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# Critical Role of the Pleckstrin Homology and Cysteine-rich Domains in Vav Signaling and Transforming Activity*

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**Vav family proteins are members of the Dbl family of guanine nucleotide exchange factors and activators of Rho family small GTPases. In addition to the Dbl homology (DH) domain important for guanine nucleotide exchange factor catalytic function, all Dbl family proteins contain an adjacent pleckstrin homology (PH) domain that serves to regulate DH domain activity. Although the role of the PH domain in Vav function has been evaluated extensively, its precise role and whether it serves a distinct role in different Vav proteins remain unresolved. Additionally, the precise role of an adjacent cysteine-rich domain (CRD) in regulating DH domain function is also unclear. In this study, we evaluated the contribution of these putative protein-protein or protein-lipid interaction domains to Vav signaling and transforming activity. In contrast to previous observations, we found that the PH domain is critical for Vav transforming activity. Similarly, the CRD was also essential and served a function distinct from that of the PH domain. Although mutation of either domain reduced Vav membrane association, addition of plasma membrane targeting sequences to either the CRD or PH domain mutant proteins did not restore Vav transforming activity. This result contrasts with other Dbl family proteins, where a membrane targeting sequence alone was sufficient to restore the loss of function caused by mutation of the PH domain. Furthermore, green fluorescent protein fusion proteins containing the PH domain or CRD, or both, failed to target to the plasma membrane, suggesting that these two domains also serve regulatory functions independent of promoting membrane localization. Finally, we found that phosphatidylinositol 3-kinase activation may promote Vav membrane association via phosphatidylinositol 3,4,5-triphosphate binding to the PH domain.**

Vav is a member of the Dbl family of proteins that function as guanine nucleotide exchange factors (GEFs)[1] (1–3) and activators of Rho family small GTPases (4, 5). Rho family protein function is controlled by a regulated GDP/GTP cycle. Whereas Dbl family proteins function as GEFs and stimulate the formation of the active GTP-bound protein, GTPase activating proteins promote formation of the inactive GDP-bound protein. The GTP-bound protein is the active form and interacts with a spectrum of downstream effector targets to stimulate signaling pathways that regulate actin cytoskeletal organization, gene expression, and cell cycle progression (6). Vav was first identified as a transforming protein where $NH_2$-terminal truncation of negative regulatory sequences converted Vav into a constitutively activated GEF. Subsequently, two closely related proteins, Vav2 and Vav3, were identified that shared strong sequence similarity (approximately 70%) and structural topography with Vav. Overlapping, as well as very distinct, functions have been described for the different Vav proteins (7). All Dbl family proteins share a catalytic Dbl homology (DH) that is followed invariantly by a pleckstrin homology (PH) domain (1, 3). The DH domains of Vav proteins function as GEFs for multiple Rho family proteins, including RhoA, RhoG, Rac1, and Cdc42 (8–14). Although PH domains can be found on signaling molecules other than Dbl family proteins (15), the invariant DH/PH domain organization suggests that the PH domain serves as a critical regulator of DH domain function. Current evidence suggests two roles for the PH domain in regulating DH domain function. First, the mutation of the PH domains of several Dbl family proteins (Lfc and Dbs) has been shown to abolish their transforming activity, and the addition of the plasma membrane targeting sequence of Ras proteins can restore transforming activity (16, 17). In contrast, the critical function of the PH domain of Dbl could not be restored by addition of a plasma membrane targeting sequence (18). Thus, the PH domain can serve simply as a membrane targeting sequence for some but not all Dbl family proteins. Whether the PH domain of Vav serves such a role has not been determined.

A second function of the PH domain may be to serve as a negative, intramolecular regulator of DH domain function. Broek and colleagues found that the PH domain of Vav, like other PH domains (19), can bind phosphatidylinositol 4,5-biphosphate ($PIP_2$) and phosphatidylinositol 3,4,5-triphosphate ($PIP_3$) (20, 21). Whereas $PIP_2$ association with proto-Vav did not promote its GEF function, $PIP_3$-mediated dissociation of $PIP_2$ binding enhanced its GEF function *in vitro*. Mutations in the PH domain that impaired $PIP_2$/$PIP_3$ binding caused increased GEF function. Thus, a model was proposed where $PIP_2$ association with the PH domain served as a negative regulator of DH domain function, and the loss of $PIP_2$ binding caused by $PIP_3$ binding relieved this negative regulation. Consistent with a negative regulatory role, Abrams and colleagues (22) found that mutation of the PH domain of full-length Vav led to a constitutively activated protein. In contrast, the PH domain

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[1] The abbreviations used are: GEF, guanine nucleotide exchange factor; DH, Dbl homology; PH, pleckstrin homology; SH2, Src homology 2; SH3, Src homology 3; CRD, cysteine-rich domain; GFP, green fluorescent protein; PI3K, phosphatidylinositol 3-kinase; $PIP_2$, phosphatidylinositol 4,5-bisphosphate; $PIP_3$, phosphatidylinositol 3,4,5-trisphosphate; PKC, protein kinase C; HA, hemagglutinin; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline.

was found to be dispensable for Vav3 biological activity (13), whereas we recently determined that the PH domain served a critical positive regulatory role for Vav2 function (23). These observations suggest the possibility that the PH domain serves divergent functions for the closely related Vav proteins.

Beyond the DH/PH domain structure, Dbl family proteins show little or no sequence similarities in their remaining sequences (1, 3). In addition to the DH and PH domains, Vav family proteins also contain a diverse array of putative protein-protein or protein-lipid interaction domains that can potentially serve as negative or positive regulators of Vav function (reviewed in Refs. 7 and 24). The $NH_2$ terminus of Vav contains a leucine-rich, calponin homology and an acidic amino acid-rich domain. Because $NH_2$-terminal truncation of these sequences converts Vav to a potent transforming protein, these sequences serve as negative regulators of Vav function (25, 26). Following the PH domain, Vav contains a cysteine-rich domain (CRD), two Src homology 3 (SH3) domains, and a SH2 domain at its COOH terminus (27). This domain organization is conserved in all three Vav family proteins. Although introduction of missense mutations into the SH2 and SH3 domains caused a loss of Vav transforming activity (28, 29), subsequent deletion analyses showed that all the SH2/SH3 domains were dispensable for the transforming activity of all three Vav proteins (10, 13, 23). The basis for these contrasting observations is not known.

In contrast to the SH domains, either missense mutation or deletion of the CRD has shown that this domain is critical for the biological function of all three Vav proteins (10, 13, 23, 30). Although the precise role of the CRD in Vav function still remains to be elucidated, CRDs in other proteins have been shown to facilitate translocation to the plasma membrane, *e.g.* the CRDs of protein kinase C or RasGRP proteins bind diacylglycerol and phorbol esters, which in turn promote their translocation to the plasma membrane (31–33). Mutation of the CRD of RasGRP causes a loss of transforming activity, and addition of a plasma membrane targeting sequence can restore the loss of CRD function. Although the CRD of the Raf-1 serine/threonine kinase does not bind diacylglycerol or phorbol esters, it can interact with Ras to facilitate Raf-1 association with the plasma membrane to allow activation of its kinase function (34). The CRD of Raf-1 also binds the 14-3-3 adaptor protein as well as phospholipids. Thus, although the CRD of Vav does not bind diacylglycerol, it may still serve as a protein-protein or protein-lipid interaction domain that facilitates Vav association with the plasma membrane.

