Control of Intramolecular Interactions between the Pleckstrin Homology and Dbl Homology Domains of Vav and Sos1 Regulates Rac Binding

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Vav and Sos1 are Dbl family guanine nucleotide exchange factors, which activate Rho family GTPases in response to phosphatidylinositol 3-kinase products. A pleckstrin homology domain adjacent to the catalytic Dbl homology domain mediates the effects of phosphoinositides on guanine nucleotide exchange activity. Here we tested the possibility that phosphatidylinositol 3-kinase substrates and products control an interaction between the pleckstrin homology domain and the Dbl homology domain, thereby explaining the inhibitory effects of phosphatidylinositol 3-kinase substrates and stimulatory effects of the products. Binding studies using isolated fragments of Vav and Sos indicate phosphatidylinositol 3-kinase substrate promotes the binding of the pleckstrin homology domain to the Dbl homology domain and blocks Rac binding to the Dbl domain, whereas phosphatidylinositol 3-kinase products disrupt the Dbl homology/pleckstrin homology interactions and permit Rac binding. Additionally, Lck phosphorylation of Vav, a known activating event, reduces the affinities of the Vav Dbl family homology and pleckstrin homology domains and permits Rac binding. We also show Vav activation in cells, as monitored by phosphorylation of Vav, Vav association with phosphatidylinositol 3,4,5-trisphosphate, and Vav guanine nucleotide exchange activity, is blocked by the phosphatidylinositol 3-kinase inhibitor wortmannin. These results suggest the molecular mechanisms for activation of Vav and Sos1 require disruption of inhibitory intramolecular interactions involving the pleckstrin homology and Dbl homology domains.

The Dbl family of guanine nucleotide exchange factors (GEFs)1 regulate the activity of Rho-GTPases by stimulating the release of bound guanine nucleotides; the Rho proteins are then activated upon the binding of GTP and subsequent release from these GEFs (1, 2). The activated Rho proteins modulate changes in the actin cytoskeleton as well as gene expression (2). Nearly all known Dbl-related GEFs have a pleckstrin homology (PH) domain on the C-terminal side of the catalytic domain (Dbl homology (DH) domain) responsible for GEF function suggestive of an integrated function of the tandem DH-PH domains (2). Recent reports have indicated that phosphoinositide (PI) 3-kinase products binding to the PH domain activate the GEF activity of Vav and Sos1 (3–5). In addition to the control by PI 3-kinase, Vav is also under the control of a Src-related tyrosine kinase activity (5–7).

PH domains are found in approximately 100 molecules with critical roles in signal transduction (8). These include enzymes involved in regulation of the Ras and Rac families of GTPases, serine/threonine and tyrosine kinases, regulators of lipid second messengers, and GTPases (8). Many PH domains interact with phosphoinositides, some binding predominantly to the PI 3-kinase substrate, phosphatidylinositol (PI)-4,5-P2, whereas others bind PI 3-kinase products with higher affinities than the substrate (9). The high affinities of these PH domains for phosphoinositides are thought to anchor the proteins to membranes rich in the phosphoinositides to which they bind (8–11). Indeed, several PH domain-containing proteins that are normally found in the cytoplasm translocate to plasma membranes upon receptor stimulation that changes the phosphoinositide composition of these membranes (8–11). Thus, some PH domains play a role in cellular regulation by translocating enzymes to membranes containing their substrates and regulators. However, in addition to translocating enzymes to membranes, PH domains may also confer allosteric regulation of enzymatic activities in response to changes in phosphoinositides.

Studies of the Vav PH domain have failed to find evidence for a role in regulating recruitment to membranes (5). Instead, biochemical evidence suggests the PH domain of the Vav GEF could affect allosteric regulation of the adjacent catalytic DH domain (3, 4). Biochemical studies using water-soluble derivatives of phosphoinositides and purified Vav and Rac found that C8-PI-4,5-P2 potently inhibits Vav GEF activity whereas C8-PI-3,4,5-P3 activates GEF activity (3). It is unlikely these results reflect the activation of GEF activity by concentrating enzyme and substrate onto the surface of lipid vesicles. First, these water-soluble phosphoinositides do not readily form lipid vesicles. Second, these studies used Escherichia coli expressed DMEM, Dulbecco’s modified Eagle’s medium; PAGE, polyacrylamide gel electrophoresis.
Rac protein, which lacks the fatty acid modification necessary for efficient membrane association. A phosphoinositide-induced conformational change in Vav is also suggested by the enhanced Lck-dependent phosphorylation of Vav in the presence of C8-PI-3,4,5-P3. Although the role of the Vav PH domain in regulating catalytic activity can be argued to be an allosteric effect, direct evidence supporting this has not been reported.

