Vav2 Is an Activator of Cdc42, Rac1, and RhoA*

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Vav and Vav2 are members of the Dbl family of proteins that act as guanine nucleotide exchange factors (GEFs) for Rho family proteins. Whereas Vav expression is restricted to cells of hematopoietic origin, Vav2 is widely expressed. Although Vav and Vav2 share highly related structural similarities and high sequence identity in their Dbl homology domains, it has been reported that they are active GEFs with distinct substrate specificities toward Rho family members. Whereas Vav displayed GEF activity for Rac1, Cdc42, RhoA, and RhoG, Vav2 was reported to exhibit GEF activity for RhoA, RhoB, and RhoG but not for Rac1 or Cdc42. Consistent with their distinct substrate targets, it was found that constitutively activated versions of Vav and Vav2 caused distinct transformed phenotypes when expressed in NIH 3T3 cells. In contrast to the previous findings, we found that Vav2 can act as a potent GEF for Cdc42, Rac1, and RhoA in vitro. Furthermore, we found that NH₂-terminally truncated and activated Vav and Vav2 caused indistinguishable transforming actions in NIH 3T3 cells that required Cdc42, Rac1, and RhoA function. In addition, like Vav and Rac1, we found that Vav2 activated the Jun NH₂-terminal kinase cascade and also caused the formation of lamellipodia and membrane ruffles in NIH 3T3 cells. Finally, Vav2-transformed NIH 3T3 cells showed up-regulated levels of Rac-GTP. We conclude that Vav2 and Vav share overlapping downstream targets and are activators of multiple Rho family proteins. Therefore, Vav2 may mediate the same cellular consequences in nonhematopoietic cells as Vav does in hematopoietic cells.

DbⅯ family proteins serve as guanine nucleotide exchange factors (GEFs)† and activators of specific Rho family small GTPases (reviewed in Refs. 1 and 2). Rho family proteins function as GDP/GTP-regulated molecular switches that cycle between an active GTP-bound state and an inactive GDP-bound state (reviewed in Refs. 3–5). Whereas Dbl family proteins stimulate GDP dissociation to promote the formation of the active GTP-bound protein, Rho family proteins are inactivated by GTPase-activating proteins (GAPs) that stimulate the intrinsic rate of GTP hydrolysis. A wide array of extracellular stimuli has been shown to promote the activation of Rho family proteins, most likely through the activation of Dbl family proteins.

Fourteen mammalian members of the Rho family of proteins have been identified: RhoA, RhoB, RhoC, RhoD, RhoE, Rnd1/Rnd6, Rnd2/Rho7, RhoG, Rac1, Rac2, Rac3, Cdc42, TC10, and TTF (5). Rho family proteins have been shown to regulate actin cytoskeletal organization, thus influencing cell shape, adhesion, and motility (reviewed in Refs. 3–5). For example, RhoA promotes the formation of actin stress fibers and focal adhesions, whereas Rac1 promotes the formation of lamellipodia and membrane ruffling, and Cdc42 causes actin microspike formation and filopodia development.

Rho family proteins are also activators of signaling pathways that regulate gene expression and cell growth (3–5). For example, Rac1 and Cdc42 but not RhoA activate the Jun NH₂-terminal kinase (JNK) mitogen-activated protein kinase cascade, and JNK in turn phosphorylates and activates the Jun, ATF-2 and Elk-1 transcription factors (6, 7). RhoA, Rac1, and Cdc42 are also activators of the NF-κB and serum response factor transcription factors (8, 9). These transcription factors in turn regulate genes that promote cell growth. Rho family proteins are also required for progression through the G1 phase of the cell cycle, in part, by up-regulating the expression of cyclin D1 (10, 11). Thus, it is not surprising that the aberrant activation of Rho family proteins can promote cellular transformation, and the proteins are required for the transforming actions of Ras and other oncoproteins.

To date, over 30 mammalian Dbl family members have been identified. All Dbl family proteins share a tandem Dbl homology (DH) and pleckstrin homology (PH) domain structure (reviewed in Refs. 1 and 2). The DH domain serves as the GTPase binding and GEF catalytic domain. Whereas some Dbl proteins show very specific substrate specificity, others show broad substrate specificity. For example, the Dbl family proteins Lsc, Fdg1, and Tiam1 are specific for RhoA, Cdc42, and Rac1, respectively (12–14). In contrast, Vav has been found to activate multiple Rho family proteins (Rac1, RhoA, RhoB, RhoG, and Cdc42) (15–18). Although the structural basis for substrate specificity of DH domains remains to be delineated, we have observed that the overall sequence identities of DH domains does broadly correlate with their GTPase targets (2).

Although PH domains are found in other signaling proteins, a PH domain is invariably positioned just COOH-terminal to the DH domain within Dbl family proteins (1, 2). Thus, the tandem DH/PH association suggests that these domains may functionally interdependent. Presently, it is believed that the PH domain facilitates the membrane localization of Dbl family proteins, where their GTPase substrates reside (19, 20). A second function involves the positive or negative regulation
of the catalytic function of the DH domain (21–23). Outside the DH domains, Dbl family members generally share little sequence similarity. Because NH2-terminal deletion of sequences upstream of the DH domain creates transforming versions of various Dbl family proteins (e.g. Vav, Dbl, and Tiam1), the NH2-terminal sequences may serve a negative regulatory role for these proteins.

A majority of Dbl family proteins have been shown to exhibit transforming potential in NIH 3T3 focus formation assays: Dbl, Vav, Dbs, Ost, Ect2, Net1, Tim, Lbc, Lsc, Lfc, Tiam1, Trio, and Fdg1 (1, 2). Several lines of evidence indicate that Dbl family transformation is the result of their deregulation of Rho GTPase activity. First, where it has been analyzed, mutation of the DH domain causes a loss of transforming activity. Second, Dbl family proteins cause the same changes in actin cytoskeletal organization as their small GTPase substrates. Third, constitutively activated Rho GTPases and Dbl family proteins cause very similar transformed phenotypes when assayed in rodent fibroblasts and other cell types. Fourth, Dbl family members can activate similar signaling pathways as their Rho GTPase substrates. Thus, the activation of Rho GTPases plays an important role in Dbl transformation.

Vav and Vav2 are members of the Dbl family of proteins. These two proteins are highly related showing 63% sequence similarity and 55% identity (24, 25). In particular, among all Dbl family members, the DH domain of Vav shares the strongest sequence identity with that of Vav. The transforming activity of both Vav and Vav2 can be activated by NH2-terminal truncation (25, 26). Both proteins also contain the same spectrin-like tetramerization domain and tandem Src homology 3 (SH3) domains split by an intervening SH2 domain. However, Vav and Vav2 are also distinct in several ways. First, whereas Vav expression is restricted to hematopoietic cells, Vav2 is expressed ubiquitously. Second, although Vav has shown GEF activity on RhoA, RhoG, Rac1, and Cdc42, and Rho GAP p190 have been described and characterized previously (28), cDNAs encoding wild-type p190 Rho GAP (29) and C3 transferase were provided by R. Weinberg and J. Settleman, respectively. pCMV-D3 WASP-GBD encodes the NH2-terminal sequences of WASP that contains the Cdc42-GBD and WASP-GBD has been shown to function as an inhibitor of Cdc42 function (provided by M. Symons) (10, 30, 31).

**Vav2 Specificity, Signaling, and Transformation**

**Cell Culture, Transfection, and Transformation Assays**

NIH 3T3 cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% bovine calf serum. