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Lck Regulates Vav Activation of Members of the Rho Family of GTPases

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Vav is a member of a family of oncogene proteins that share an approximately 250-amino-acid motif called a Dbl homology domain. Paradoxically, Dbl itself and other proteins containing a Dbl domain catalyze GTP-GDP exchange for Rho family proteins, whereas Vav has been reported to catalyze GTP-GDP exchange for Ras proteins. We present *Saccharomyces cerevisiae* genetic data, in vitro biochemical data, and animal cell biological data indicating that Vav is a guanine nucleotide exchange factor for Rho-related proteins, but in similar genetic and biochemical experiments we fail to find evidence that Vav is a guanine nucleotide exchange factor for Ras. Further, we present data indicating that the Lck kinase activates the guanine nucleotide exchange factor and transforming activity of Vav.

vav is a proto-oncogene expressed primarily, if not exclusively, in hematopoietic cells (22, 23). Recent studies have yielded conflicting results concerning activities associated with Vav. Several reports suggest that Vav is a guanine nucleotide exchange factor (GEF) capable of converting Ras to its active GTP-bound state (14–16). Other reports conclude that Vav is not a Ras GEF and provide evidence that Ras and Vav cooperate in the activation of mitogenic pathways (5, 24). While Vav is not structurally related to other well-characterized Ras GEFs, it contains a Dbl homology domain and SH2 and SH3 domains (1, 9, 29). The Dbl protein and several structurally related molecules are known to regulate CDC42Hs (or members of the Rho family) by acting as GEFs (18, 19).

While several reports indicate that Vav is a GEF for Ras, several other reports provide circumstantial evidence suggesting that Vav is a GEF for Rho-related proteins but not for Ras (14, 24). Vav-transformed cells closely resemble Dbl-transformed cells, exhibiting phenotypes distinct from those of Ras-transformed cells (5, 24). First, Vav- and Dbl-transformed cells exhibit well-developed stress fibers and focal adhesions, which are lacking in Ras-transformed cells (24). Second, in contrast to Ras transformation, Vav transformation and Dbl transformation fail to activate transcription from Ras-responsive elements (24). Third, in contrast to Ras transformation or Ras GEF transformation, Vav- or Dbl-transformed cells do not exhibit an increase in cellular Ras GTP levels (24). Those investigators concluded that Vav transformation is not a consequence of Ras activation, but they speculated that Vav transformation, like Dbl transformation, is a consequence of activation of Rho family members. Vav has a Dbl homology domain, and, by analogy with the Dbl protein, it has been postulated to have GEF activity towards the Rho family of GTPases (19, 24). The morphology of Vav-transformed cells is similar to that of Rho-transformed cells (25, 36). This observation is consistent with the role of Vav as an activator of Rho-related proteins involved in cytoskeletal reorganization. In the present study, we provide direct evidence for the role of Vav as a GEF for Rho family members.

The Lck tyrosine kinase plays a role in T-cell activation, which is triggered by receptor ligation (26, 33, 44). Previous reports demonstrate that cross-linking of B- or T-cell antigen receptors results in the rapid tyrosine phosphorylation of a number of proteins, including Vav (26, 33, 43, 44). Further, the Vav protein can serve as a substrate for Lck kinase in vitro, suggesting that Lck kinase may play a critical role in T-cell signaling by activating Vav signaling (14, 15, 43). Using *Saccharomyces cerevisiae* genetic, biochemical, and biological assays, we tested whether Lck is also involved in the Vav-mediated signaling pathway of Rho-related GTPases. In this paper, we demonstrate that Lck kinase is involved in the Vav signaling pathway by modulating the GEF activity of Vav for Rho family proteins. These are the first data concerning the regulation of the activity of Dbl-related proteins.

MATERIALS AND METHODS

Construction of yeast expression plasmids. The pAD4-Vav(wt) plasmid was constructed by subcloning the *Spe*I-*Sal*I fragment encoding full-length mouse Vav from the *vav* clone pMB24 (9) into the *Hin*dIII-*Sal*I site of the yeast expression vector pAD4 (47). pAD4-Vav(onc) was made by subcloning the *Nru*I-*Sal*I fragment of *vav* from pMB24 into *Hin*dIII-*Sal*I-digested pAD4. pAD4-Vav(DH) was made by digesting pAD4-Vav(onc) with *Nco*I and *Hin*dIII and self-ligating the 9.6-kb fragment. To construct the YEp24ADH-Lck plasmid, a 1.5-kb *Stu*I fragment encoding mouse Lck was cut out from m-*lck* clone NT18 (30) and subcloned into the *Sal*I site of YEp24ADH. YEp24ADH was constructed by subcloning the *Bam*HI-*Sal*I ADH promoter fragment of pAD4 into the *Bam*HI-*Sal*I-digested YEp24 vector (40). YEp103-cdc24 (8) was provided by Alan Bender. Plasmid pRS315(CDC42Sc) (50) was provided by Douglas Johnson.

The 2.4-kb *Nru*I-*Ssp*I Vav fragment from pMB24 was subcloned into the *Sma*I site of pAD4caax to create plasmid pVav(caax). pAD4caax was created by inserting an oligonucleotide encoding the CAAX box of H-ras into *Sal*I-*Sac*I-digested pAD4 (37).

Molecular constructs. Molecular constructs encoding mouse proto-Vav (pJC11), N-terminally truncated and transforming ΔVav (JC12), proto-Dbl (pZIP-pDbl), N-terminally truncated and transforming Dbl (pZIP-Dbl), constitutively activated human RhoA [pZIP-RhoA(63L)], and wild-type (pLXSN-Lck) and constitutively activated [pLXSN-Lck(505F)] Lck have been described previously (24, 46).

Yeast strains and complementation assay. The temperature-sensitive yeast strain ABY147 (MAT**a** *cdc24*-4 *ura3 leu2 his3*) was kindly provided by Alan Bender (2). Strain ABY178 (also referred to as DJTD2-16D) (MAT**a** *cdc42*-1

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ura3 leu2 trp1 his4) was kindly provided by Alan Bender (21). Yeast transformations were performed by the lithium acetate method with the modification that 25 mM dithiothreitol was included in the transformation buffers (40). Complementation assays were performed as described in the legend of Fig. 2.

Preparation of Vav and small GTPases. The *vav* cDNA was subcloned into the bacterial expression vector pRSET-B (Invitrogen) by using PCR-amplified copies of the *vav* cDNA flanked by restriction endonuclease sites (*Xho*I and *Hin*dIII). The fragments used corresponded to *vav* codons 170 through 481 and 170 through 845. Plasmids constructed were transformed into *Escherichia coli* BL21 (DE3). Induction and purification of the His-tagged fusion proteins were performed essentially as described previously (35). The final concentration and purity of the proteins were determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). The apparent molecular sizes of the His_6-tagged Vav proteins are 35 and 75 kDa. Glutathione-agarose beads were incubated with *E. coli* extract of the glutathione *S*-transferase (GST)-RhoA, GST-Rac1, GST-CDC42Hs, GST-Ran, or GST–H-Ras expression systems for 1 h and then extensively washed in buffer A (50 mM Tris-HCl [pH 7.5], 5 mM $MgCl_2$, 20 mM KCl, 1 mM dithiothreitol) containing 1% Triton X-100 to remove all unbound materials. Beads bound with proteins were then incubated in a solution containing 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, and 10 mM glutathione, and the resulting dissociated proteins were dialyzed overnight against 300 volumes of buffer A. GST fusion protein expression systems for RhoA, Rac1, and CDC42Hs were kindly provided by Gary Bokoch. Expression vector for GST-Ran was a generous gift from Linda Van Aelst. A restriction fragment of wild-type H-Ras (*Bam*HI and *Eco*RI) was subcloned into pGEX-2T (Pharmacia) to generate the fusion expression vector for GST–H-Ras.

In vitro binding of Vav and small GTPases. Nickel-agarose or His_6-tagged-Vav-coated nickel-agarose beads (50 pmol) were resuspended in 250 μl of buffer A containing 5 mM imidazole and 500 μg of bovine serum albumin (BSA) per ml; then, 500 pmol of GST-GTPases (RhoA, Rac1, CDC42Hs, Ran, and H-Ras) in their nucleotide-free state, GDP-bound state, or GTP-bound state was added, and the mixture was incubated with shaking at room temperature for 1 h. After the incubation period, the beads were pelleted and washed five times in a solution containing 50 mM Tris-HCl (pH 7.5), 1% Triton X-100, 0.5 M NaCl, and 60 mM imidazole. Precipitated proteins were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membrane. GST fusion protein was detected by blotting with anti-GST antibody (Santa Cruz Biotechnology) and reagents from the Immun-Lite chemiluminescent blotting kit (Bio-Rad, Richmond, Calif.). In control experiments GST-CDC25 failed to bind Rho, Rac, or CDC42 under conditions where GST-Vav(L) bound significant amounts of these GTPases.

In vitro GEF activity of Vav directed toward various GTPases. GST-GTPase proteins were loaded with [^{3}H]GDP (10 Ci/mmol; DuPont) as described previously (11). Forty picomoles of [^{3}H]GDP-GST-GTPase was incubated with 10 pmol of His-tagged Vav alone or His-tagged Vav phosphorylated by Lck kinase in 200 μl of buffer A containing 1 mM GTP. After 15 min of incubation at room temperature, 50 μl of reaction product was removed and binding of [^{3}H]GDP to GST-GTPase protein was quantified by filtration on nitrocellulose filters and liquid scintillation counting as previously described (4).

Phosphorylation of Vav by Lck kinase. Five micrograms of His_6-Vav protein bound to nickel-agarose beads was washed with kinase buffer (10 mM HEPES [pH 7.0], 10 μM ATP, 12 mM $MgCl_2$) and incubated with 5 ng of recombinant Lck tyrosine kinase for 30 min at 37°C. Phosphorylated Vav protein bound to nickel-agarose beads was then washed with either buffer A containing 5 mM imidazole and 500 μg of BSA per ml for in vitro binding experiments or with buffer A for GEF assay.

Cell culture and transformation assays. NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum. DNA transfections were done by the calcium phosphate precipitation technique as described previously (7). Cells were transfected with plasmid constructs encoding the indicated proteins at 1 μg per dish for proto-Vav, ΔVav, proto-Dbl, and RhoA(63L) and at 10 ng per dish for ΔDbl alone or were cotransfected with 500 ng of c-Lck or 100 ng of activated Lck(505F) per dish. Transformed foci were quantitated after 14 days. Transfected cultures were stained with 0.4% crystal violet to better visualize transformed foci.

Transfection and immunofluorescence. Rat embryo fibroblast (REF-52) cells were grown to 80% confluence in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and transfected as previously described (13) with 1 μg of total DNA per 60-mm-diameter dish for 16 h. After 16 h, cells were prepared for immunofluorescence as previously described (41). Tagged V12Cdc42, V12Rac1, and oncogenic Vav were visualized with anti-T7 antibody (Novagen) and rhodamine-conjugated goat anti-mouse secondary antibody (Molecular Probes). Filamentous actin was visualized with fluorescein isothiocyanate-phalloidin. Fluorescent images were photographed on a Zeiss Axiophot microscope using a 63× oil-immersion lens and Kodak P3200 Tmax film. Plasmid construction for this purpose was done as follows: CDC42Hs(Val12), VRac1(Val12), and oncogenic Vav were amplified by PCR from cDNA clones (generous gifts of Alan Hall) and subcloned as an *Xba*I-*Sal*I fragment in pCGT. pCGT is derived from pCGN (42) with a replacement of the Lerner epitope by the T7 epitope (generous gift from Angus Wilson, Cold Spring Harbor Laboratory).

Jnk assays. Jun nuclear kinase (Jnk) assays were done as previously described (45). Briefly, transiently transfected COS cells were lysed in modified radioimmunoprecipitation assay buffer. Lysates containing approximately 30 μg of protein were incubated with 1 to 5 μg of GST–c-Jun(1-79) substrate in a kinase assay. Proteins were then separated by SDS-PAGE, and the phosphorylation of the substrate was observed after autoradiography.

