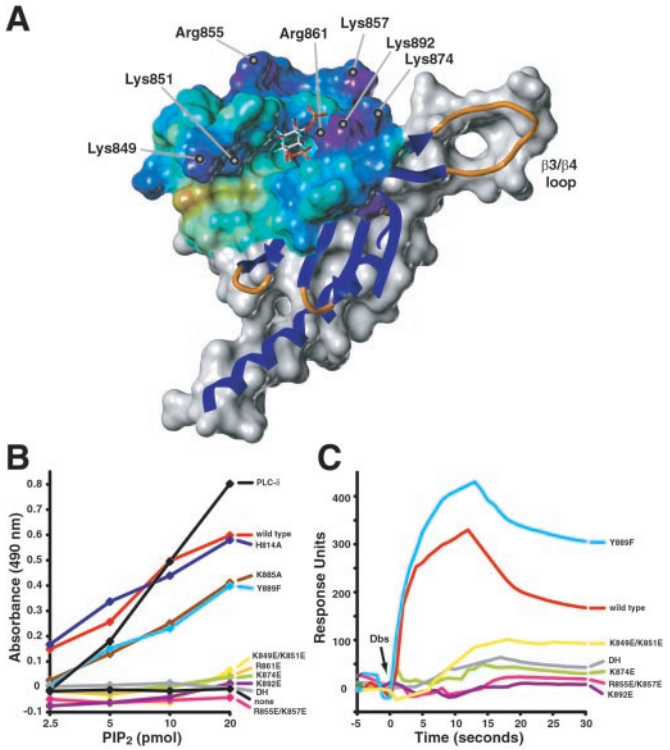

FIG. 2. **Mutational analysis of the phosphoinositide-binding pocket.** *A*, model of the headgroup of phosphatidylinositol 4,5-bisphosphate docked onto the PH domain of Dbs. The inositol ring of phosphatidylinositol 4,5-bisphosphate was docked onto the crystal structure of the PH domain of Dbs (PDB code 1KZ7) using the program DOCK (22). The electropositive surface (*blue*) was used during docking, and the top-scoring complex is shown with residues that impair binding of phosphoinositides upon mutation labeled. *B*, all substitutions within the putative phosphoinositide-binding pocket of Dbs DH/PH abrogate binding of phosphatidylinositol 4,5-bisphosphate as measured by microtiter-based ELISA. Conversely, mutations within the DH domain (H814A) or the β3/β4 loop of the PH domain have insignificant effects on phosphatidylinositol 4,5-bisphosphate binding. Also shown is the binding profile for the PH domain of PLC-δ1 known to bind phosphatidylinositol 4,5-bisphosphate with high affinity (40) as well as the isolated DH domain of Dbs. *C*, similar binding results were obtained using small unilamellar vesicles doped with phosphatidylinositol 4,5-bisphosphate at 3% mol fraction and measuring interactions by surface plasmon resonance. Raw data were normalized to the signal achieved from nonspecific binding to an empty flow cell surface.

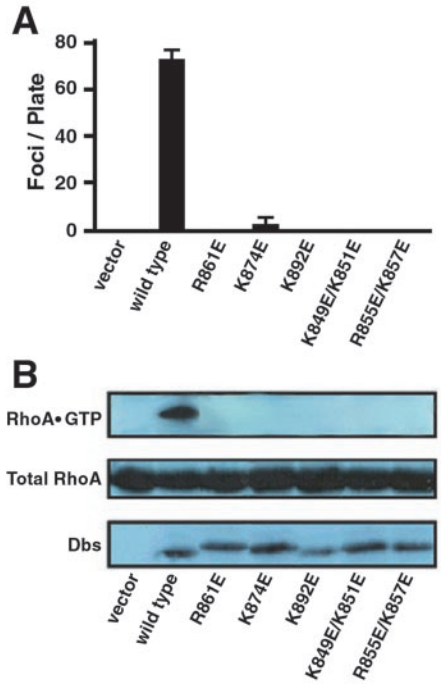

FIG. 3. ***In vivo* activation of RhoA and associated transformation require phosphoinositide binding to the PH domain.** *A*, mutations within the PH domain of Dbs that destroy binding to phosphoinositides also prevent transformation of NIH 3T3 cells as measured in primary focus formation assays. Data shown are representative of three independent assays performed on triplicate plates. *B*, the identical panel of mutants also fails to activate RhoA in NIH 3T3 cells. Lysates were prepared from cell lines stably expressing either Dbs-HA6, the various mutations within a Dbs-HA6 background, or the cognate pCTV3H vector and examined by Western blot for expression of RhoA (*Total-RhoA*) and the HA-tagged Dbs proteins (*anti-HA*). Each lysate was subsequently normalized for expression of total RhoA and subjected to affinity purification using immobilized GST-C21 to isolate RhoA-GTP also visualized by Western blot. All experiments were performed a minimum of three times, and data shown constitute a representative set.

other PH domains largely through residues in and surrounding the β1/β2 and β3/β4 loops of the PH domain. Like most PH domains, the β1/β2 and β3/β4 loop region of Dbs exhibits a large envelope of positive electrostatic potential, compatible with binding the electronegative headgroups of phosphoinositides (17). To further define the lipid-binding pocket within the Dbs PH domain, we used a computational approach to dock various inositol phosphates onto the surface of the protein (Fig. 2*A*). These analyses suggest that residues within the PH domain of Dbs that are predicted to contact phosphoinositides within this site, and/or contribute to the positive electrostatic potential include: Lys-849, Lys-851, Arg-855, and Lys-857 (within the β1/β2 loop); Arg-861 (at the base of β2); Lys-874 (within β3); and Lys-892 (within β4).

To determine if regions comprising β1/β2 and β3/β4 of the PH domain of Dbs are responsible for mediating binding to phosphoinositides, we substituted the identified lysine and arginine residues with glutamate and assessed the effect of each mutation upon the ability of Dbs to bind phosphatidylinositol 4,5-bisphosphate. Initially, an enzyme-liked immunosorbent assay (ELISA) was used to assess binding to phosphatidylinositol 4,5-bisphosphate in the absence of secondary lipids (Fig. 2*B*). These results were supported by a more detailed analysis using surface plasmon resonance and small unilamellar vesicles containing a low molar fraction of phosphatidylinositol 4,5-bisphosphate within a background of negatively charged lipids (Fig. 2*C*). As we observed previously (15), wild-type Dbs DH/PH domain binds significantly to phosphatidylinositol 4,5-bisphosphate, whereas the isolated DH domain of Dbs shows no binding. In comparison, Dbs proteins harboring either single or double glutamate substitutions within the putative phosphoinositide binding site of the PH domain do not significantly bind phosphatidylinositol 4,5-bisphosphate in either assay. In contrast, other mutations within the extended β3/β4 loop (K885A) or β4 loop (Y889F) of the PH domain, at sites distinct from the phosphoinositide-binding pocket, do not significantly affect binding of phosphoinositides. Therefore, as observed within the structures of other PH domains bound to inositol phosphates, the PH domain of Dbs binds lipids through portions of β1/β2 and β3/β4, and mutations within this pocket selectively abolish lipid binding.

RhoA Activation and Cellular Transformation Require Engagement of Phosphoinositides by the PH Domain—To assess if phosphoinositide binding to the PH domain is critical for Dbs transformation of NIH 3T3 cells, we placed each mutation into the Dbs-HA6 construct and compared transformation potentials in primary focus formation assays (Fig. 3*A*). Strikingly, except for the low level of foci formation exhibited by Dbs(K874E), transformation was completely eliminated by all other mutations within the pocket of the PH domain responsi-

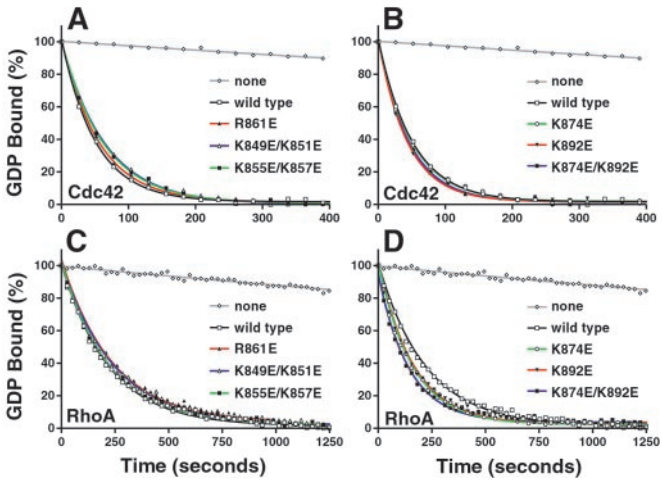

FIG. 4. ***In vitro* GEF activity of Dbs is unaffected by loss of phosphoinositide binding to the PH domain.** *In vitro* guanine nucleotide exchange of Cdc42 (*A* and *B*) or RhoA (*C* and *D*) by each phosphoinositide binding site mutant of Dbs was carried out as described under "Experimental Procedures." Guanine nucleotide exchange reactions were performed by stimulating 1 μM of GTPase by 0.2 μM of wild-type or mutant Dbs DH/PH domain. "*None*" refers to the intrinsic exchange data of wild-type Cdc42 or RhoA in the absence of GEF.

ble for binding phosphoinositides. Although exact identification of the endogenous phosphoinositide targeted by Dbs awaits more detailed analyses, these data resoundingly highlight that phosphoinositide binding by the PH domain is essential for Dbs-mediated transformation.

Dbs transformation of NIH 3T3 cells is closely correlated to the activation of endogenous pools of RhoA and not Cdc42 (25). Because the mutations within the PH domain destroy transforming activity in primary focus assays, an associated decrease in the levels of activated RhoA in these cells is expected. To measure endogenous levels of activated RhoA, the Rho-binding domain of Rhotekin was used to precipitate GTP-bound RhoA from lysates of NIH 3T3 cells expressing either wild-type Dbs-HA6 or Dbs-HA6 harboring the previously characterized mutations within the PH domain (Fig. 3*B*). Although Dbs-HA6 exhibited robust activation of RhoA, activation of RhoA was not detectable in cells expressing the mutant versions of Dbs that were previously shown to be defective in binding phosphatidylinositol 4,5-bisphosphate and transformation. The loss of transformation and RhoA activation was not a result of poor expression or stability of the various Dbs mutants, because the expression levels for all Dbs proteins were found to be equivalent (Fig. 3*B*, *lower panel*). These data demonstrate that cellular transformation of NIH 3T3 cells is dependent upon RhoA activation and both processes require Dbs to functionally engage phosphoinositides.

However, the formal possibility exists that the observed loss of *in vivo* transforming activity and RhoA activation results from reduction of the inherent GEF activity of Dbs and not the loss of phosphoinositide binding upon introducing substitutions within the Dbs PH domain. To determine the functional consequences of substitutions within the lipid binding site of Dbs upon guanine nucleotide exchange, we analyzed the ability of each mutant to exchange Cdc42 and RhoA *in vitro* (Fig. 4 and Table II). As expected, none of the mutations within the PH domain of Dbs were detrimental to Dbs-stimulated exchange of Cdc42 or RhoA, and both the K874E and K892E mutations within Dbs actually resulted in a modest, but reproducible, increase in exchange activity of the GEF (Fig. 4, *B* and *D*, and Table II). In summary, removing the ability of Dbs to interact with phosphoinositides results in the complete loss of both transforming ability as well as the ability of Dbs to activate endogenous pools of RhoA.

Proper Subcellular Localization of Dbs Does Not Require the PH Domain to Bind Phosphoinositides—It is widely assumed that PH domains generally promote association of their host proteins to membranes dependent upon specific phosphoinositides. However, only high affinity interactions of PH domains with phosphoinositides are capable of inducing efficient membrane localization (11), and the relatively low affinity between the PH domain of Dbs and phosphatidylinositol 4,5-bisphosphate (15) argues against a critical role for phosphoinositides in localizing Dbs to cellular membranes. To assess potential linkage between the binding of phosphoinositides to the PH domain and the subcellular localization of Dbs, the previously characterized mutations within the PH domain shown to abrogate binding of phosphoinositides were assessed for affects on the cellular distribution of Dbs determined by membrane fractionation (Fig. 5*A*). Contrary to the straightforward idea of the PH domain as a simple membrane-tethering device, none of the mutations within the PH domain grossly altered the subcellular distribution of an extended fragment of Dbs (Dbs-HA6) stably expressed in NIH 3T3 cells and containing both DH and PH domains as well as flanking sequences. The lack of observable changes in membrane localization of the Dbs mutants were not due to the insensitivity of our assay, because a membrane-targeted version of the Dbs DH domain (DH-C*AAX*) was nearly entirely associated with the insoluble, membrane fraction. In contrast, a non-targeted DH domain is distributed largely within the cytoplasm of NIH 3T3 cells as previously reported (18). These data demonstrate that, although the PH domain and surrounding regions are necessary for the recruitment of Dbs to cellular membranes, this recruitment is independent of the need for the PH domain to bind phosphoinositides.

Results arising from the cellular fractionations are supported by more detailed immunofluorescence studies (Fig. 5*B*). When transiently expressed in NIH 3T3 cells, Dbs-HA6 localizes predominantly in membrane structures that coincide with lamellipodia and dense cytoplasmic pockets of indeterminate identity. Importantly, the entire panel of mutations within the PH domain previously tested by cellular fractionations similarly have no effect on subcellular localization; Dbs-HA6(K892E) is shown as a representative example. Similarly, stable expression of the panel of mutant proteins shows no alterations in subcellular distribution relative to wild-type Dbs, although staining is weaker and more diffuse (data not shown). Fragments of Dbs, truncated before the PH domain (wild-type Dbs DH, Fig. 5*A*) as well as the isolated PH domain,[2] are predominantly cytosolic; therefore, regions carboxyl-terminal of the PH domain are likely to direct subcellular localization of Dbs. Future studies will test this idea. Given that the entire panel of mutants is defective in binding phosphoinositides through the PH domain yet retains wild-type ability to localize within cells as determined by both subcellular fractionation and immunofluorescence, it seems unequivocal that localization of Dbs to cellular compartments is independent of any requirement of the PH domain to bind phosphoinositides.

DISCUSSION

The invariant positioning of PH domains immediately carboxyl-terminal to DH domains strongly implies a unique functional interrelationship. Recent structures of several DH/PH fragments bound to Rho GTPases highlight a highly conserved

[2] K. L. Rossman, L. Cheng, G. M. Mahon, R. J. Rojas, J. T. Snyder, I. P. Whitehead, and J. Sondek, unpublished data.

TABLE II

Rate constants of guanine nucleotide exchange reactions catalyzed by wild-type and mutant Dbs proteins on Cdc42 and RhoA

k_{obs} values and -fold stimulation were estimated as in Table I using data from Fig. 4.

Dbs	Cdc42		RhoA	
	k_{obs}	Stimulation	k_{obs}	Stimulation
	$s^{-1} \times 10^{-3}$	*-fold*	$s^{-1} \times 10^{-3}$	*-fold*
None	0.28 ± 0.00	1	0.12 ± 0.00	1
DH/PH (wt)	17.39 ± 0.98	63	4.22 ± 0.35	35
K849E/K851E	15.16 ± 0.31	55	4.17 ± 0.11	34
K855E/K857E	16.09 ± 0.23	58	4.41 ± 0.14	36
R861E	16.90 ± 0.78	61	4.00 ± 0.09	33
K874E	20.48 ± 1.30	74	6.35 ± 0.07	52
K892E	23.37 ± 0.90	84	6.35 ± 0.14	52
K874E/K892E	23.57 ± 1.12	85	7.09 ± 0.09	58

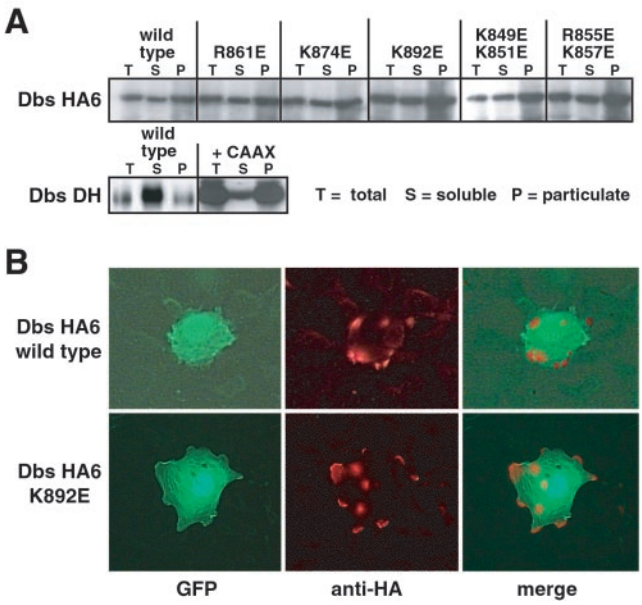

FIG. 5. **Mutations within the PH domain of Dbs that destroy lipid binding do not affect subcellular localization.** *A*, NIH 3T3 cells that stably express Dbs-HA6, Dbs-HA8 (DH), Dbs-HA7 (DH plus C*AAX*), or the various lipid binding mutants were lysed and fractionated as described under "Experimental Procedures." 30 μg of protein from total (*T*), soluble (*S*), and particulate (*P*) fractions were resolved by SDS-PAGE. Protein expression was determined with an anti-HA epitope antibody (BAbCO). Data shown are representative of three independent assays. *B*, NIH 3T3 cells, which were transiently cotransfected with pEGFP and either Dbs-HA6 or Dbs-HA6 harboring mutants defective in lipid binding, were examined for cellular distribution of Dbs. GFP was used as both a counterstain and a marker for transfected cells. Cells expressing only GFP were examined for expression of the HA epitope. GFP and HA images were merged (*merge*) to orient the Dbs staining with respect to cellular structures.

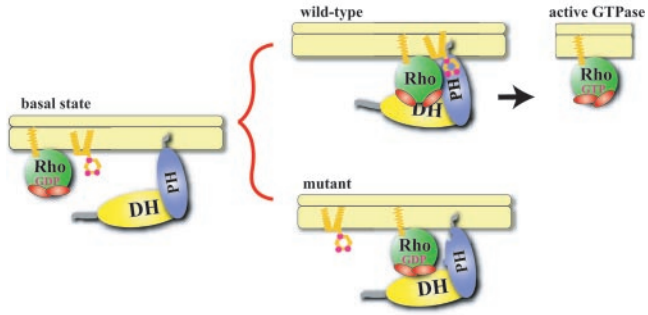

FIG. 6. **Model linking the binding of phosphoinositides to activation of Rho GTPases.** Prior to GTPase activation (*left panel*), local accumulation of specific phosphoinositides and membrane-resident, inactive GTPases favor interactions with GEFs located in close approximation to the relevant cellular membranes. For wild-type GEFs (*upper panel*), interactions with phosphoinositides and GTPases promote ejection of GDP and the subsequent loading of GTP to activate GTPases. For Dbs, exchange requires the direct interactions of both the DH and PH domains with Rho GTPases at the membrane surfaces to allow both domains to participate effectively in GTPase activation. Mutations (*lower panel*) that destroy the ability of the Dbs PH domain to bind either phosphoinositide (*dimple* in the PH domain) or GTPase (*bump*) consequently destroy GTPase activation without altering the sub-cellular localization of Dbs. Most significantly, this model invokes the PH domain in directly regulating GTPase activation by detecting both GTPases and phosphoinositides, making the PH domain an active participant in the exchange process and not simply a membrane-anchoring device.

mechanism of exchange by DH domains (15, 17, 26). However, there is mounting evidence that PH domains associated with DH domains can cooperate in the exchange process. For example, relative to the isolated DH domain, the DH/PH fragment of Trio is more efficient at catalyzing nucleotide exchange upon Rac1 *in vitro* (16). Similarly, DH/PH fragments of p115 RhoGEF, Dbl, and Dbs are more proficient GEFs relative to their isolated DH domains (17, 27, 28).

Importantly for Dbs, the underlying mechanism for the PH domain-associated increase in the rate of nucleotide exchange has been revealed within the structure of Dbs·Cdc42. The structure shows that the PH domain of Dbs interacts with Cdc42 through interactions within β1, β4, and the β3/β4 loop of the PH domain to contact switch 2 and α3b within Cdc42, and biochemical studies show these interactions are critical for efficient guanine nucleotide exchange *in vitro* (17). In particular, mutation of Tyr-889 to phenylalanine within the Dbs PH domain was particularly detrimental to guanine nucleotide exchange. Here we show that Tyr-889 likely acts to encourage interactions between Asp-65 within Cdc42 (Asp-67 in RhoA) and His-814 within the Dbs DH domain. Consistent with the related *in vitro* results, Dbs harboring Y889F (or H814A) failed to transform NIH 3T3 cells. These effects are not due to an inability to bind phosphoinositides, because Dbs(Y889F) possesses wild-type affinity toward phosphoinositides (Fig. 2) and maintains a three-dimensional structure essentially identical to wild-type Dbs (17). Therefore, both the DH and PH domains of Dbs are critical for directly catalyzing the activation of Rho GTPases in cells.

Pleckstrin homology domains typically associate with phosphoinositides, and the lipid binding sites are predominantly composed of residues in and around the β1/β2 and β3/β4 loops (11, 29). Based on docking experiments between inositol 4,5-bisphosphate (the phosphoinositol ring of phosphatidylinositol 4,5-bisphosphate) and the Dbs PH domain, basic residues within the Dbs PH domain that participate in phosphatidylinositide binding have been predicted. Consistent with these predictions, substitutions to glutamate at these sites abolish the ability of Dbs to productively bind to phosphatidylinositol 4,5-bisphosphate *in vitro*. When the mutants of Dbs that are deficient in binding lipid are analyzed for *in vivo* function, they are completely devoid of transformation potential and do not

detectably activate RhoA in NIH 3T3 cells, despite suffering no loss in exchange activity *in vitro*. Therefore, lipid binding by the PH domain of Dbs is critical to both the transformation potential of Dbs and the ability of Dbs to activate Rho GTPases *in vivo*.

The binding of phosphoinositides to PH domains is often invoked to explain the translocation of cytosolic proteins to membrane surfaces. However, for Dbs, this function does not appear to be the main role for the PH domain. For instance, the PH domain of Dbs binds phosphatidylinositol 4,5-bisphosphate with relatively low affinity (K_D of ~10 μM) (15) typically considered insufficient to recruit proteins to plasma membranes (11). Therefore, not surprisingly, mutants of Dbs deficient in binding phosphoinositides through the PH domain continue to associate with distinct cellular compartments similar to wild-type Dbs as assessed by membrane fractionation and immunofluorescence studies. Other Dbl family proteins behave similarly. For example, the affinity of phosphatidylinositol 4,5-bisphosphate binding to the PH domain of Sos1 is ~2 μM (30, 31) and while the PH domain of Sos1 translocates to plasma membranes in response to serum-treatment of cells, this recruitment is not due to association with phosphoinositides but likely involves association with other ligands (31). In fact, whenever it has been measured (12, 15, 32, 33) affinities between DH-associated PH domains and phosphoinositides are sufficiently low as to suggest a role other than membrane targeting as the principal function for these PH domains.

An alternative model consistent with the available data postulates that binding of phosphoinositides by the PH domain reorients the DH and PH domains to engage effectively Rho GTPases (Fig. 6). In the simplest scenario, binding of phosphoinositides to the PH domain results in the *en bloc* reorientation of the DH and PH domains relative to membrane surfaces. However, a plethora of structural data (17, 20, 26, 34, 35) highlights a large degree of conformational flexibility between PH domains and associated DH domains. In the extreme case of Sos1, the PH domain must necessarily move out of the way to allow access of Rho GTPases to the major binding determinant within the DH domain subsequent to Rac activation dependent upon phosphoinositide 3-kinase (36). Therefore, perhaps it is more likely that binding of membrane-resident phosphoinositides to PH domains serves to alter the relative orientation of DH and PH domains to engage effectively Rho GTPases. These conformational rearrangements could relieve inhibitory constraints in the case of Sos1 and has been proposed for Vav (12) and Dbl (14, 37) or promote activation as has been suggested for Tiam1 (13, 38), Vav (12), and P-Rex1 (39). As previously discussed, Dbs·Cdc42 is organized to allow simultaneous engagement of membranes by the PH domain of Dbs and the geranylgeranyl group of Cdc42 (17). The structure of the complex further suggests that membrane binding by the PH domain would not preclude and, indeed, may favor concomitant interactions between GTPases and the PH domain. Therefore, the PH domain of Dbs, in concert with the DH domain, possibly cooperates to integrate information regarding local fluctuations in both Rho GTPase concentrations and the phosphoinositide composition of nearby membranes.

In conclusion, we have used the recently determined structure of Dbs·Cdc42 to design mutations within the PH domain of Dbs that specifically impair either guanine nucleotide exchange or phospholipid binding *in vitro* and assessed their functional effects *in vivo*. In agreement with both the structure and *in vitro* biochemistry, the PH domain is an integral part of the interface with GTPases required for efficient exchange *in vivo* and mutations at sites within the PH domain that directly perturb this interface severely impair cellular transformation by Dbs. Similarly, cellular transformation and GTPase activation *in vivo* require the PH domain of Dbs to properly engage phosphoinositides, but this requirement manifests only in the presence of cellular membranes and membrane-associated GTPases. Moreover, the PH domain clearly does not serve as a simple membrane anchor or subcellular trafficking device dependent upon binding phosphoinositides, because a variety of forms of Dbs deficient in binding phosphoinositides to the PH domain still localize correctly within cells. Although the details await clarification, it seems likely that the invariant placement of PH domains downstream of DH domains must serve an invariant function. One intriguing possibility consistent with these data is that the two domains function cooperatively to detect specific membrane-resident GTPases and phosphoinositides produced under controlled stimuli.

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