Despite extensive analyses, the precise contribution and functions of the PH domain and CRD to Vav signaling and transformation remain unclear. Conflicting observations have been made that may reflect differences in experimental analyses. To attempt to clarify the role of these two domains in regulating Vav DH domain function, we have evaluated their functions within the context of the minimal functional unit of Vav, the DH/PH/CRD fragment. This approach avoids potential complications that may arise from the presence of other interaction domains present in Vav. Another issue that we have addressed is whether these domains serve similar or distinct functions for other Vav proteins. To accomplish this, we have taken the identical approaches that we have used recently to study the role of the PH domain and CRD in Vav2 function (23). We found that, identical to our observations with Vav2, the PH domain and CRD of Vav are both critical for Vav biological activity. Thus, we find no isoform differences in PH domain or CRD function for Vav proteins. Additionally, whereas both domains do facilitate DH domain association with membranes, alone they do not function as membrane targeting sequences nor can their functions be replaced by plasma membrane targeting sequences. Finally, we determined a limited role for PI3K activation in promoting Vav membrane association and activity.

## EXPERIMENTAL PROCEDURES

*Molecular Constructs*—cDNA sequences encoding mutations in the SH3 domains were made using the Chameleon site-directed mutagenesis kit (Stratagene). Mouse proto-*vav* cDNA sequences subcloned into the mammalian expression vector pCTV3HA, which contains sequences from a hemagglutinin (HA) epitope tag at its $NH_2$ terminus, has been described previously (16, 30). A 730-base pair (bp) *Hpa*I fragment containing a unique *Bam*HI site at the COOH-terminal end of Vav, just prior to the start of the first SH3 domain, was generated by polymerase chain reaction (PCR). This fragment was ligated to the pCR 2.1 vector from the TA cloning kit (Stratagene), sequenced, and then used as the template DNA for mutagenesis. To generate the single mutations within each SH3 domain (W636K and W820K), mutagenic primers (5′-ATT CCT TCC CTC CCA CTT GTT GTG CTC AGC CTC and 5′-GAT CTC CCC ACG CCA CTT GCC TTG CTG TCC) were used, respectively, along with a *Xho*I → *Cla*I selection site primer (5′-CCC TCT AGA TGC ATG ATC GAT CGG CCG CCA GTG). When the mutations were verified by sequencing, the double mutant was constructed by using the W820K mutant sequence as a template, the W636K mutagenic primer, and a second *Hin*dIII → *Sal*I selection site primer (5′-GCT CGG TAC CGT CGA CGG CGT AAT CAT GG). The pCR 2.1 plasmids containing the correct mutations were then digested with *Hpa*I and ligated to pCTV3HA. This plasmid was then digested with *Bam*HI and a 2.3-kilobase (kb) fragment containing the appropriate mutations, and 1.5 kb of the COOH terminus of pCTV3HA was isolated. To complete the sequences with the $NH_2$-terminal portion of *vav*, pCTV3HA ΔN-186 *vav* (described in Ref. 26), was digested with *Bam*HI and the $NH_2$-terminal sequences were ligated to the SH3 domain mutant *Bam*HI fragments. ΔN-186 *vav* ΔC was constructed in a similar fashion as was done for proto-*vav*ΔC (described in Ref. 26), except that a *Fse*I/*Bsi*WI ΔN-186 *vav* fragment was used instead of a *Fse*I/*Bsi*WI proto-*vav* fragment.

cDNA sequences encoding amino acid substitutions in the PH domain were generated by PCR-mediated oligonucleotide site-directed mutagenesis. These constructs all terminated just prior to the first SH3 domain similar to the ΔN-186 VavΔC mutants. Because the lysine residue at position 404 lies close to the unique *Fse*I site in Vav, the K404A PH mutant could be made directly from the 5′ primer (5′-CA GTT AAC GGC CGG CCC GCG ATT GAC GGT GAG CTC). The 3′ primer corresponding to amino acids 594–600 (3′-CA GTT AAC CTA CTA GGA TCC CTT AGG CAG ACC CAA TTC) contains two stop codons flanked by a *Bam*HI site and a *Hpa*I site at the 3′ end. The W495L mutant was generated by four primer PCR using the unique *Fse*I site. Two fragments were generated, a 280-bp fragment (5′-CA GTT AAC GGC CGG CCC AAG ATT GAC GGT GAG and 3′-CG AAC TGT TCC ATC AGC TTC TTC TTC AGC) and a 330-bp fragment (5′-GAG CTG AAG AAG AAG CTG ATG GAA CAG TTC G and 3′-CAG GCC GGC CAG GAT CCC ATA GTA TTC CTG AAA CAC TT), which were then annealed together and amplified by PCR. Sequences encoding both PH mutants K404A and W495L were contained in a 610-bp fragment, which was then digested with *Hpa*I and ligated into the pCTV3HA plasmid. This plasmid was digested with *Fse*I/*Bsi*WI and ligated to the pCTV3HA ΔN-186 *Fse*I/*Bsi*WI fragment to complete the construct.

The pCTV3HA ΔN-186 *vav*ΔCRD mutant was constructed by PCR utilizing the same 5′ primer used for the W495L PH domain mutant and a 3′ primer (3′-CA GTT AAC CTA CTA GGA TCC CCC ATT GGC TGT AGC ATT) corresponding to residues 509–515 with two stop codons flanked by a *Bam*HI and a *Hpa*I site at the 3′ end. This 333-bp fragment was digested with *Hpa*I and ligated into pCTV3HA. To add the remaining $NH_2$-terminal sequences, the pCTV3HA construct was digested with *Fse*I/*Bsi*WI and ligated to the *Fse*I/*Bsi*WI fragment from pCTV3HA ΔN-186 *vav*.

To make membrane targeting versions of ΔN-186 *vav*ΔC PH(K404A), ΔN-186 *vav*ΔC PH(W495L), and ΔN-186 *vav*ΔCRD, sequences encoding the last 18 remaining amino acids from K-ras4B (includes a polylysine-rich region and the C*AAX* prenylation signal CVIM) were ligated to each construct at the *Bam*HI restriction enzyme site just prior to the tandem stop codons. Briefly, two primers corresponding to the aforementioned K-ras4B sequences (5′-CA GTT AAC GGA TCC AGC AAA GAT GGT AAA AAG AAG AAA AAG AAG TCA AAG ACA AAG TGT GTA ATT ATG TAA; 3′-CA GTT AAC TTA CAT AAT TAC ACA CTT TGT CTT TGA CTT CTT TTT CTT CTT TTT ACC ATC TTT GCT) containing *Hpa*I restriction enzyme sites at each end and a novel

*Bam*HI at the 5′ end were annealed together and then digested with *Hpa*I. The 75-bp fragment was ligated to a *Hpa*I-digested pCTV3HA expression vector (pCTV3HA *vav*-C*AAX*). Because the cognate vector pCTV3HA contains a unique *Bam*HI restriction enzyme site downstream from its multiple cloning site, digestion of pCTV3HA *vav*-C*AAX* with *Bam*HI derives a 1.6-kb fragment that can be ligated to the *Bam*HI site introduced into the mutated PH domain and ΔCRD constructs.

The green fluorescent protein (GFP) fusion expression constructs of the *vav* PH, CRD, and PH/CRD domains were constructed by PCR amplification of these regions from the cDNA sequence in ΔN-186 *vav* in pAX142. The PH domain containing amino acids 368–517 was amplified with primers incorporating an in-frame 5′ *Xho*I restriction site and a stop codon adjacent to a 3′ *Hin*dIII site. The CRD and PH/CRD encoding fragments were created in similar fashions to include amino acids 507–573 and 368–573, respectively. The digested fragments were ligated into the *Xho*I/*Hin*dIII sites of the pEGFP-C3 mammalian expression vector (Clontech). The products were verified by sequencing.

*Cell Culture and Transformation Assays*—NIH 3T3 mouse fibroblasts and 293T human kidney epithelial cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum or fetal calf serum, respectively. DNA transfections were performed by calcium phosphate precipitation method as described previously (35). For transformation studies, the cognate empty vector was used as a control. The transfected cultures were maintained in culture for 14 days, fixed, and stained with crystal violet (0.5%), and then the number of foci of transformed cells was quantitated. To establish cell lines that stably expressed the Vav mutants, transfected NIH 3T3 cells were selected in growth medium supplemented with hygromycin (400 μg/ml) and multiple drug-resistant colonies (>100) were pooled together after 10 days of selection to establish mass populations of stably transfected cells.

*Transient Expression Reporter Gene Assays*—Transient transfections using various reporter plasmids to determine transcriptional activity were performed as described previously (36). NIH 3T3 cells were transfected by calcium phosphate precipitation, allowed to recover for 30 h, and were then serum-starved (0.5% calf serum) for 14–15 h before lysing with luciferase lysis buffer (Amersham Biosciences). For the ΔN-186 Vav and p110-C*AAX* cooperation assays, NIH 3T3 cells were transfected with LipofectAMINE Plus reagent (Invitrogen) according to the protocol from the manufacturer. After transfection, these cells were treated exactly as described above. The p110-C*AAX* construct was described previously (37). The lysates were analyzed using enhanced chemiluminescent reagents and a Monolight 2010 luminometer (Analytical Luminescence). All the assays were performed at least in triplicate.

*Membrane Fractionation Analyses*—Subcellular fractionation was performed as described previously (26). 293T cells were transiently transfected with 3 μg of the pAX142 empty vector or pAX142 constructs encoding ΔN-186 Vav, ΔN-186 VavΔC, the ΔN-186 VavΔC PH domain mutants, and the ΔN-186 VavΔCRD mutants. After transient transfection, 293T cells were grown for 48 h, then gently washed twice with ice-cold phosphate-buffered saline (PBS), and resuspended in 1 ml of cold TSA buffer (2 mM Tris, pH 8.0, 0.14 M NaCl, 0.25 M sucrose, 1 mM EDTA, 100 μg/ml phenylmethylsulfonyl fluoride, 25 μg/ml leupeptin). The cells were homogenized and centrifuged (15,000 × *g*) to acquire total (T) protein (200 μl of supernatant). The remaining supernatant was then centrifuged (100,000 × *g*) in a Beckman TLS-55 rotor for 2 h to separate the supernatant into crude cytosolic (S100) and membrane and cytoskeletal protein containing (P100) fractions. The protein concentrations of the total, cytosolic, and membrane samples were determined using a BCA protein assay kit (Pierce). Protein fractions (25 μg) were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to Immobilon-P membranes, and probed with anti-HA epitope antibody (Babco).

*Protein Analysis*—Protein concentrations of the transiently transfected 293T cells or in the NIH 3T3 cells stably expressing the Vav mutants were determined by the BCA kit (Pierce). Protein expression was determined by Western blot analysis and detected by chemiluminescence (Amersham Biosciences) and the anti-HA epitope antibody as described previously (38).

*Confocal Microscopy*—NIH 3T3 cells were transiently transfected with pEGFP-C3 vector or pEGFP-C3 constructs encoding GFP-Vav PH, GFP-Vav PH/CRD, or GFP-Vav CRD, or the previously reported pEGFP-C1 Akt-PH and pEGFP-N1 PKCγ-C1A-(26–89) (39, 40). Transfections were carried out with LipofectAMINE Plus reagent (Invitrogen) following the suggested protocol. After transfection, cells were grown in DMEM with 10% calf serum for 6 h and then serum-starved in DMEM for 18 h. The cells were washed once with PBS and kept in PBS for visualization of live cells. For dishes that were serum-stimulated, images were taken 60 s after addition of PBS supplemented with 10% calf serum. Microscopy was performed on an Olympus Fluoview FV 300 laser scanning biological microscope. Images are stacks of serial scans taken through each cell at 0.4-μm thickness.

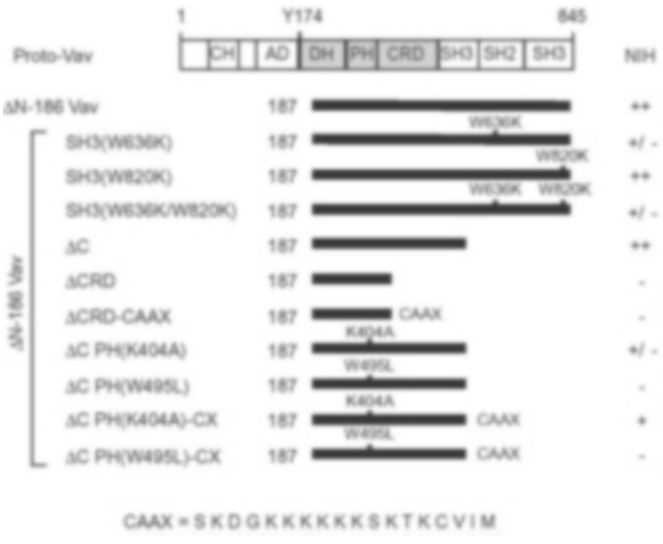


FIG. 1. **Domain structure of the ΔN-186 Vav COOH-terminal point and deletion mutants.** Shown are the domain structure of full-length Vav (proto-Vav), and the SH3 domain, CRD, and PH domain mutants. Figure shows calponin homology (*CH*) domain, acidic domain (*AD*), DH domain, PH domain, CRD, SH3 and SH2 domains, and 18-amino acid plasma membrane targeting sequence from K-Ras4B (C*AAX*). The *lines below* proto-Vav show the predicted translational products of the various Vav mutants. The amino acid substitutions are shown *below* the *lines*. All the mutants were constructed within ΔN-186 Vav and fused in-frame to a HA epitope tag at the $NH_2$ terminus. *NIH*, focus-forming activity in NIH 3T3 transfection assays: ++, 100% or more of activity of ΔN-186 Vav (~4 × 10 (3) foci/pmol); +, 25–50% of ΔN-186 Vav; +/− < 10% of ΔN-186 Vav; −, no focus-forming activity.

## RESULTS

*The DH/PH/CRD Is the Minimal Functional Transforming Fragment of Vav*—Previous studies showed that point mutations in the SH2 domain or the COOH-terminal SH3 domain decreased Vav transforming activity (28–30), whereas other studies found that deletion of the entire SH3/SH2/SH3 domains of Vav proteins did not cause loss of transforming activity (10, 13, 23). The basis for these opposite observations is not clear but may reflect different consequences of missense and deletion mutagenesis, or alternatively, differences in Vav constructs used. To distinguish between these possibilities, we compared the consequences of missense and deletion mutations of the SH3 domains of the $NH_2$-terminal truncated and highly transforming ΔN-186 Vav mutant protein.

Mutation of the conserved tryptophan present in a majority of SH3 domains to lysine has been shown to disrupt the function of other SH3 domains (41, 42). We made the analogous mutations at residue 636 (W636K) in the $NH_2$-terminal SH3 domain or at residue 820 (W820K) in the COOH-terminal SH3 (Fig. 1). We also made a Vav mutant with mutations in both SH3 domains (W636K/W820K) as well as a ΔN-186 Vav mutant that lacked the entire SH3/SH2/SH3 region (ΔN-186 VavΔC).

First, we determined whether the different point or deletion mutations caused any significant alterations in protein stability. Western blot analyses of cells transiently (Fig. 2) or stably (data not shown) expressing each SH3 mutant showed that each HA epitope-tagged Vav proteins was expressed at comparable levels. Thus, differences in biological activity would not be influenced by differences in protein stability or level of expression. We next performed focus formation transformation assays in NIH 3T3 cells to evaluate the effect of the SH3

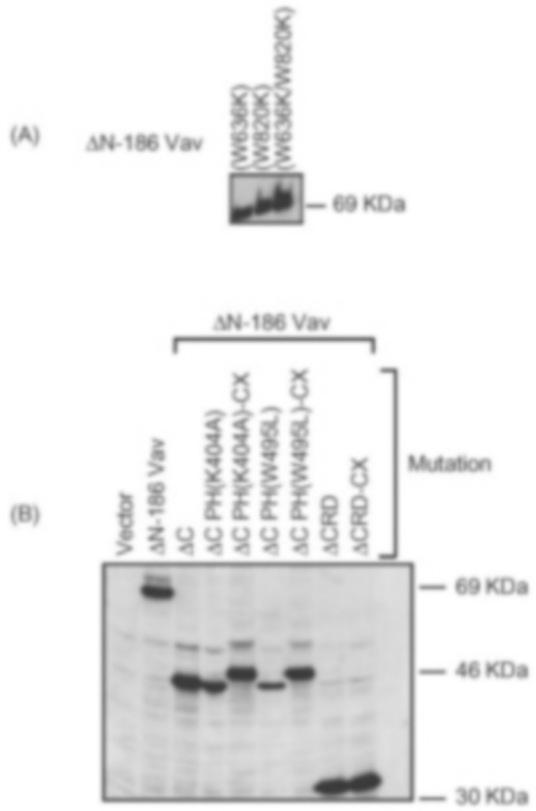


FIG. 2. **Expression of Vav SH3 mutants (*panel A*) and Vav PH domain and ΔCRD mutants (*panel B*) in 293T cells.** 293T cells were transiently transfected with the cognate empty pAX142 mammalian expression vector or pAX142 encoding the indicated proteins. Cell lysates (25 $\mu$g) were assayed for protein expression by Western blot analyses with the anti-HA epitope antibody.

domain mutations, as well as removal of the entire SH3/SH2/SH3 region, on Vav transforming activity (Fig. 3). We found that the transforming activities of the ΔN-186 Vav SH3(W636K) and ΔN-186 Vav SH3(W636K/W820K) mutants were decreased 3–6-fold, respectively. In contrast to the decreased activity described in a previous study (28), we found that the ΔN-186 Vav SH3(820K) mutant showed an enhanced focus-forming ability. However, when we deleted the entire SH3/SH2/SH3 domain (ΔN-186 VavΔC), we were surprised to see that it was enhanced significantly in transforming activity. These results suggest that a functional $NH_2$-, but not COOH-, terminal SH3 domain, within the context of the intact SH3/SH2/SH3 domains, is required for full Vav transforming activity. However, the potent transforming activity of ΔN-186 VavΔC clearly demonstrates that all three SH domains are dispensable for Vav transforming activity. Thus, we suspect that the different consequences of the SH3 domain point mutations may be the result of indirect consequences on adjacent domains. Finally, no transforming activity was detected with the SH domains were deleted from proto-Vav (proto-VavΔC), indicating that, unlike deletion of the $NH_2$-terminal sequences, deletion of COOH-terminal sequences does not result in constitutively activated Vav proteins.

*The PH Domain Alone Is Not Sufficient to Support DH Domain Transformation*—Point mutations that disrupt the structure of the CRD of Vav have been shown previously to decrease the transforming activity of onco-Vav (30). However, because we observed that point mutations and deletion mutants in the SH3/SH2/SH3 domain region of Vav had different consequences on Vav transforming activity, we determined whether complete deletion of the CRD would also cause a loss of Vav function. The previously described CRD mutations were made in a version of Vav that retained the SH3/SH2/SH3 domains. Therefore, we generated a mutant of the ΔN-186 VavΔC mutant that lacked the CRD (ΔN-186 VavΔCRD) (Fig. 1). Similar to previous observations made with point mutations in the CRD of Vav, we found that ΔN-186 VavΔCRD lacked any detectable focus-forming activity (Fig. 3).

By analogy to the CRDs of PKC and Raf-1, the CRD may bind phospholipids and serve to promote Vav membrane association (32, 43). To address this possibility, we determined whether removal of the CRD altered Vav membrane association and whether addition of a plasma membrane-targeting sequence could restore the transforming activity of ΔN-186 VavΔCRD. A chimeric protein was generated, where the 18 COOH-terminal amino acids of K-Ras4B were added onto the COOH terminus of ΔN-186 VavΔCRD (designated ΔN-186 VavΔCRD-CX) (Fig. 1). This sequence signals for the farnesylation and other post-translational modifications of K-Ras4B and can target heterologous proteins to the plasma membrane (44). Protein expression was determined by Western blot analysis using cell lysates from 293T cells transiently transfected with the ΔCRD mutants (Fig. 2) and from cell lysates of NIH 3T3 cells stably expressing these mutants (data not shown) and verified that these CRD variants were not altered in protein stability.

We first evaluated whether Vav membrane association was altered upon removal of the CRD and whether addition of the membrane targeting sequence restored membrane association. We performed crude subcellular fractionation (100,000 $\times$ *g*) with cell lysates from 293T cells transiently transfected with the Vav ΔCRD mutants (Fig. 4). We saw significant reduction in membrane association with the deletion of the CRD. Whereas ΔN-186 VavΔC was found predominantly in the P100 membrane (and cytoskeletal protein-containing) fraction (80%) (Fig. 5), the ΔN-186 VavΔCRD mutant was found predominantly in the S100 cytosolic fraction (66%), and showed a 40% shift from membrane to cytosolic fractions (Fig. 4). These results support a role for the CRD in facilitating Vav association with membranes or cytoskeletal components. However, the ΔN-186 VavΔCRD-CX mutant was found equally distributed between the S100 and P100 fractions. Therefore, we saw only a partial restoration of membrane association with the membrane-targeted version of the Vav ΔCRD mutant. These experiments were repeated with NIH 3T3 cells stably expressing the Vav ΔCRD mutants, and similar results were observed, suggesting the high degree of overexpression in the transient assays did not interfere with localization of the mutants (data not shown). Finally, the addition of a membrane targeting sequence to the DH/PH domains of Vav did not restore transforming activity (Fig. 3). These results suggest that the CRD does serve a critical role in Vav transformation that is not substituted for by a plasma membrane targeting sequence. These observations contrast with our previous studies of the CRD of RasGRP, where a plasma membrane targeting sequence did restore the loss of function caused by mutation of the CRD (33).

We showed previously that the highly transforming mutant ΔN-186 Vav could stimulate the transcriptional activity of the Elk-1, Jun, and SRF transcription factors (26). Therefore, we next assessed the ability of the Vav ΔCRD mutants to stimulate these transcriptional activities to determine whether the loss of transforming activity was associated with the loss of a particular signaling pathway. However, we found that deletion of the CRD abolished the ability of Vav to activate any of these signaling pathways (data not shown). Furthermore, we did not see any restoration of these activities with the membrane-targeted ΔN-186 VavΔCRD-CX mutant. Therefore, our data suggest that the CRD is critical for mediating the Vav signaling

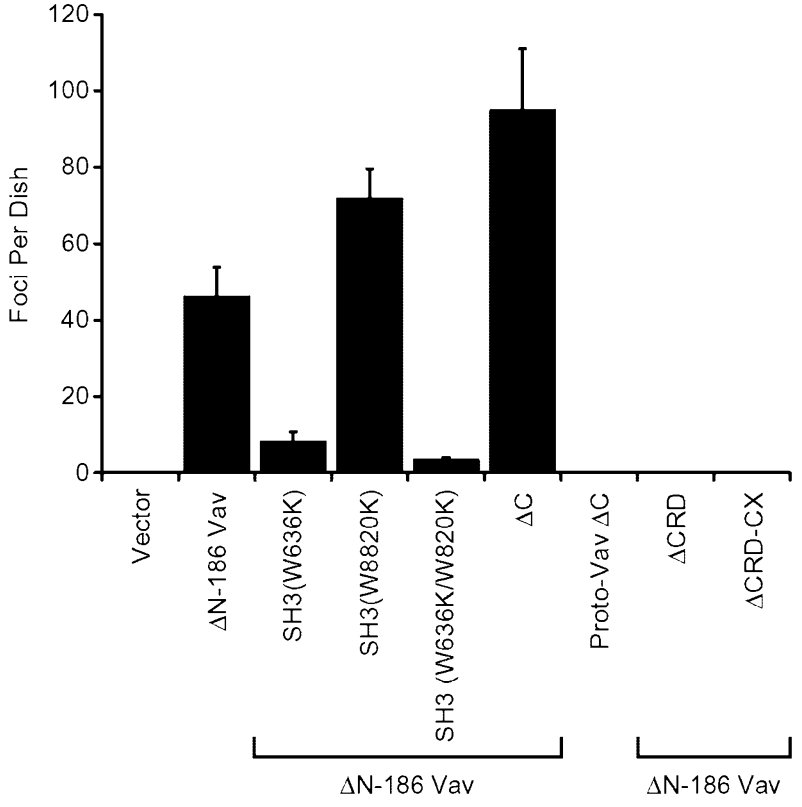


FIG. 3. **The SH2 and SH3 domains, but not CRD, are dispensable for Vav transformation.** NIH 3T3 cells were transfected with pAX142 expression plasmids encoding the various SH3 domain mutants of Vav (30 ng of DNA/60-mm dish). Focus-forming activity was scored 14 days after transfection. The values represent the average ± standard error of three dishes. These results were normalized to the number of foci per picomole of transfected DNA to account for differences in plasmid size, but this did not significantly alter the results (data not shown). Data shown are representative of three independent assays.

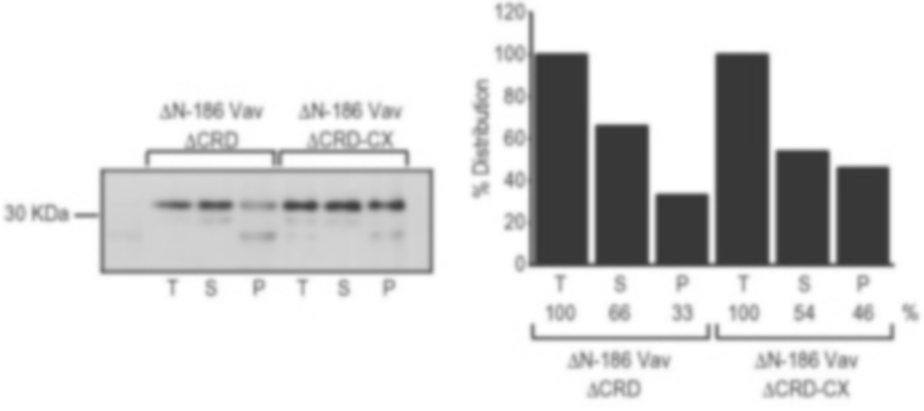


FIG. 4. **Subcellular distribution of the Vav ΔCRD mutants.** 293T cells were transiently transfected with the empty pAX142 mammalian expression vector or pAX142 plasmids encoding the ΔN-186 VavΔCRD mutant proteins. Forty-eight hours after transfection, the cells were lysed and subjected to high speed centrifugation (100,000 × *g*) to separate into crude cytosolic (S100) and membrane (P100) fractions. Twenty-five ng protein of the total (T), cytosolic (S100), and membrane (P100) fractions were resolved by SDS-PAGE and protein expression was determined by Western blot analysis using the anti-HA epitope antibody.

required for its transforming activity. Thus, Vav contrasts with the majority of other Dbl family proteins where the tandem DH/PH domains alone are sufficient for activity *in vivo*.

*Mutation of the PH Domain Disrupts Vav Signaling and Transforming Activity*—In contrast to the essential role of the PH domain in the function of most Dbl family proteins, mutational analyses showed that the PH domain of Vav family proteins serves as a negative regulator of DH domain function (22) and, hence, was found to be dispensable for Vav and Vav3 transforming activity (13, 20). In light of our recent determination that the PH domain is critical for the transforming activity of the related Vav2 protein (23), suggesting distinct functional roles for the PH domain in Vav isoforms, we initiated studies to further evaluate the importance of the PH domain in Vav function. We performed these studies with the equivalent DH/PH/CRD fragment of Vav (ΔN-186 VavΔC) as we did with Vav2 to avoid complications from other adjacent interaction domains and to allow us to compare our results with Vav and Vav2.

We introduced two PH domain mutations into ΔN-186 VavΔC (Fig. 1). First, we mutated the tryptophan residue present in the Vav PH domain (W495L) that corresponds to the conserved tryptophan residue found in all PH domains (designated ΔN-186 VavΔC PH(W495L)). This mutation is expected to cause a drastic reduction in PH domain structure and function (41, 42). Second, we also introduced a mutation at a lysine residue (K404A) that was shown previously to be important for Vav PH domain binding of phosphoinositides (designated ΔN-186 VavΔC PH(K404A)) (20). Broek and colleagues (20) showed previously that the K404A mutation greatly impaired $PIP_2$ binding *in vitro*. In anticipation that disruption of PH domain function will impair Vav membrane association, we also generated membrane-targeted versions of these PH domain mutants (Fig. 1). Western blot analyses of transiently transfected 293T cells (Fig. 2) or stably transfected NIH 3T3 cells (data not shown) were done to verify that the introduced mutations did not perturb protein stability.

Because the PH domain has been shown to contribute to the membrane association of at least some Dbl family proteins (16, 17), we performed crude subcellular fractionations to determine the effects of the PH domain mutations on Vav membrane association (Fig. 5). Although both PH domain mutations

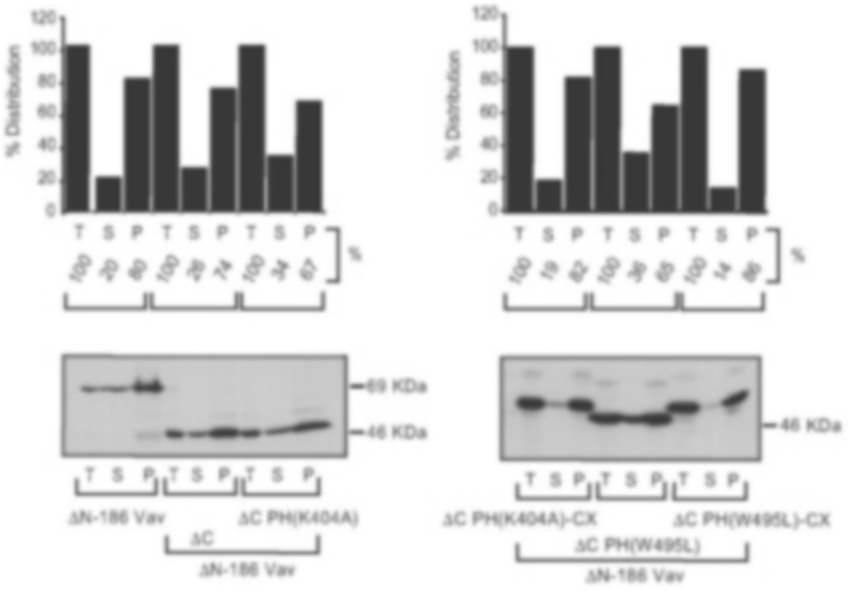


FIG. 5. **Subcellular distribution of the Vav PH domain mutants.** 293T cells were transiently transfected with the empty pAX142 mammalian expression vector and pAX142 plasmids encoding the various PH domain mutants. The cultures were lysed 48 h after transfection and subjected to high speed centrifugation (100,000 × *g*) to separate cytosolic and membrane fractions. Twenty-five ng total protein for total (T), cytosolic (S100), and membrane (P100) fractions were resolved by SDS-PAGE. Protein expression was determined by Western blot analysis using the anti-HA epitope antibody.

caused a slight reduction in the amount of protein found in the P100 fraction, both retained predominant association with the P100 membrane-containing fraction (~65%). The membrane-targeted versions of both proteins, designated ΔN-186 VavΔC PH(K404A)-CX and ΔN-186 VavΔC PH(W495L)-CX, showed greatly increased association with the P100 crude membrane fractions (>80%) that was equivalent to that of the non-mutated ΔN-186 VavΔC protein. Fractionation studies done with NIH 3T3 cells stably expressing the ΔN-186 VavΔC PH mutants yielded similar results (data not shown). These results suggest that the PH domain may make a minor but significant contribution to Vav membrane association.

We next performed NIH 3T3 focus formation assays to assess the consequences of the PH domain mutations on Vav transforming activity. For these analyses, we used pAX142 expression vectors encoding each of the PH domain mutants and compared their activities with the oncogenic ΔN-186 VavΔC mutant protein (Fig. 6). Whereas the ΔN-186 VavΔC PH(K404A) mutant showed a greater than 30-fold reduction in focus-forming activity, the ΔN-186 VavΔC PH(W495L) mutant showed a complete loss of transforming activity. Addition of a membrane targeting sequence (ΔN-186 VavΔC PH(K404A)-CX) caused a partial 5-fold increase in transforming activity that was still approximately only 30% of the activity seen with the non-mutated protein. Thus, our data indicate the loss of Vav transforming activity in ΔN-186 VavΔC PH(K404A) may be caused by a loss, in part, of PH domain-mediated membrane association. In contrast, despite its strong association with membranes, the membrane-targeted ΔN-186 VavΔC PH(W495L)-CX protein still lacked any detectable focus-forming activity (Fig. 6). Finally, we compared the ability of the PH domain mutants to stimulate transcriptional activation of c-Jun, Elk-1, SRF, or NF-κB, and we found that the degree of stimulation seen with all transcription factors correlated with transforming activity (Fig. 7). These observations suggest that the PH domain may serve an additional role distinct from facilitation of Vav association with membranes. Additionally, because the PH domain mutants retained the CRD, these two COOH-terminal domains must serve distinct roles in facilitating Vav signaling and transformation.

*Vav PH Domain and CRD Are Not Sufficient for Membrane Targeting*—Our analyses above indicated that the requirement for the PH domain and the CRD in Vav transformation may be caused, in part, by facilitating membrane association. This

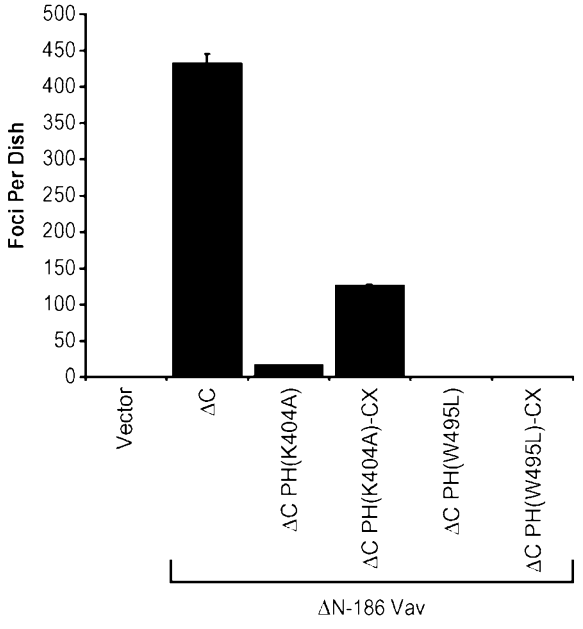


FIG. 6. **Mutation of the PH domain of Vav decreases Vav transforming activity.** NIH 3T3 cells were transfected with the empty pAX142 plasmid or pAX142 plasmids encoding the various PH domain mutants (1 μg/60-mm dish). The appearance of transformed foci was quantitated on day 14. The values represent the average ± standard error of three dishes and are representative of three independent assays.

possibility is consistent with observations that the PH domain (*e.g.* Akt) or CRD (*e.g.* PKC) of some signaling proteins do function as membrane-targeting domains. Therefore, we evaluated the possibility that the isolated PH domain or CRD of Vav could promote association with the plasma membrane.

For these analyses, we generated GFP-tagged fusion proteins that contained either the isolated PH domain or the CRD, or both (40). These were designated GFP-Vav PH, GFP-Vav CRD, and GFP-Vav PH/CRD, respectively. We utilized GFP fusion proteins containing the PH domain of Akt (GFP-AKT PH) and the first CRD of PKCγ (GFP-PKC CRD) as positive controls for these analyses. GFP-AKT PH has been shown to localize to the plasma membrane in response to PI3K activation and formation of $PIP_3$ (39, 45), whereas GFP-PKC CRD

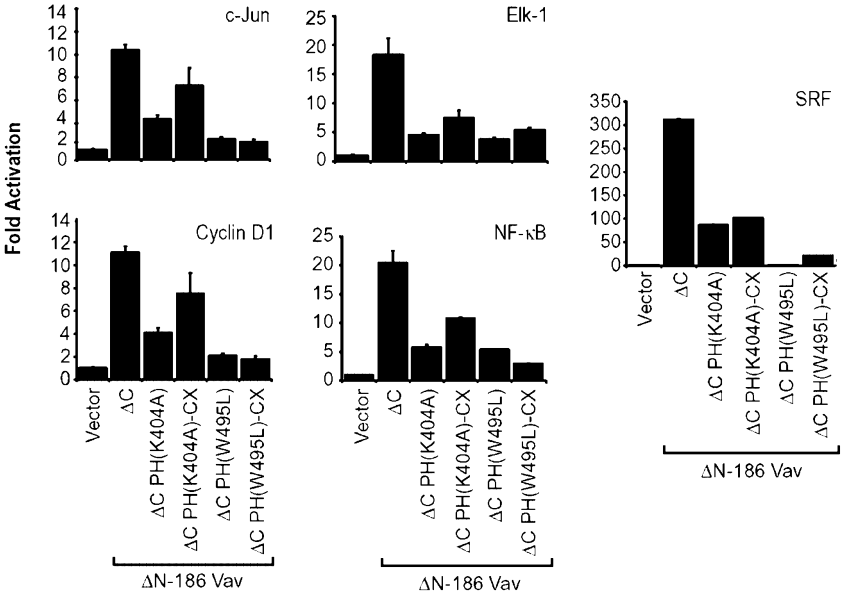


FIG. 7. **Mutation of the PH domain of Vav decreases Vav signaling activity.** NIH 3T3 cells were transiently transfected with the empty pAX142 mammalian expression vector or pAX142 plasmids encoding the various PH domain mutants (500 ng/30-mm dish), together with luciferase reporter plasmids to determine the activation of c-Jun, Elk-1, SRF, NF-κB, and expression from the cyclin D1 promoter. Data shown are the average of duplicate plates and are representative of at least three independent assays.

localized to the plasma membrane upon treatment with the diacylglycerol analog, phorbol myristic acid (40). We transiently transfected NIH 3T3 cells with the expression plasmid DNAs encoding the different GFP fusion proteins. In agreement with previous studies, we found that both GFP-AKT PH and GFP-PKC CRD showed strong localization to the plasma membrane (Fig. 8). In contrast, cells expressing GFP-Vav PH, GFP-Vav CRD, or GFP-Vav PH/CRD showed a diffuse cytoplasmic staining and lacked significant plasma membrane staining. Although it is possible that these domains may be misfolded in the GFP fusion proteins, their significant level of expression in NIH 3T3 cells argue that this is not the case. Thus, in contrast to related domains in other signaling proteins, the PH domain and CRD of Vav, when expressed as isolated domains, are not sufficient to function as plasma membrane-targeting sequences. These results are also consistent with our fractionation studies that showed that each domain had limited roles in promoting Vav membrane association.

*Phosphatidylinositol 3-Kinase Does Not Greatly Enhance Vav Transformation or Signaling*—Previous studies showed that the products of PI3K, such as $PIP_3$, can interact with the PH domain and promote Vav catalytic activity *in vitro* (20, 21). Additionally, it was shown that the PI3K substrate $PIP_2$ promoted the interaction of the PH domain with the DH domain, thus inhibiting DH domain interaction with its GTPase substrate (21). In contrast, $PIP_3$ association with the PH domain disrupted this association, leading to GTPase association with the DH domain. These observations suggest that the activation of PI3K should enhance the transforming and signaling properties of Vav.

Next, we wanted to determine whether co-expression of activated PI3K would cause synergistic enhancement of Vav signaling or transforming activity. These studies used p110-C*AAX*, which is a plasma membrane-targeted fusion protein containing the p110 catalytic subunit of PI3K. ΔN-186 Vav cooperated with p110-C*AAX* only modestly, resulting in ~1.7-fold increase over ΔN-186 Vav alone (Fig. 9). Luciferase reporter assays were performed with SRF-Luciferase to determine whether signals downstream of Vav exchange activity were enhanced in the presence of p110-C*AAX*. Again, only a 1.7-fold activation of SRF-luciferase was observed with ΔN-186 Vav and p110-C*AAX* over ΔN-186 Vav alone (Fig. 9). Together, these data indicate that PI3K activation can cause a limited enhancement of Vav function *in vivo*.

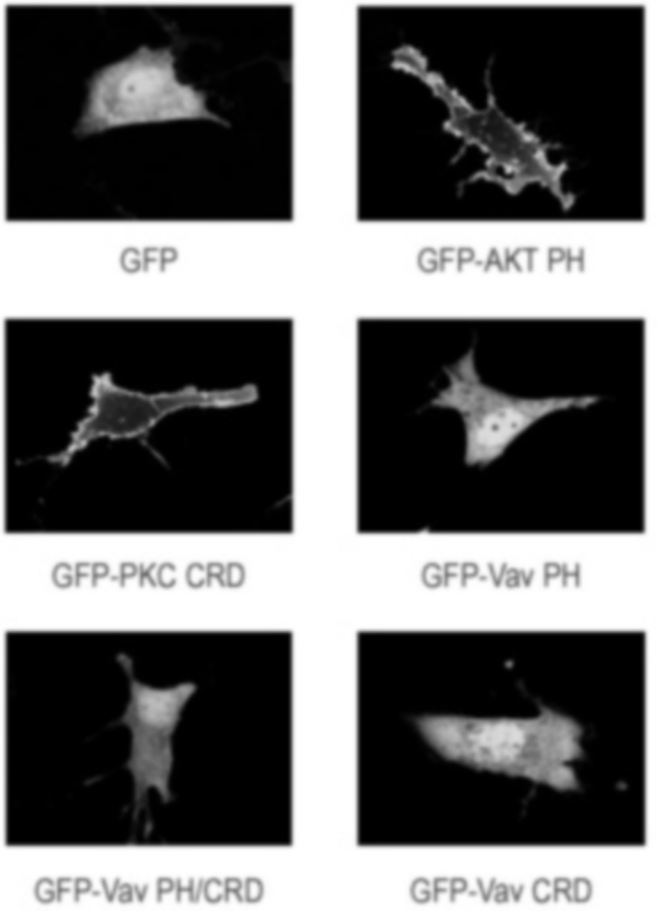


FIG. 8. **The Vav PH domain and CRD are not sufficient to target Vav to the plasma membrane.** Serum-starved NIH 3T3 cells transiently transfected with the empty pEGFP-C3 mammalian expression vector or pEGFP-C3 plasmids encoding Vav PH, Vav CRD or Vav PH/CRD and pEGFP-C1/Akt PH or pEGFP-N1/PKC CRD as positive controls were visualized with confocal microscopy.

## DISCUSSION

The invariant association of a PH domain with all DH domains argues that the PH domain is critical for DH domain function. Recent crystallographic determination of the structures of three different DH/PH domain fragments in complex

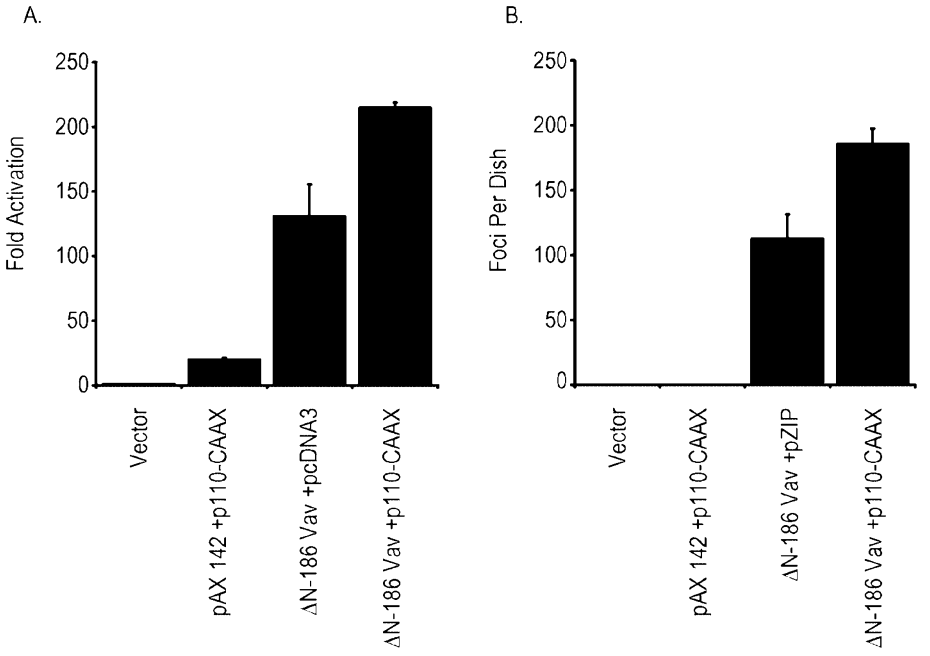


FIG. 9. **PI3K activation causes limited enhancement of ΔN-186 Vav transformation or signaling.** *A*, SRF-luciferase assays were done by transient transfection of NIH 3T3 cells with pAX 142 empty vector or encoding ΔN-186 Vav (100 ng) and pcDNA3 empty vector or encoding p110-C*AAX* (500 ng) and SRF-luciferase (250 ng). Values shown are averages ± standard error of three dishes and are representative of four independent experiments. *B*, NIH 3T3 focus formation assays were done with empty pAX 142 vector or encoding ΔN-186 Vav (50 ng) and the empty pZIP-NeoSV(x)1 vector or encoding p110α-C*AAX* (500 ng). The number of foci of transformed cells was quantitated after 14 days. Values are the average ± standard error of three dishes and are representative of four independent assays.

with their respective Rho GTPase substrates indicates that this role will vary significantly with different DH domains (46–48). Whereas the PH domain is dispensable for DH domain GEF activity for Tiam1 and intersectin, it is necessary for the DH domain GEF function of Dbs. Previous studies argued that the PH domain was dispensable for the function of the Vav and Vav3 DH domain and suggested that an adjacent CRD may substitute for PH domain function. Therefore, in this study, we have evaluated the roles of the PH domain and CRD in Vav function. We found that, in contrast to the majority of other DH domains, the Vav DH domain depends on the PH domain as well as an adjacent CRD for biological activity. Although both domains contribute to Vav membrane association, their functions cannot be replaced with a plasma membrane targeting sequence. This result contrasts observations made with two other Dbl family proteins, where the PH domain was essential for transforming activity and PH domain function could be replaced by a plasma membrane targeting sequence. Furthermore, in contrast to the PH domain or CRD found in other signaling proteins, the isolated or tandem PH domain or CRD of Vav does not possess such membrane targeting activity. Although there is evidence for functional differences between Vav isoforms, our observations in this study, together with our recent observations on Vav2 (23), argue that the PH domain and CRD will have conserved functions in all Vav proteins. Finally, we found that phosphoinositide binding may facilitate Vav membrane association as well as modulate DH domain function *in vivo*. Previous studies reached conflicting conclusions regarding the importance of SH domains for Vav transforming activity. Whereas missense mutation of the SH domains caused a loss of activity (28, 29), deletion of the SH domains indicated that these domains were dispensable for transforming activity. The basis for these different observations is not known. In this study, we found that a missense mutation in the COOH-terminal SH3 domain did impair Vav activity, whereas a complete deletion of the entire SH3/SH2/SH3 region did not abrogate Vav transformation. Bustelo and colleagues (10) also made similar observations with Vav3, and we recently found that the SH domains are dispensable for Vav2 transforming activity as well (23). Therefore, our analyses reveal the different consequences on biological activity when a domain is mutated within the context of other domains *versus* when the domains are deleted completely.

We found that Vav transforming and signaling were ablated upon deletion of the CRD. Thus, in contrast to most other Dbl family proteins, the minimal functional unit is not defined by the tandem DH/PH domains alone. What role does the CRD play in Vav function? We determined previously that the CRD was also critical for the transforming activity of RasGRP, a GEF for Ras proteins, and that a plasma membrane targeting sequence could replace the CRD and restore RasGRP transforming (33). However, we found that the loss of Vav transforming activity caused by deletion of the CRD could not be restored by addition of a plasma membrane targeting sequence. Additionally, in contrast to the CRD of PKC, GFP-tagged Vav CRD did not translocate to membranes in response to extracellular stimuli. Thus, although these negative observations do not define a role for the CRD in Vav function, they do show that the CRD has a function distinct from the CRDs of other signaling proteins. If the Vav CRD does not play a role in membrane targeting, what other function might the CRD serve? Recently, Bustelo and colleagues (13) observed that the CRD of Vav3 could bind to its GTPase substrates *in vitro*, and suggested that the CRD may facilitate DH domain interaction with its substrates. Consistent with this possibility, we recently observed that mutation of the CRD impaired Vav2 catalytic activities *in vitro* (23). However, because we have not been able to express recombinant forms of the DH/PH and DH/PH/CRD peptides of Vav, we have not been able to extend these observations to Vav. Nevertheless, because our studies have determined that the SH and PH domains, as well as the CRD, play identical roles in Vav and Vav2 function, we speculate that the CRD of Vav will also be important in facilitating DH domain interaction with and activation of Rho GTPases.

We found that mutation of the PH domain, within the context of an intact CRD, caused a loss of Vav signaling and transforming activity. Similarly, we also found that the PH domain is critical for the transforming activity of a constitutively activated mutant of human Vav2 (23). Therefore, the PH domain shares a common function in at least two different Vav family proteins, and this function is distinct from that facilitated by the CRD. However, our results contrast with previous studies that showed a negative regulatory (20, 22) or dispensable function (13) for the PH domain of Vav and Vav3. One

possible explanation for these different observations may be that the previous studies mutated the PH domain in Vav constructs that retained other flanking domains such as the SH2 and SH3 domains. We assessed PH domain function in the minimal functional unit of Vav proteins, the DH/PH/CRD fragment, to avoid complications from other domains. Thus, what we can conclude from our studies is that, similar to other Dbl family proteins, the PH domain is a critical component of DH domain function. It remains possible that, within the context of full-length Vav, other domains may compensate for PH domain function.

The PH domain has also been shown to be critical for the function of other Dbl family proteins as well as other signaling proteins. For some Dbl family proteins (Lfc and Dbs), deletion or truncation of PH domain sequences resulted in complete loss of transforming activity, and this defect could be restored by the addition of the Ras COOH-terminal plasma membrane targeting sequence (16, 17). In contrast, we found that the Ras plasma membrane targeting sequence did not restore the loss of function caused by the loss of function of the PH domain of Vav. Thus, although this observation does not exclude the possibility that the Vav PH domain does have membrane targeting activity that is not accurately mimicked by the Ras membrane targeting sequence, it does show that such a function is distinct from the membrane targeting function of the PH domains of other Dbl family proteins. Finally, in contrast to the PH domain of Akt, which alone can promote membrane targeting as a GFP fusion protein, we found that the PH domain of Vav alone is not sufficient for plasma membrane targeting. Although one trivial explanation for this negative result is that the GFP fusion protein of the PH domain is misfolded, the lack of a membrane targeting function is consistent with our fractionation analyses that showed that the PH domain had a limited role in facilitating Vav association with membranes. It is also consistent with observations by us (49) and others that the critical structural determinants required for high affinity binding to phosphoinositides are absent from the PH domains of Dbl family proteins (50, 51). Thus, these PH domains are not expected to function as efficient membrane targeting domains. Therefore, we feel that our results do suggest that the PH domain of Vav has a function that is distinct from that of the PH domain of Akt.

The issue of whether PI3K-generated phosphoinositides can promote the activation of Dbl family proteins is controversial. Although there are reports in the literature that $PIP_3$ can activate some Dbl family proteins, our recent analyses *in vitro* indicates that this will not involve regulation of the intrinsic catalytic function of the DH domain (49). We observed that the reduction in transforming activity caused by the K404A PH domain mutation, which impairs $PIP_3$ binding (20), could be restored partially by the addition of a membrane targeting sequence. Thus, although the Vav PH domain alone is not sufficient to promote membrane association, it may still facilitate $PIP_3$-dependent membrane association in conjunction with the DH domain and CRD interaction with membrane-associated Rho GTPases. Based on the limited stimulation of Vav activity seen with PI3K activation, this membrane association may further promote DH domain activity *in vivo*. However, because the complete loss of transforming activity seen with a second PH domain mutant (W495L) could not be restored by the addition of a membrane targeting sequence, it argues that the PH domain must contribute a second essential function independent of membrane association.

The Vav PH domain may also be required for the full catalytic function of the DH domain. A comparison of the catalytic activity of the DH and DH/PH domains derived from several Dbl family proteins (*e.g.* Trio, Dbs) showed that the PH domain-containing protein exhibited up to 100-fold greater GEF activity than measured for the DH domain alone *in vitro* (52, 53). However, we found that mutation of the PH domain of Vav2 impaired its function *in vivo*, but did not impair DH domain catalytic activity *in vitro* (23). Thus, the PH domain of Vav must serve some other function, in addition to facilitating membrane association, *in vivo*.

In summary, although Vav has been one of the most intensely studied Dbl family proteins, much remains unresolved concerning its regulation. In particular, there have been considerable discrepancies in the literature concerning the role of the COOH-terminal domains of Vav in regulation of GEF catalytic activity and biological function. Our studies have certainly not reached a clear conclusion regarding their functions. However, we feel that our detailed analyses of both Vav and Vav2, using similar assays and experimental approaches, have provided more definitive information with regards to how the PH domain and CRD act in regulating the catalytic activity of the DH domain. What is also clear from our studies is that, when compared with our similar analyses of other Dbl family proteins, the PH domain serves functions in Vav, which are very different than what has described for the PH domains for other Dbl family members. For Vav proteins, it is now clear that membrane localization is not the only role of the PH domain and CRD. In addition, whether activation of PI3K will cause regulation of Dbl proteins via the PH domain is not a simple issue, and PI3K is likely to have very different roles in regulating the activities of other Dbl family proteins. It is now clear that simple and general models will not be adequate to explain the complex and diverse mechanisms by which DH domain function will be regulated in different Dbl family proteins.

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