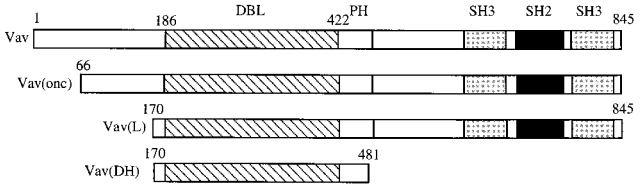

FIG. 1. Schematic representation of the fragments of Vav used for these studies. Full-length Vav encodes a protein of 845 amino acids including a domain homologous to the Dbl protein (DBL), a plekstrin homology (PH) domain, and SH2 and SH3 domains. Full-length Vav or the indicated fragments of Vav were cloned into the yeast expression vector pAD4 (32), into the *E. coli* expression vector pRSET (Invitrogen) for the production of His_6-tagged fusion proteins, or into the animal cell expression vector pCGT (42).

Other materials and methods. Oligonucleotides were purchased from the University of Southern California Microchemical Core Laboratory. *E. coli* transformations were performed by electroporation as described by the manufacturer (Bio-Rad). Yeast strains were grown in yeast extract-peptone-dextrose medium or synthetic complete (SC) medium (40). *E. coli* strains were grown in Luria-Bertani medium containing 100 μg of ampicillin per ml. DNA sequencing was performed by the method of Sanger et al. (39) with a Sequenase version 2.0 kit from United States Biochemical Corp., Cleveland, Ohio.

RESULTS

Vav suppresses a *cdc24* temperature-sensitive defect in *S. cerevisiae*. Because Vav contains a domain structurally related to the GEF domain of Dbl and other Dbl family proteins, we predicted that Vav might be a positive regulator of CDC42, Rho, or Rac molecules by functioning as a GEF. The yeast *S. cerevisiae CDC42* gene product is homologous to the vertebrate CDC42Hs protein. The yeast CDC42-GTPase is regulated by a Dbl-related GEF encoded by *CDC24*. Temperature-sensitive mutations in the yeast genes *cdc24* and *cdc42* result in similar lethal phenotypes at the restrictive temperature. We tested whether Vav can suppress the temperature-sensitive defect of a *cdc24* strain of yeast. The biological activity of Vav has been reported to be activated by an N-terminal deletion or by Lck phosphorylation of the full-length Vav (14, 23). Consequently, we tested full-length Vav and fragments of Vav (Fig. 1) for suppression of the *cdc24* temperature-sensitive defect. From these experiments we have obtained genetic data suggesting that Vav functions like Dbl, regulating a CDC42 family member.

We find that oncogenic Vav (lacking the N-terminal 66 amino acids) can partially suppress the loss of the *S. cerevisiae* Dbl homolog CDC24 (6, 12) (Fig. 1 and 2a). This suppression is due to the Dbl homology domain of Vav as judged by the ability of residues 66 through 566 of Vav (encompassing the Dbl homology domain) to complement the loss of CDC24 function (Fig. 1 and 2a). In contrast, full-length Vav (Fig. 1 and 2a) as well as a fragment of Vav lacking a portion of the Dbl homology domain (data not shown) failed to suppress the loss of CDC24 function. These results suggest that the Dbl homology domain of Vav, like the yeast CDC24-GEF, can activate the endogenous yeast CDC42-GTPase. In support of this suggestion, Vav failed to suppress the loss of CDC42 function in yeast, indicating that Vav does not function downstream of CDC42 (Fig. 2c). Further, if Vav was a GEF for Ras proteins as previously suggested (14–16), we predicted that Vav should suppress the loss of the yeast Ras-specific GEF encoded by *CDC25*. When a *cdc25*^{ts} mutant strain was used, all fragments

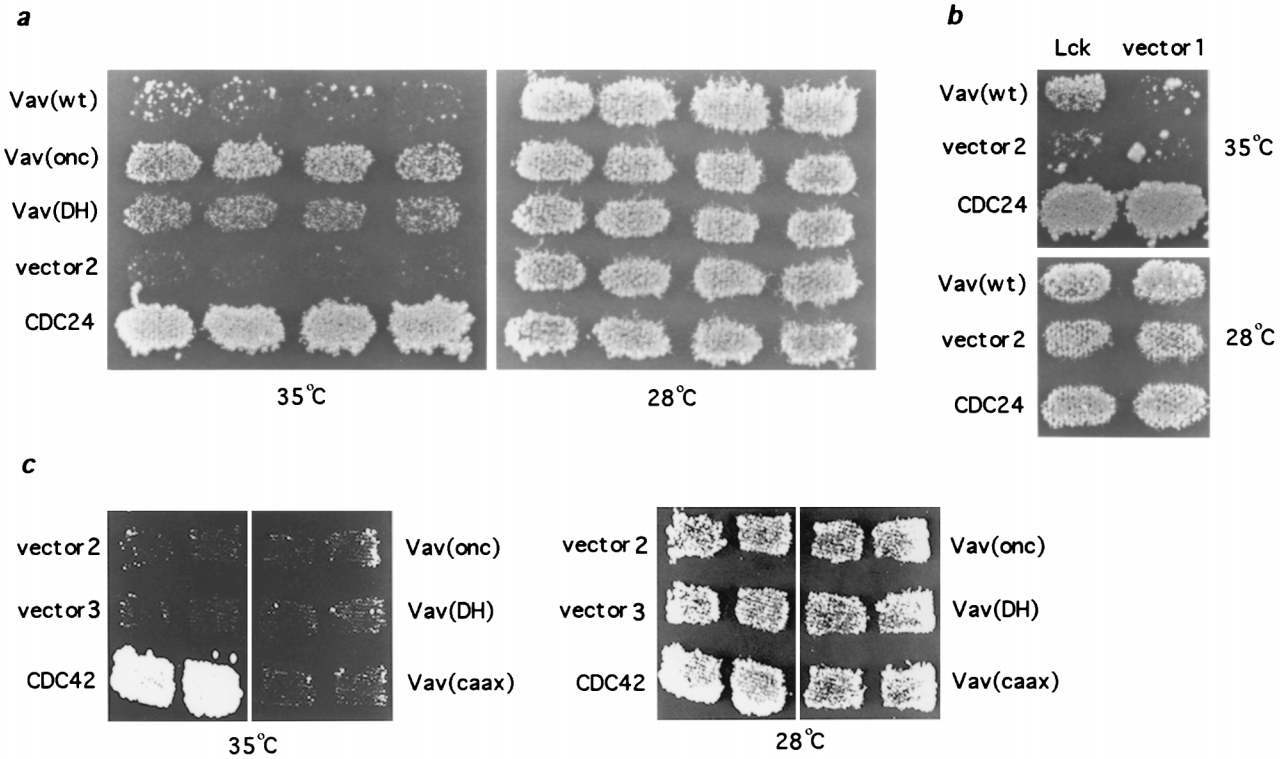

FIG. 2. Vav can suppress the loss of CDC24 function in *S. cerevisiae*. (a) The *S. cerevisiae cdc24* temperature-sensitive strain ABY147 (2) was cotransformed with pAD4-based vectors containing fragments of Vav (Fig. 1) together with a YEp103-based vector. Transformants were selected by plating onto SC media lacking leucine (for selection of the pAD4-based vectors) or lacking uracil (for selection of the YEp103-based vectors). For a positive control, the strain ABY147 was cotransformed with pAD4 (vector 2) and a YEp103-based vector containing the wild-type yeast *CDC24*. Four independent transformants from each of the transformations were patched onto $SC^{-Leu,-Ura}$ plates and incubated for 2 days. The resulting master plate was replica plated onto two $SC^{-Leu,-Ura}$ plates; one plate was incubated at 35°C (the nonpermissive temperature) for 3 days, while the other plate was incubated at 28°C (the permissive temperature) for 3 days. (b) The *S. cerevisiae cdc24* temperature-sensitive strain ABY147 (2) was cotransformed with a full-length (wt) Vav-pAD4 expression vector together with a YEp24-based vector expressing the mouse Lck cDNA under the control of the yeast ADH promoter. Also, the Lck expression vector was cotransformed with the empty vector pAD4. For a positive control, the strain ABY147 was cotransformed with a yeast YEp103-CDC24 expression vector together with vector pAD4 (vector 2). Transformants for each of these cotransformations were patched onto $SC^{-Leu,-Ura}$ plates. For a negative control, strain ABY147 was cotransformed with vectors pAD4 (vector 2) and YEp24 (vector 1). The resulting master plate was replica plated onto two $SC^{-Leu,-Ura}$ plates; one plate was incubated at 35°C for 3 days while the other was incubated at 28°C for 3 days. (c) The *S. cerevisiae* CDC42 temperature-sensitive strain ABY178 was transformed with pAD4-based vectors expressing oncogenic Vav, the Dbl homology domain of Vav, or oncogenic Vav fused to a CAAX box. ABY178 cells transformed with a CDC42-expressing vector, pRS315(CDC42Sc), were used as positive controls. Cells transformed with pAD4 (vector 2) and pAD5 (vector 3) were used as negative controls. Two independent transformants from each transformation were patched onto SC^{-Leu} plates and incubated at 28°C for 2 days. The resulting master plate was replica plated onto two SC^{-Leu} plates. One plate was incubated at 35°C and the other at 28°C for 3 days.

of Vav described here (Fig. 1) failed to complement the loss of CDC25 function (data not shown).

Previous reports have proposed that the Lck tyrosine kinase is a positive regulator of Vav function (14). We examined whether coexpression of full-length Vav and Lck affected the ability of Vav to complement yeast CDC24 function. Although full-length Vav failed to suppress the *cdc24* temperature-sensitive defect, coexpression of full-length Vav and Lck complemented the loss of CDC24 function (Fig. 2b). Further, Lck enhanced the ability of oncogenic Vav to suppress the loss of CDC24 function (data not shown). These results show that Vav's ability to activate the yeast CDC42-GTPase is enhanced either by an N-terminal deletion or by Lck-phosphorylation of Vav.

Vav preferentially binds to the nucleotide-free form of CDC42-related GTPases. We examined whether Vav could bind to various GTPases in a manner similar to that reported for other GEFs, such as Dbl, SOS, and CDC25 (32). A biochemical hallmark of GEFs is their ability to bind significantly more tightly to the nucleotide-free form of their corresponding GTPases than to their GTPases when complexed to guanine nucleotides (32). His_6-tagged fusions of fragments of Vav (residues 170 through 845 [Vav(L)] and residues 170 through 481 encompassing the Dbl homology domain [Vav(DH)] (Fig. 1) were purified from *E. coli* expression systems. The fragments of Vav bound to Ni-agarose were incubated with purified GST fusions of GTPases in their nucleotide-free state, GDP-bound state, or GTP-bound state. After incubation, the Ni-agarose beads were washed and the resulting pellets were analyzed by immunoblotting with antibodies specific for GST. GST-CDC42Hs, GST-Rac1, and GST-RhoA were initially tested for precipitation by Ni-agarose bound with His_6-Vav(L), His_6-Vav(DH), or Ni-agarose alone. As seen in Fig. 3, CDC42Hs and Rac1 in their nucleotide-free state bound well to both His_6-Vav(L) and His_6-Vav(DH). In contrast, GDP-bound and GTP-bound forms of CDC42Hs and Rac1 bound to the fragments of Vav only weakly compared to the nucleotide-free proteins (Fig. 3). In similar binding reactions, His_6-Vav(L) bound equally well to nucleotide-free RhoA, GDP-bound RhoA, or GTP-bound RhoA (Fig. 3).

Previous reports have indicated that the Lck tyrosine kinase positively regulates Vav signaling (14). We tested whether phosphorylation of His_6-Vav(L) by recombinant Lck altered the former's ability to bind to CDC42Hs, Rac1, and RhoA.

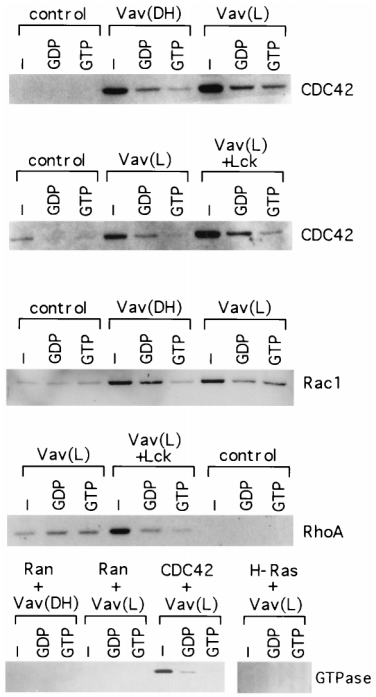

FIG. 3. Binding of Vav protein to CDC42, Rac1, or RhoA proteins. Fifty picomoles of His_6-Vav(DH) or His_6-Vav(L) bound to nickel-agarose beads was incubated with 500 pmol of the GST fusions of the indicated GTPase (CDC42Hs, Rac1, RhoA, Ran, or H-ras) in their nucleotide-free state, GDP-bound state, or GTP-bound state. All incubations were carried out for 1 h at room temperature in 300 μl of buffer A containing 5 mM imidazole and 500 μg of BSA per ml. Where indicated, the Vav protein was preincubated with the Lck kinase under conditions where saturation of phosphorylation was achieved. For a negative control, nickel-agarose beads not complexed with Vav protein were used in similar reactions. After the incubation period, the nickel-agarose beads were washed five times to remove material not bound to the beads and the resulting pellets were analyzed by immunoblotting with antibodies specific for GST protein.

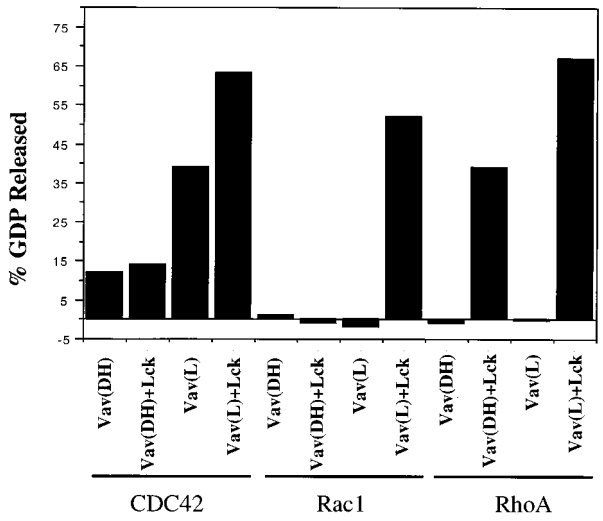

FIG. 4. Vav stimulates release of GDP from CDC42Hs, Rac1, and RhoA. Forty picomoles of the indicated GTPase (CDC42Hs, Rac1, or RhoA) bound with [^{3}H]GDP was incubated with 10 pmol of His_6-Vav(DH) or His_6-Vav(L) or without addition of Vav protein. Where indicated, the Vav proteins were preincubated with Lck kinase under conditions where saturation of phosphorylation of Vav was achieved. All reactions were carried out in 200 μl of buffer A containing 1 mM GTP. After 15 min incubation, the amount of [^{3}H]GDP remaining bound to the GTPases was determined by a nitrocellulose filter binding assay. The values shown for percentage of GDP released reflect the percentage of nucleotide released in the reaction mixture containing Vav relative to the negative-control reaction mixtures (i.e., without Vav). They were calculated as follows: % GDP release = [(cpm released in the presence of Vav) − (cpm released in the absence of Vav)]/initial cpm of [^{3}H]GDP bound to the GTPases. In all experiments, the counts per minute (cpm) of [^{3}H]GDP released from the GTPases in the absence of Vav was less than 20% of the amount bound at time zero. The values shown are the averages of duplicate data points which did not differ by more than 8%. Similar results were obtained from three independent experiments.

His_6-Vav(L) complexed to Ni-agarose beads was phosphorylated by incubation with recombinant Lck kinase and ATP for 30 min. At completion of the kinase reaction, the Lck kinase was removed by extensive washing of the Ni-agarose beads. The phosphorylated His_6-Vav(L) was then incubated with GST-CDC42Hs, GST-Rac1, or GST-RhoA in their nucleotide-free, GDP-bound, or GTP-bound states. After washing of the Ni-agarose beads, the resulting pellets were analyzed by immunoblotting with anti-GST antibodies. As seen in Fig. 3, CDC42Hs and RhoA in their nucleotide-free forms bound well to the Lck-phosphorylated Vav(L). In contrast, Lck-phosphorylated Vav(L) bound significantly less well to the GDP- and GTP-bound versions of each GTPase (Fig. 3). Similarly, nucleotide-free Rac1, but not nucleotide-bound Rac1, bound well to the Lck-phosphorylated Vav(L) (data not shown). The preferential binding of these GTPases to Vav when in their nucleotide-free state is consistent with the suggestion based on the yeast genetic data presented above that Vav is a GEF for Rho-related molecules and that Lck phosphorylation of Vav affects its GEF activity. Under similar reaction conditions, the Ran GTPase and the human H-ras protein failed to bind each of the Vav fragments (with [data not shown] or without [Fig. 3] Lck-phosphorylation) tested here.

In vitro Vav promotes GDP release from CDC42Hs-related GTPases. We tested whether Vav could promote GDP release from CDC42Hs, Rac1, and RhoA. For this purpose, GST fusions of CDC42Hs, Rac1, and RhoA proteins bound to [^{3}H]GDP were incubated with either His_6-Vav(DH), Lck-phosphorylated His_6-Vav(DH), His_6-Vav(L), Lck-phosphorylated His_6-Vav(L), or Ni-agarose alone. After 15 min of incubation, the stimulation of GDP release (relative to the Ni-agarose control) was determined. GDP release from CDC42Hs was accelerated by each of the fragments of Vav tested, although Vav(L) was more potent than Vav(DH), and Lck-phosphorylation of Vav(L) enhanced its activity (Fig. 4). Stimulation of GDP release from Rac1 was observed only with Lck-phosphorylated Vav(L), while acceleration of GDP release from RhoA was observed with Lck-phosphorylated Vav(DH) and Lck-phosphorylated Vav(L) (Fig. 4). In similar reactions using H-Ras–GDP as the substrate, no acceleration of GDP release was observed (data not shown). Taken together, these results suggest that Vav is a GEF for the Rho family of GTPases and that the Dbl homology domain of Vav is responsible for this activity.

Vav induces filopodium formation when used to transfect REF-52 fibroblasts. We predicted that Vav might induce phenotypes in fibroblasts consistent with activation of one of the members of the Rho family of GTPases (38). In fibroblasts, Rac proteins have been shown to induce actin polymerization at the plasma membrane to form membrane ruffles and lamellipodia followed by the formation of stress fibers (27). The stress fiber formation occurs in a RhoA-dependent manner (27). More recently, a third member of the Rho family, human CDC42 (CDC42Hs), has been shown to trigger the formation of filopodia followed by induction of lamellipodia and stress fibers (34). To test whether Vav affects cytoskeletal reorgani-

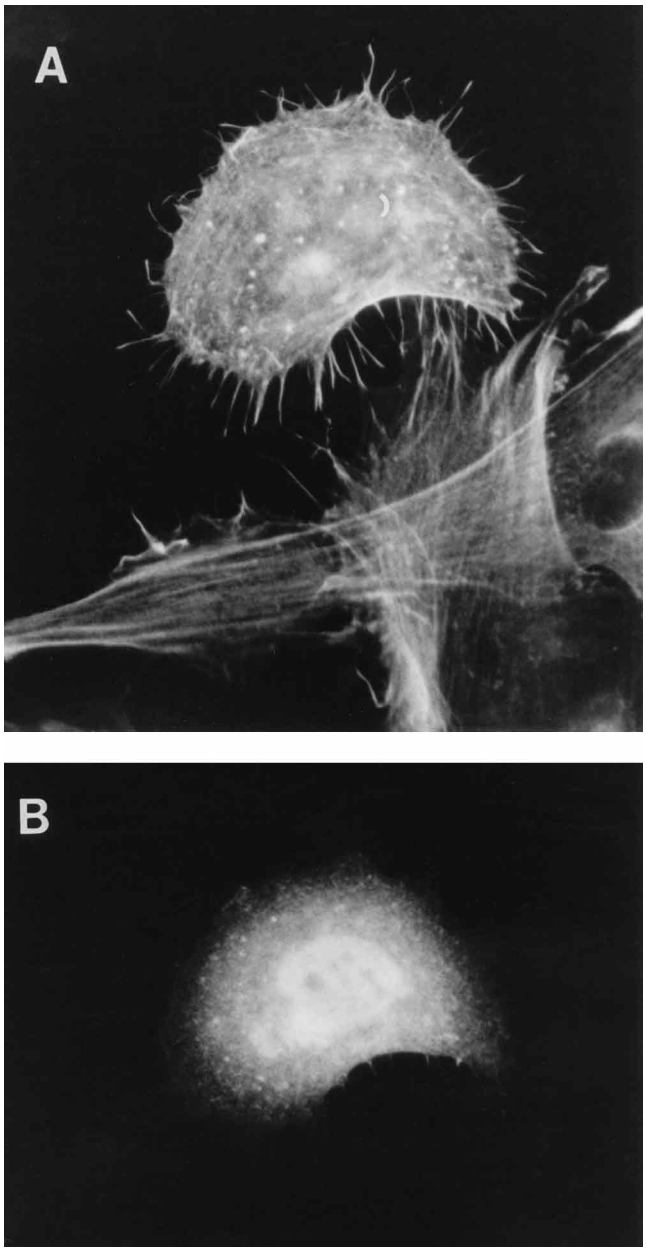

FIG. 5. Vav induces filopodium formation in fibroblasts. Rat embryo fibroblasts (REF-52) were grown to 80% confluence in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and transfected with 1 μg of total DNA per 60-mm-diameter dish (13, 41). A vector expressing a T7-tagged oncogenic Vav (residues 66 through 845) was constructed and used for this purpose. Sixteen hours after transfection, the cells were prepared for immunofluorescence and light microscopy (34). (A) Filamentous actin was visualized with fluorescein isothiocyanate-phalloidin. (B) Cells expressing Vav were identified by use of the T7 tag, which was visualized with anti-T7 antibody (Novagen) and rhodamine-conjugated goat anti-mouse secondary antibody (Molecular Probes).

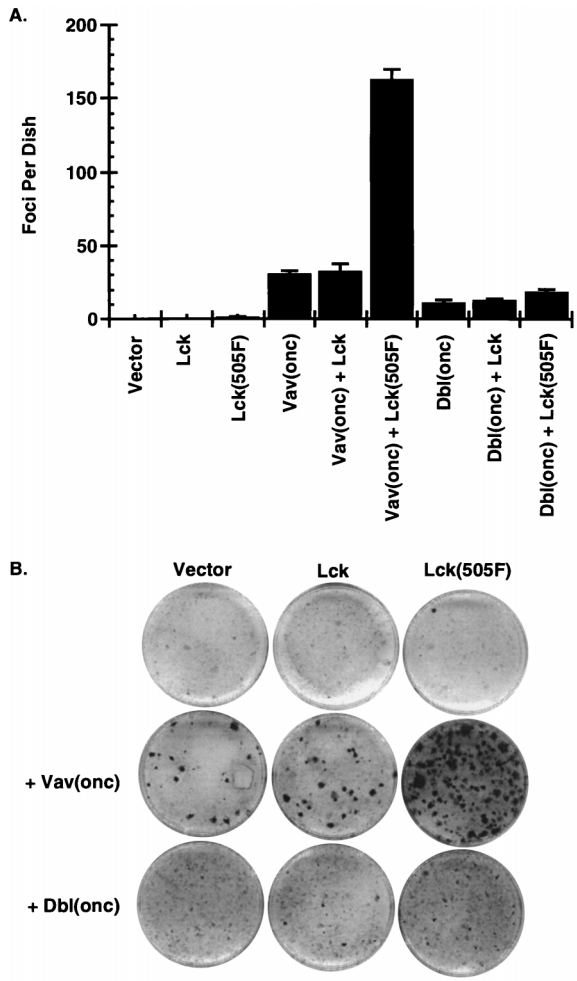

FIG. 6. Coexpression of activated Lck(505F) caused synergistic enhancement of Vav, but not Dbl, focus-forming activity. Cotransfection focus formation analyses were done as described in Materials and Methods. (A) Data are the averages of four dishes for each determination and are representative of two independent assays. (B) Representative dishes were stained with crystal violet to better visualize transformed foci.

zation at the plasma membrane, we transfected oncogenic Vav into REF-52 fibroblasts and monitored for one of the cytoskeletal-reorganization phenotypes, namely, filopodium formation. Figure 5A shows multiple filopodia projecting from a fibroblast which stained positive for the Vav protein but not from fibroblasts lacking exogenous Vav expression. The formation of filopodia in the Vav-transfected fibroblast is consistent with our proposal that Vav is a GEF for the Rho/Rac/CDC42Hs family of GTPases. We are currently examining whether Vav affects lamellipodium and stress fiber formation.

Coexpression of Lck synergistically enhances Vav but not Dbl or RhoA transforming activity. Our in vitro analysis suggested that Lck phosphorylation of Vav enhanced its GEF activity. Since we previously showed that the Vav-induced transformed phenotype was consistent with the activation of Rho proteins (24, 25), we evaluated the possibility that Lck would enhance Vav transforming activity. We find that coexpression of activated Lck caused a 10-fold enhancement of Vav focus-forming activity (Fig. 6 and 7). In contrast, Lck failed to enhance the Dbl (Fig. 6) or Rho (Fig. 7) focus-forming activity. These results suggest that Lck phosphorylation of Vav promotes its Rho GEF activity, which in turn mediates its transforming activity.

An alternate explanation for the synergistic transforming activity seen when Lck is coexpressed with Vav is that Lck and Vav show cooperation by activating parallel but otherwise independent signaling pathways. For example, we and others recently showed that coexpression of activated RhoA or Rac with activated Raf-1 caused a synergistic transforming activity

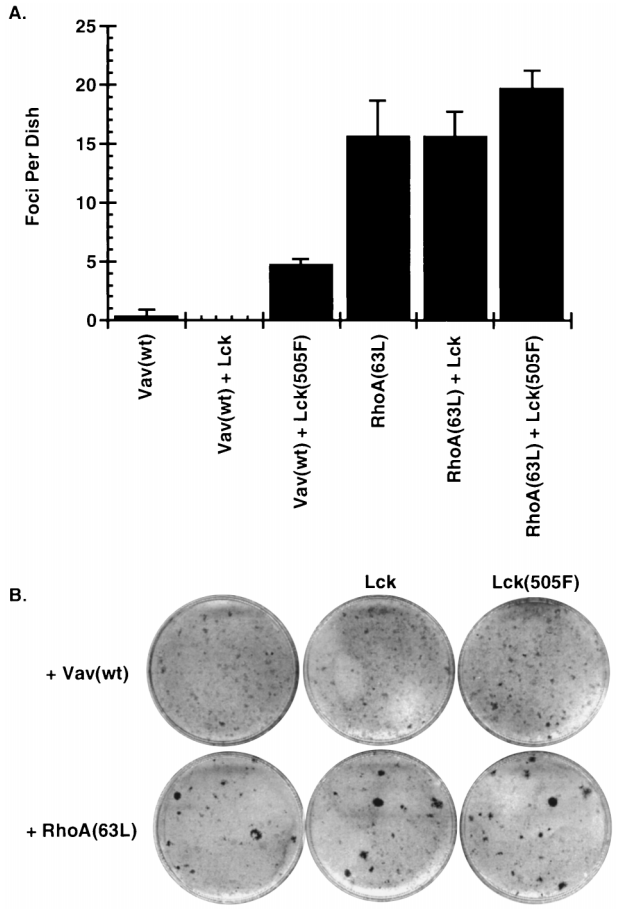

FIG. 7. Coexpression of activated Lck(505F) caused synergistic enhancement of Vav, but not RhoA, focus-forming activity. Cotransfection focus formation analyses were done as described in Materials and Methods. (A) Data shown are the averages of four dishes for each determination and are representative of two independent assays. (B) Representative dishes were stained with crystal violet to better visualize transformed foci.

that was more than 10-fold above their additive focus-forming potentials (25, 36). However, the inability of Lck to cause synergistic enhancement of activated Dbl or RhoA argues against this possibility (Fig. 6 and 7). Finally, we observed that activated Lck alone caused the appearance of transformed foci that were indistinguishable from those caused by Vav or Dbl (data not shown) but different from those caused by activated Ras or Raf. These transformation data are consistent with our genetic and biochemical data and further support a Rho GEF function for Vav.

Vav activates Rho-associated signaling events. Recent studies have shown that Rac1 and CDC42Hs, as well as Dbl, are activators of Jnk (10, 31). Jnk in turn phosphorylates and activates the Jun ATF-2 transcription factors (17). Additionally, RhoA, Rac1, and CDC42Hs cause activation of the serum response factor (20). Thus, in addition to causing changes in actin organization, activation of Rho proteins may promote changes in gene expression via upregulation of transcription factor function. Our observation that Vav functions as a Rho family GEF suggests that Vav should also modulate the activities of Jnk and the ATF-2 transcription factor. We observe that oncogenic Vav, but not wild-type Vav, results in a more-than-twofold activation of both Jnk and ATF-2 activities in transient-transfection analyses of COS and NIH 3T3 cells (data not shown).

DISCUSSION

We have examined the ability of Vav to serve as a GEF for the Rho/Rac/CDC42 family members, using several distinct biological and biochemical assays to assess the regulation of these GTPases. These assays either directly or indirectly measure the ability of Vav to interact with and activate Rho/Rac/CDC42-related GTPases. From each of these distinct assays we have obtained evidence supporting our conclusion that Vav is a GEF for Rho/Rac/CDC42-related GTPases. Further, these studies raise several new questions concerning signaling of Vav and other Dbl-related molecules.

The yeast Dbl homolog encoded by *CDC24* has been demonstrated to act as a GEF for the yeast CDC42 (49). The yeast CDC42, like its mammalian counterpart, regulates cytoskeletal reorganization (50). CDC42 is activated by the CDC24-GEF, and the activated CDC42 in turn transmits its signal (at least in part) through the downstream target, the PAK1 kinase (28). Recently, a mammalian PAK1 has been shown to be a downstream target of CDC42 in vertebrates (48). We demonstrate that Vav is functionally homologous to the CDC24-GEF by showing the ability of fragments of Vav to complement a *cdc24* temperature-sensitive defect in yeast. We observed several fragments of Vav, each with an intact Dbl homology domain, showing biological activity. First, full-length Vav in the presence of the Lck kinases is a potent suppressor of the loss of CDC24 function (Fig. 2). Second, a fragment of Vav lacking the N-terminal 66 amino acids as well as a fragment of Vav encompassing the Dbl homology domain suppresses the loss of CDC24 function (Fig. 2). In contrast, each of these biologically active fragments of Vav failed to suppress the loss of CDC42 function, thus indicating that Vav likely functions upstream of CDC42.

Recently, we have obtained additional genetic data supporting a role for Vav as a regulator of CDC42 function. We have identified several mutants of Vav which, when expressed in yeast, are lethal (17a). We compared the terminal lethal phenotypes of these dominant lethal mutants with the lethal phenotypes previously reported as blocking CDC42 function and producing constitutive activation of CDC42. Loss of CDC42 function is lethal with an unbudded, large-cell phenotype (50). In contrast, constitutive activation of CDC42 function leads to a terminal lethal phenotype characterized by elongated buds and to a multibudded phenotype (50). Interestingly, each of the Vav lethal mutants exhibited one of these two distinct phenotypes, one of which is indistinguishable from that of the loss of CDC42 function and one of which is indistinguishable from that of the gain of CDC42 function. Thus, these dominant lethal Vav mutants likely represent two distinct classes of mutants: one class of dominant interfering mutants which block CDC42 function and a second class of dominant lethal mutants which enhance CDC42 function. The lethality of the first type, the dominant interfering mutants, can be suppressed by overexpression of wild-type CDC42, indicating that these Vav mutants block CDC42 function by sequestering available CDC42 in the cell.

From the genetic analysis of Vav in yeast, we conclude that Vav has the potential to activate the CDC42 pathway. In contrast, from our yeast genetics analysis, we fail to find any evidence that Vav activates Ras function in yeast (unpublished data). The yeast genetics results suggest Vav is a GEF for CDC42-like molecules. However, to assess the significance of

these observations for Vav function in animal cells, we sought to characterize Vav using additional assays for Vav function.

The biochemistry of GEFs has revealed that they exhibit specificity for a subset of small GTPases. GEFs functionally related to the yeast CDC25 GEF catalyze nucleotide exchange on Ras but not on other small GTPases (3). Similarly, the Dbl-GEF catalyzes nucleotide exchange on CDC42-related GTPases but not on Ras (18). Another biochemical characteristic of GEFs is their preferential binding to nucleotide-free GTPases rather than to GDP- or GTP-bound versions of the GTPases (32). We observed several fragments of Vav, each containing a complete Dbl homology domain, exhibiting GEF activity directed towards Rho, Rac, and CDC42. In contrast, these fragments of Vav lack the ability to catalyze nucleotide exchange on the Ras or Ran GTPases.

We note that the GEF activity of Vav directed towards RhoA and Rac1 is strictly dependent on Vav being phosphorylated by the Lck kinase. CDC42 serves as a substrate for both unphosphorylated and phosphorylated Vav, although phosphorylated Vav is the more potent GEF. The ability of Lck to modulate Vav activity is significant in several respects. First, Vav has been reported to be a substrate of Lck, and both of these molecules are expressed in similar cell types (14). Second, Lck appears to activate wild-type Vav expressed in yeast, as indicated by the ability of wild-type Vav coexpressed with Lck, but not wild-type Vav or Lck alone, to suppress *cdc24*^{ts}. Third, the in vitro GEF activity of Vav fragments directed towards RhoA and Rac1 is strictly dependent on Vav being phosphorylated by Lck. Taken together, these observations lead us to suggest that Lck is an activator of Vav GEF activity. To our knowledge, this represents the first known mechanism for the activation of the GEF activity of a Dbl-related GEF. We are currently examining whether other src-related tyrosine kinases activate Vav's GEF activity or the GEF activity of other Dbl-related molecules. In this regard, we observe that Lck and Dbl do not cooperate in transformation of fibroblasts whereas Vav and Lck act synergistically in transformation of fibroblasts. This may reflect the ability of Lck to activate Vav but not Dbl.

Under conditions where Vav binds to Rho, Rac, and CDC42 GTPases, Vav fails to bind Ras or Ran. Vav binds preferentially to nucleotide-free CDC42 and Rac1. In contrast, Vav binds equally well to nucleotide-free or nucleotide-bound RhoA. However, when Vav is phosphorylated by Lck, it binds preferentially to nucleotide-free RhoA. The preferential binding of Vav to these GTPases is reminiscent of other GEFs which preferentially bind to the reaction intermediate in the nucleotide exchange reaction, i.e., nucleotide-free GTPase. The biochemical characterization of Vav thus yields results consistent with our yeast genetic studies, indicating that Vav is an activator of CDC42-like GTPases. Further, both the yeast genetic data and in vitro biochemical analysis of Vav indicate that Lck phosphorylation of Vav enhances its GEF activity directed towards CDC42-like GTPases. We are currently mapping phosphorylation sites in Vav to better understand the involvement of Lck in the Vav signaling pathway.

While the yeast genetic analysis and in vitro biochemical analysis of Vav strongly suggest that Vav is a GEF for CDC42-related GTPases, these experimental approaches are not well suited for evaluating signaling occurring in animal cells. The mammalian CDC42/Rac/Rho pathways are known to induce cytoskeletal changes as well as changes in gene expression. We present several lines of evidence suggesting that Vav activates CDC42/Rac/Rho signaling in animal cells. First, Vav induces filopodium formation when transfected into REF-52 fibroblasts. Filopodium formation is known to be triggered by activation of CDC42 (34). It will be interesting to determine whether Vav also regulates other cytoskeletal-mediated events such as membrane ruffling, stress fiber formation, and lamellipodium formation. Second, Rac1 and CDC42 are activators of the Jun nuclear kinase (10, 31). We observed that oncogenic Vav activates Jun nuclear kinases. Third, Rac1 and CDC42 indirectly lead to the activation of the ATF-2 transcription factor (17, 20). We observed that Vav also activates ATF-2 in transient transfections. Thus, in each of these cell biological assays for CDC42/Rac/Rho signaling we found evidence indicating that Vav also has the potential to regulate these events.

From results obtained from any one of the experimental approaches we employed to assess Vav's involvement in Rho/Rac/CDC42 signaling, we cannot make a compelling case for Vav being a GEF for these GTPases. Taken together, the four distinct lines of evidence presented here strongly suggest that the physiological role of Vav is to activate Rho/Rac/CDC42-related GTPases and that the Lck kinase modulates Vav activity.

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