We sought to define the mechanism by which phosphoinositides regulate Vav and Sos1 function. We find that isolated protein fragments encompassing the PH domains of Sos1 and Vav when bound to the GEF inhibitor, C8-PI-3,4,5-P3, bind to purified DH fragments of Sos1 and Vav, respectively. However, when these PH domains are bound with the GEF activator, C8-PI-3,4,5-P3, they bind their respective DH fragments significantly less well. Fragments of these exchange factors encompassing both the DH and PH domains bind to Rac proteins in the presence of PI-3,4,5-P3, but not in the presence of PI-4,5-P2. Binding analysis also reveals that Lck phosphorylation of the isolated DH domain of Vav reduces its ability to bind to an isolated Vav(PH) fragment. The results presented here provide direct evidence that a PH domain can affect the allosteric regulation of an enzymatic activity. This regulation involves the inhibitory effect of intramolecular interactions between the PH and DH domains of the Vav and Sos1 GEFs. PI-4,5-P2 promotes the inhibitory effects of the PH domain, whereas PI-3,4,5-P3 disrupts these intramolecular interactions and hence abolishes the inhibitory effects of the PH domain. In addition, Lck phosphorylation of Vav, which is promoted by PI-3-kinase products, destabilizes the inhibitory effects of the PH domain on the DH domain. Previous studies have provided support for a model whereby Vav activation occurs in response to PI-3-kinase and a Src-related kinase (3, 5). Here we extend those studies by examining Vav activation in cells with regard to the molecular events identified by biochemical analysis (Vav phosphorylation, phosphoinositide binding, and GEF activity).

We find by analysis of immunoprecipitates from Vav-expressing cells that: 1) phosphorylation of Vav, 2) PI-3,4,5-P3 association with Vav, and 3) Vav GEF activity increase following serum stimulation. Importantly, each of these increases are blocked by pretreatment of cells with the PI-3 kinase inhibitor, wortmannin.

**EXPERIMENTAL PROCEDURES**

**Reagents and Plasmids**—The mammalian expression vector for wild-type Vav, pCDNA3 Vav(wt), has been described (3). His-tagged VavDH(PH) was expressed from pSETBvavL, which has been described (7). For expression of GST-Vav(PH), codons 379–505 of mouse Vav were amplified by PCR and subcloned into the EcoRI/HindIII site of pGEX-KG vector. For expression of His-Vav(DH), a SacII/BglII fragment of mouse Vav sequence encoding amino acids 173–436 of Vav was subcloned into the HindIII/BamHI site of the Eco coli expression vector pET28C. For expression of GST-Sos(DH), sequences encoding amino acids 211–502 of mouse Sos1 were PCR-amplified and subcloned into the BamHI/EcoRI sites of the pGEX2TK vector (Amersham Pharmacia Biotech). The inserts of each of the plasmids described above were sequenced after completion of the plasmid construction finding the sequenced plasmids to be identical in the laboratory (3, 7).

**In Vitro Binding of Vav to 125I-PI-4,5-P2**—Two cultures of cells were treated with 15% fetal calf serum for 10 min after transfection, the transfection medium was replaced with fresh serum-free medium, and the cells were grown to 75% confluence in DMEM (Life Technologies, Inc.) containing 15% calf serum. Cells were then incubated in DMEM containing 0.5 mCi/ml [32P]orthophosphate. Cells were stimulated with 15% calf serum. Two cultures of cells were treated with 100 nM wortmannin for 40 min prior to serum addition. Cell lysates and Vav immunoprecipitates were prepared as described above and analyzed by SDS-PAGE and autoradiography.

**Phosphorylation of Vav in Vivo by Cotransfected Kinases**—COS7 cells were cotransfected with mammalian expression vectors for wild-type Vav (5 μg) and the kinases Syk-B or Fyn (1 μg of pSVcIISykB or pXSmyc-Fyn, respectively). The cells were transfected with Superfect reagent (Qiagen Inc.) according to the manufacturer’s protocol. At 2.5 h after transfection, the transfection medium was replaced with fresh culture medium followed by further incubation for 5 h. The cells were then serum-starved for 18 h in medium containing 0.5% calf serum. Two cultures of cells were treated with 15% fetal calf serum for 10 min at 37 °C. One set of serum-stimulated cultures was treated with 100 nM wortmannin for 25 min prior to addition of serum. Following the 10 min serum stimulation, cells were lysed in radiolmmune precipitation buffer (200 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 10 μM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin) for 30 min. Anti-Vav antibody conjugated to agarose beads (Santa Cruz Biotechnology) were used for Vav immunoprecipitates. The bead-bound immunocomplexes were washed five times in lysis buffer and resuspended in lysis buffer (200 μl) without Triton X-100. Lipids were extracted with 600 μl of CHCl3:MeOH:acetic acid (100:50:1) and the organic phase dried under a nitrogen stream. The lipids, resuspended in CHCl3:MeOH (10 mM) HCl (20:10:1), were separated by thin layer chromatography (TLC) (14) on Silica gel 250F plates (Baker Scientific) pretreated with 1% potassium oxalate solution (and dried at 120 °C for 40 min). [32P]-Labeled phospholipids were visualized after autoradiography. Similar results were obtained in three independent experiments. Visualization of PI-4,5-P2 was not possible due to a high background of non-specific material detected in Vav immunoprecipitates from cells not expressing Vav.

**Vav Phosphorylation in Serum-stimulated NIH-3T3 Cells**—NIH-3T3 cells were grown and induced to enter quiescence and labeled with [32P]orthophosphate as described above. Cells were stimulated with 15% calf serum. Where indicated, cells were treated with 100 nM wortmannin for 40 min prior to serum addition. Cell lysates and Vav immunoprecipitates were prepared as described above and analyzed by SDS-PAGE and autoradiography.

**Vav-GEF Activity in Serum-stimulated NIH-3T3 Cells**—Vav immunoprecipitates were resolved by SDS-PAGE and prepared for immunoblotting with anti-phosphotyrosine antibody or anti-Myc antibody.
the band intensity in the absence of phosphoinositides (i.e. the band intensity in the absence of phosphoinositides is 1). Densitometry analysis of the bands produced by anti-GST immunoblots of a range of predetermined amounts of GST-Vav(PH) showed that, in the range analyzed in Fig. 1B, the band intensity determined by this method changes linearly with the amount of GST-Vav(PH) present in the lane.

In Vitro Kinase Reactions—GST- or His-tagged fragments of Vav immobilized on glutathione-agarose or nickel-agarose beads, respectively, were washed with kinase buffer (20 mM HEPEs (pH 7.5), 20 mM MgCl2, 20 mM β-glycerophosphate, 0.1 mM Na3VO4, 2 mM dithiorthiol, 20 mM ATP) and resuspended in 450 μl of kinase buffer. The 45-min kinase reactions at 37 °C were initiated by addition of bacterially expressed Lck (1:1 molar ratio of Lck to His-Vav(DH/PH)). The bead-bound His-Vav(DH/PH) was then washed several times prior to binding or GEF reactions. Under these conditions (which lack a PI 3-kinase product necessary for efficient phosphorylation), we determined that 45 min of incubation allowed phosphorylation of Vav to a similar extent as with kinase reactions containing C8-PI-3,4,5-P3 and 100-fold less Lck.

In Vitro Interaction of DH and PH Domains—Fifty picomoles of His-Sos(PH) or GST-Vav(PH) was preincubated in binding buffer phosphate-buffered saline, 500 μg/ml bovine serum albumin, and 0.1% Triton X-100 with either C8-PI-4,5-P3, C8-PI-3,4-P2, or C8-PI-3,4,5-P3 at room temperature for 30 min, then 50 pmol of immobilized GST-Sos(DH) or His-Vav(DH) were added to the reaction (250 μl final volume). The reactions were incubated for an additional 30 min at room temperature. Nucleotide-free GST-Rac was prepared by addition of 5% EDTA to a final concentration of 7.5 mM to GST-Rac protein in binding buffer and incubation at room temperature for 15 min. The binding of magnesium by EDTA dramatically destabilizes the Rac-bound guanine nucleotides. The free nucleotide and EDTA were removed by dilution with binding buffer and centrifugation through a Centricon-10 column (Amicon Corp.). Fifty picomoles of nucleotide-free GST-Rac was then added to the binding reaction (250 μl final volume) and further incubated at room temperature for 2 h. After the incubation, the nickel-agarose beads were extensively washed with buffer (50 mM Tris-HCl (pH 7.5), 1% Triton X-100, and 50 mM imidazole) and the bound Rac protein was detected by immunoblotting to detect GST.

RESULTS

Phosphoinositides Regulate PH Domain Interactions with DH Domain—We have begun to explore the mechanisms involved in PI 3-kinase product-mediated activation of Vav and Sos1 as well as their inhibition by PI-4,5-P2. We hypothesized that the PH domain when bound to PI-4,5-P2 but not PI-3,4-P2 or PI-3,4,5-P3, contacts the DH domain, masking the Rac binding site and thereby explaining the inhibitory effects of PI-4,5-P2 on GEF activity. This model assumes that PI-4,5-P2 and PI-3,4,5-P3 compete for a single site (or overlapping sites) on the PH domains. In support of this, we find that 125I-labeled C8-PI-4,5-P2 (approximately 10–15 μCi) binding to recombinant histidine-tagged (His) Vav protein is competed by addition of PI-4,5-P2 or PI-3,4,5-P3 (Fig. 1A). We note that C8-PI-4,5-P2 is more efficient at blocking 125I-labeled C8-PI-4,5-P2 binding, the same concentration of C8-PI-4,5-P2 blocks approximately 40% of the 125I-labeled C8-PI-4,5-P2 binding. Similar results were obtained in two independent experiments. These results indicate that C8-PI-3,4,5-P3 blocks for binding at a single or overlapping site and that, as we suggested previously, C8-PI-3,4,5-P3 binds with a modestly higher affinity than does C8-PI-4,5-P2.

Binding experiments with a His Vav(DH) domain (residues 173–436) and a GST-tagged Vav(PH) domain (residues 397–505) were carried out to determine whether phosphoinositides have the potential to regulate intramolecular interactions involving these two domains. As seen in Fig. 1B (top panel), His-Vav(DH) immobilized on nickel-agarose beads was found to precipitate significantly more GST-Vav(PH) domain when the latter was bound to C8-PI-4,5-P2 or without phosphoinositide as compared with that bound to C8-PI-3,4,5-P3. Concentrations of C8-PI-3,4,5-P3 as low as 1 μM significantly prevented the interaction of DH and PH domains when compared with binding reaction in the absence of phosphoinositides (Fig. 1B, second panel). Analysis of the Vav(PH) band intensities bound to the Vav(DH) fragment as a function of C8-PI-3,4,5-P3 concentration is presented in Fig. 1. This analysis and that of two additional films indicates the majority (80–96%) of the reduction in PH binding occurs over the range of 0–10 μM C8-PI-3,4,5-P3. The two PI 3-kinase products, C8-PI-3,4-P2 and C8-PI-3,4,5-P3, were equally effective in blocking the interaction of the isolated DH and PH domains (data not shown). Similar binding studies failed to detect an interaction of the PH domain of Sos with the DH domain of Vav and vice versa (data not shown). Together, these results indicate the affinity of the Vav(DH) domain for the PH domain is higher in the presence of phosphoinositides that inhibit Vav-GEF activity when compared with that of the GEF-activating phosphoinositides.

Because Vav GEF activity measured in vitro is under dual regulation by Lck-dependent phosphorylation and binding to PI 3-kinase substrate and products, we examined the abilities of phosphorylated and unphosphorylated Vav(DH) fragments to bind the Vav(PH) domain bound to C8-PI-4,5-P2 or C8-PI-3,4,5-P3 (see “Experimental Procedures”). This analysis reveals that tyrosine-phosphorylated Vav(DH) binds less efficiently to the Vav(PH) domain than does unphosphorylated Vav(DH) (Fig. 1B, third panel). However, both unphosphorylated and Lck-phosphorylated DH fragments of Vav(DH) and Vav(DH)-Y-P bound more Vav(PH) when complexed with C8-PI-4,5-P2 as compared with that complexed with C8-PI-3,4,5-P3 (Fig. 1B, third panel). Thus the two events that activate Vav-GEF activity, Lck phosphorylation and PI-3,4,5-P3 binding, contribute to a lessening of the affinity between the PH and DH domains.

Similar binding experiments were carried out using a GST-Sos(DH) fragment immobilized on glutathione-agarose beads and a soluble His-Sos(PH) fragment in the presence and absence of saturating concentration (50 μM) of C8-PI-3,4,5-P3. Following the binding reaction, the washed pellets were analyzed by immunoblotting to detect the His-tagged PH fragment of Sos. We find GST-Sos(DH) reproducibly binds to His-Sos(PH) in the presence of C8-PI-4,5-P2 but not in the presence of C8-PI-3,4,5-P3 (Fig. 1B, bottom panel). Similar results were obtained using concentration of C8-PI-3,4,5-P3, C8-PI-3,4-P2, and C8-PI-3,4,5-P3 less than 10 μM (data not shown). These results demonstrate that, in vitro, the PH domain of Sos1 interacts with the DH domain and this interaction is regulated by the opposing action of PI 3-kinase substrate and product.

The PI 3-kinase substrate-dependent interaction of the DH and PH domains of Vav and Sos suggest a mechanism explaining the inhibitory effects of PI-4,5-P2 on GEF activity. The inhibition of Vav GEF activity by PI 3-kinase substrate could be due to the PH domain blocking Rac from binding to the catalytic DH domain. Activation of Vav might then occur by PI 3-kinase products causing the PH domain to break these inter-
His-tagged fragments of Sos1 encompassing the PH domain. The PH domain was bound to C8-PI-4,5-P2 or C8-PI-3,4,5-P3, or not bound with a GST fusion protein containing the DH domain of Sos1 (black bars), 5 or 10 μM PI-3,4,5-P3 (gray bars), or without the addition of phosphoinositides (open bar). After 1 h of incubation, the agarose beads were washed and the amount of 125I-PI-4,5-P2 associated determined by γ counting. The amount of radioactivity associated with the Vav-coupled agarose beads is expressed as a percentage of that bound in the absence of phosphoinositides. Similar results were obtained in two independent experiments. B, top panel, a fragment of Vav encompassing the PH domain (GST-Vav(PH)) was preincubated with saturating concentrations (50 μM) of C8-PI-4,5-P2 (PIP2) or C8-PI-3,4,5-P3 (PIP3) or without phosphoinositide. These soluble GST-Vav(PH) molecules were incubated with a His-tagged fragment of Vav encompassing the DH domain (His-Vav(DH)) immobilized on nickel-agarose beads. The nickel-agarose beads were washed to remove unbound material and analyzed by SDS-PAGE and immunoblotting to detect GST-Vav(PH). Second panel, a similar binding assay as described above is presented except a concentration range (0–50 μM) of C8-PI-3,4,5-P3 was analyzed. The relative intensities of bands detected after immunoblot analysis (see “Experimental Procedures”) are presented for the film shown (exp1) as well as two other analyses (exp2 and exp3). Third panel, His-Vav(DH) fragments immobilized on nickel-agarose beads were either phosphorylated by the Lck kinase (Vav(DH)-Y-P) or not phosphorylated (His-Vav(DH)). As described for the second panel, the immobilized Vav DH fragments were incubated with soluble Vav(PH) domain bound to the indicated C8-derivative of phosphoinositides. The washed nickel-agarose beads were analyzed for bound PH domain as described above. Bottom panel, a GST fusion protein containing the DH domain of Sos1 (GST-Sos(DH)) immobilized on glutathione-agarose beads was incubated with His-tagged fragments of Sos1 encompassing the PH domain. The PH domain was bound to C8-PI-4,5-P2 or C8-PI-3,4,5-P3, or not bound with phosphoinositide. Following the binding reaction, Sos(PH) associated with the glutathione-agarose beads was determined by SDS-PAGE followed by immunoblotting with anti-His tag antibody. C, His-tagged fragments (50 pmol) of Vav or Sos1 encompassing both the DH and PH domains were then incubated with 50 μM of C8-PI-3,4,5-P3 (PIP3) or without phosphoinositide (none) as indicated. The Rac-binding reactions were carried out at room temperature for 2 h, after which the nickel-agarose beads were washed extensively before SDS-PAGE and immunoblotting to detect GST-Rac in the final pellets. Similar results were obtained in three independent experiments. D, effects of C8-PI-3,4,5-P3 (PIP3) on the GEF activities of Vav and PH-domain mutants of Vav. His-tagged-fragments of wild-type (wt) Vav and Vav PH-domain mutants (PH-b, PH-c, PH-m) immobilized on nickel-agarose beads were phosphorylated by Lck kinase and then washed to remove Lck. The Vav proteins were then incubated with 50 μM C8-PI-3,4,5-P3 (PIP3) or C8-PI-3,4,5-P3, without phosphoinositide (−) or with phosphoinositide (−). The indicated Vav proteins, bound to the indicated phosphoinositide, were used in a GEF assay using GST-Rac-[3H]GDP as substrate. Values are expressed as percentage of GDP released over the intrinsic release observed without Vav protein. Similar results were obtained in two independent experiments.
His-Vav(DH/PH) precipitated GST-Rac protein in the presence of C8-PI-3,4,5-P3, but not in the presence of C8-PI-4,5-P3 (Fig. 1C, top panel). In a similar experiment using Vav that had first been phosphorylated by Lck, we found C8-PI-4,5-P3 blocks Rac binding, whereas C8-PI-3,4,5-P3 permitted Rac binding (Fig. 1C, middle panel). Phosphorylated Vav (not bound to phosphoinositides) binds nucleotide-free Rac much more effectively than does the unphosphorylated Vav molecule. The differences of phosphorylated and unphosphorylated Vav in binding Rac protein reflect our previous biochemical analysis demonstrating the strict dependence of GEF activity on both tyrosine phosphorylation of Vav and the absence of C8-PI-4,5-P3 (3, 7).

Similar Rac-binding results were found for a fragment of Sos encompassing both the DH and PH domains. The PI 3-kinase substrate, C8-PI-4,5-P3, blocked Rac interaction with Sos(DH/PH), whereas the PI 3-kinase product allowed Rac binding (Fig. 1C, bottom panel). These results are consistent with the hypothesis that PI-3,4,5-P3 binding to the PH domains of Sos and Vav prevents intramolecular interactions between the PH and DH domains, thereby allowing Rac interaction with the DH domain. Conversely, PI-4,5-P3 promotes these intramolecular interactions, thereby inhibiting Rac binding.

We examined the GEF activities of Vav and Vav-PH domain mutants (each of these Lck-phosphorylated) in the presence and absence of phosphoinositides. We previously reported these PH-domain point mutants exhibit a dramatic defect in binding phosphoinositides (3). Based on the binding studies presented here showing the PH domain complexed with PI-4,5-P3 binds to the DH domain to prevent Rac binding, we predicted that these PH domain mutants might display GEF activities even in the presence of PI-4,5-P3. Using conditions we previously used to monitor Vav GEF activity, we assayed the ability of Vav and the PH domain mutants to stimulate [3H]GDP release from Rac proteins (3, 7). The Lck-phosphorylated Vav proteins were first incubated in the presence or absence of 50 μM C8-PI-4,5-P3 or C8-PI-3,4,5-P3. As seen in Fig. 1D, Vav and each of the three Vav PH-domain mutants stimulated GDP release from Rac to a similar extent in the absence of phosphoinositides. Although the presence of C8-PI-4,5-P3 profoundly inhibited GDP release stimulated by wild-type Vav, the activities of PH domain mutants of Vav were unaffected by the presence of C8-PI-4,5-P3 (Fig. 1D). The presence of C8-PI-3,4,5-P3 resulted in a 2-fold increase in wild-type Vav GEF activity, but was ineffective in stimulating GEF activities of the PH-domain mutants of Vav (Fig. 1C).

Vav Activation in Cells Is Wortmannin-sensitive—Following proliferation-inducing stimulation of T cells, endogenous Vav is rapidly phosphorylated, as is exogenously expressed Vav in serum-stimulated COS7 cells (15–17). Although the tyrosine kinase activity in non-hematopoietic cells responsible for phosphorylating Vav is unknown, it is probable that a Src-related tyrosine kinase, such as Fyn, with substrate specificity similar to that of Lck kinase is involved (5, 18). Previous in vitro biochemical analysis indicated that Lck phosphorylation of Vav was greatly stimulated by PI-3,4,5-P3 binding to the PH domain of Vav (3). If a mechanism similar to that indicated by in vitro studies is operating in cells to regulate Vav function, we predicted that the phosphorylation of Vav in response to serum would be inhibited by pretreatment of cells with the PI 3-kinase inhibitor, wortmannin.

NIH-3T3 cells overexpressing Vav were labeled with inorganic [32P]phosphate and starved for serum to induce growth arrest. Following serum addition, cultures were harvested after 0 and 15 min of incubation and prepared for immunoprecipitation with anti-Vav antibodies. Lipids were then extracted from the immunoprecipitates and prepared for TLC to resolve phospholipids. Unlabeled PI-3,4,5-P3 standard was run in this TLC analysis (to determine the migration of authentic PI-3,4,5-P3 (Fig. 2D, see arrow). From the Vav-expressing cells stimulated for 15 min with serum, we detected [32P]-labeled material com-
Cells expressing wild-type Vav, labeled with $^{32}$P-orthophosphate, were stimulated with calf serum. Where indicated, 100 nM wortmannin was added 40 min prior to serum addition. Cells were harvested at 0, 5, 10, 30, and 60 min after adding serum. Vav immunoprecipitates prepared from the cell lysates were analyzed by SDS-PAGE and autoradiography. B, COS7 cells transiently transfected with plasmids for the expression of wild-type Vav and Syk, Fyn, or empty vector were starved for serum to induce growth arrest. One set of transfectants were treated with 100 nM wortmannin for 25 min prior to serum addition. Cells were harvested at 0, 5, 10, 30, and 60 min after adding serum. Vav immunoprecipitates prepared from the cell lysates were analyzed by SDS-PAGE and autoradiography. B, COS7 cells transiently transfected with plasmids for the expression of wild-type Vav and Syk, Fyn, or empty vector were starved for serum to induce growth arrest. One set of transfectants were treated with 100 nM wortmannin for 25 min prior to serum addition. Cells were harvested at 0, 5, 10, 30, and 60 min after adding serum. Vav immunoprecipitates were analyzed for GEF activity using Rac-$^{[3H]}$GDP as a substrate. Values shown represent the average of duplicate samples that did not differ by more than 8%. Similar results were obtained in independent experiments.

DISCUSSION

Previous studies have reported that Vav GEF activity for the Rho family of GTPases is regulated by a Src-related kinase activity and substrates and products of PI-3-kinase (3, 5–7). PI 3-kinase products enhance the phosphorylation of Vav in vitro suggesting that the activation of cellular Vav requires the concerted efforts of PI 3-kinase and a Src-related kinase (3). Here, we have provided support for this model. Our studies of Vav-expressing COS7 cells indicate that serum-induced phosphorylation of Vav is dependent on a wortmannin- and LY294002-sensitive cellular activity (presumably PI 3-kinase) and that this tyrosine kinase activity is related to the Fyn and Lck kinases. Consistent with a role in stimulation of Vav activity, we find Vav associates with PI-3,4,5-P$_3$ in response to serum stimulation. Additionally, the levels of Vav GEF activity following serum stimulation are down-regulated in cells pretreated with wortmannin. Together, these results support a model whereby the activation of Vav in cells occurs by its association with PI-3,4,5-P$_3$ and that this association with a PI 3-kinase product allows a tyrosine phosphorylation event mediated by a Fyn/Lck-related tyrosine kinase.

We suggest a molecular model to explain the dual regulation of Vav by PI 3-kinase and a tyrosine kinase (Fig. 3). This model takes into account previous biochemical analysis of Vav and related molecules, as well as the studies presented here. The model proposes that the PH domain inhibits GEF activity by binding to the DH domain thereby preventing the association of Rho-GTPases with the DH domain. Activation of GEF activity occurs by events that weaken the contacts between the DH and PH domains, thereby allowing the Rho-GTPases to bind the catalytic DH domain. In its inactive state, Vav is not phosphorylated by Lck-related kinases and the PH domain of Vav is bound to a PI 3-kinase substrate such as PI-4,5-P$_2$. With PI-4,5-P$_2$ bound, the PH domain makes intramolecular contacts with the unphosphorylated DH domain and this contact is of such affinity as to prevent Rac association with the DH domain. Upon stimulation of the resting cell by engagement of mitogenic receptors, tyrosine kinases related to Lck and Fyn are activated as well as PI 3-kinase. The activation of PI 3-kinase causes a reduction in the inhibitory PI 3-kinase substrate and an increase in activating PI 3-kinase products. This change favors the association of PI 3-kinase products with the PH domain of Vav (see below). Upon PI 3-kinase product binding to Vav, the affinity of the PH domain for the DH domain is lessened, allowing an altered conformation where Vav is readily recognized by the Lck-related tyrosine kinase. Tyrosine phosphorylation of Vav further reduces the affinity of the PH domain for the DH domain. The dissociation of the phosphorylated DH domain from the PH-domain bound to PI-3,4,5-P$_3$ now

Fig. 2. Serum-induced Vav phosphorylation, Vav GEF activity, and Vav association with PI-3,4,5-P$_3$. A, quiescent NIH-3T3 cells expressing wild-type Vav, labeled with $^{32}$P-orthophosphate, were stimulated with calf serum. Where indicated, 100 nM wortmannin was added 40 min prior to serum addition. Cells were harvested at 0, 5, 10, 30, and 60 min after adding serum. Vav immunoprecipitates prepared from the cell lysates were analyzed by SDS-PAGE and autoradiography. B, COS7 cells transiently transfected with plasmids for the expression of wild-type Vav and Syk, Fyn, or empty vector were starved for serum to induce growth arrest. One set of transfectants were treated with 100 nM wortmannin for 25 min prior to serum addition. Cells were harvested at 0, 5, 10, 30, and 60 min after adding serum. Vav immunoprecipitates were analyzed for GEF activity using Rac-$^{[3H]}$GDP as a substrate. Values shown represent the percentage of GDP released above that observed without addition of immunocomplexes. Values represent the average of duplicate samples that did not differ by more than 8%. Similar results were obtained in independent experiments. C, quiescent NIH-3T3 cells expressing wild-type Vav were stimulated with calf serum. Where indicated, 100 nM wortmannin was added 40 min prior to serum addition. Cells were harvested at 0, 1, 5, 10, 30, and 60 min after adding serum. Vav immunoprecipitates were analyzed for GEF activity using Rac-$^{[3H]}$GDP as a substrate. Values shown represent the percentage of GDP released above that observed without addition of immunocomplexes. Values represent the average of duplicate samples that did not differ by more than 8%. Similar results were obtained in independent experiments. D, quiescent NIH-3T3 cells overexpressing wild-type Vav and the parental cell line not expressing Vav, labeled with $^{32}$P-orthophosphate, were stimulated with calf serum. Where indicated, 100 nM wortmannin was added 40 min prior to serum addition. Cultures of cells were harvested at 0 and 15 min after addition of serum. Lipids extracted from Vav immunoprecipitates were analyzed after thin layer chromatography and autoradiography. The arrow indicates the position of migration of a PI-3,4,5-P$_3$ standard (PIP3). Similar results were obtained in independent experiments.
permits Rac to bind to the DH domain and hence activation of the GTPases can occur.

This model cannot rule out the possibility that additional factors affect Vav activation. Indeed, activation of Vav via a heterotrimeric G-protein-coupled receptor has been reported (5); however, the mechanism for this activation remains unknown. Given that some PH domains have been found to interact with heterotrimeric G-protein, it is interesting to suggest heterotrimeric G-proteins could provide an additional mechanism for relieving the inhibitory effects of the Vav PH domain.

This model proposes the activation of Vav requires the removal of the inhibitory effects of the PH domain. Significantly, the events reported here that promote or disrupt the intramolecular interaction involving the PH and DH domains lead to inhibition or activation of Vav GEF activity, respectively (3). A negative regulatory role of the PH domain for Vav and Sos has been suggested by cell-based assays, which observed increased phenotypic responses by removal of the PH domains of these GEFs (4, 5).

A role of PI 3-kinase products in the activation of Sos1 GEF activity toward Rac has been proposed to involve disruption of the inhibitory effects of the PH domain (4). The results presented here indicate the molecular mechanism underlying activation of Sos GEF activity involves regulation of intramolecular interactions of the PH and DH domain similar to that proposed for Vav. However, in contrast to Vav, Sos1 GEF activity has not been reported to be regulated by a tyrosine kinase. Whether or not other members of the Dbl family GEFs are regulated by phosphoinositides as are Vav and Sos1 remains to be established.

The activation of Vav and Sos1 may require the control of the PI-4,5-P₂ and PI-3,4,5-P₃ levels in the immediate vicinity of these GEFs. This is likely necessary because the levels of PI-3,4,5-P₃ in total cell membranes does not exceed that of PI-4,5-P₂ even under conditions where PI 3-kinase is active. The direct association of PI 3-kinase with Vav may contribute to changes in the local concentrations of phosphoinositides (21). The recruitment by activated growth factor receptors of both Sos and PI 3-kinase may also affect the phosphoinositide composition in a local environment. The ability of Rac to activate PI 5-kinase (22), resulting in the production of the inhibitory PI-4,5-P₂ may provide feedback inhibition of Vav and Sos1.

The relative affinities of these phosphoinositides for the PH domains of Vav and Sos1 thus will determine the timing of activation of their Rac-GEF activities as PI-4,5-P₂ is converted to PI-3,4,5-P₃. The affinity of the Sos1 PH domain for PI-3,4,5-P₃ has been reported to be approximately 5-fold higher than that for PI-4,5-P₂ (23). A direct determination of the affinity of PI-3,4,5-P₃ for the PH domain of Vav has not been reported. However, PI-3,4,5-P₃ binds Vav with higher affinity than PI-4,5-P₂ (Kₐ ~ 3–4 μM). We previously reported that excess C8-PI-4,5-P₂ was significantly less efficient than excess C8-PI-3,4,5-P₃ in blocking the binding of radiolabeled-C8-PI-3,4,5-P₃ to Vav suggesting PI-3,4,5-P₃ binds with a greater affinity (3). Here we demonstrate that C8-PI-3,4,5-P₃ is more efficient than C8-PI-4,5-P₂ in blocking the binding of ¹²⁵I-C8-PI-4,5-P₂ to Vav. Further, the GEF activity of Vav is inhibited by PI-4,5-P₂, activated by PI-3,4,5-P₃, and activated when both phosphoinositides are present in equal concentration (3). Together, these results indicate that C8-PI-3,4,5-P₃ binds Vav with a modestly higher affinity than does C8-PI-4,5-P₂. These arguments assume that these two phosphoinositides bind Vav at a common or overlapping site. In support of this, we previously found that three distinct PH domain mutants of Vav significantly reduced binding to both PI-4,5-P₂ and PI-3,4,5-P₃ (3). Further, we show here that these mutants exhibit GEF activities that are resistant to the inhibitory effects of PI-4,5-P₂ or activating effects of PI-3,4,5-P₃ (24). Finally, Fig. 1A shows that C8-PI-3,4,5-P₃ can compete with ¹²⁵I-C8-PI-4,5-P₂ for binding to Vav.

This model proposing a regulated interaction of the PH domain with the DH domain (to mask the Rac-binding sequences) finds support in the recently reported structures of Dbl-related GEFs. In the crystal structure of Sos1 (without a phosphoinositide bound to the PH domain) the PH and DH domains are not intimately associated but are linked by a hingelike structure (24). The link between the DH and PH domains is likely a flexible structural element as the defraction data did not permit the determination of a fixed structure. In the crystal structure, the PH and DH domains are oriented at a right angle to each other and the proposed Rac interacting region of the DH domains lies within this right angle. The solution structure of the DH and PH regions of the PIX GEF reveals these two domains behave as independent units without a fixed orientation with respect to each other (25). This again argues that the link between these domain is flexible, allowing multiple orientations. These structural studies together with the binding data presented here suggest the DH/PH region could undergo structural changes where the PH domain could either fold onto and contact the DH domain (thus obscuring the Rac binding region) or move away from the DH domain to permit Rac binding. Our biochemical analysis of Vav indicates that phosphoinositides regulate the intramolecular interaction of the PH and DH domain. Interestingly, phosphoinositide binding to the PH domain of Sos has been shown to induce structural alteration in the region of the PH domain (β3-β4 loop) which in the crystal structure faces the DH domain (24, 26). This type of phosphoinositide-induced structural alteration could therefore
underlie the regulated intramolecular interactions proposed here.

PH domains of many molecules including GAP1(m), GRP1, ARNO, AKT (also known as PKB), PDK1, cytohesin-1, Btk, and PLC-γ have been reported to regulate their interaction with membranes (27–36). This does not exclude a role of these PH domains in allosteric regulation of enzymatic activities. Interestingly, like in the case of Vav, PI 3-kinase-dependent phosphorylation events have been observed for the PH-containing enzymes Btk, ITK, and AKT (37–39). AKT is the best characterized among these. AKT is a serine/threonine kinase that is activated by insulin or growth factors, and this activation parallels an increase in AKT phosphorylation (39). Inhibition of PI 3-kinase by wortmannin blocks the phosphorylation and activation of AKT induced by extracellular stimuli. Like Vav, AKT has a PH domain that mediates the effects of phosphoinositides. Biochemical studies have suggested that a role of the PH domain in PI 3-kinase-dependent activation of AKT involves a PI 3-kinase product (PI-3,4,5-P3)-dependent conformational change or oligomerization of AKT such that the movement of the PH domain exposes critical phosphorylation sites recognized by the kinase, PDK1 (36, 40). However, an intramolecular interactin involving the PH and kinase domains of AKT has not been reported. Therefore, whether AKT and other PH-containing enzymes are regulated in a manner akin to that proposed here for Vav remains to be determined. The inhibitory intramolecular interactions of Vav’s catalytic and PH domains that are promoted by PI-4,5-P2 and disrupted by PI-3,4,5-P3 suggests additional member of the 100 or more PH-containing family of molecules may be similarly regulated.

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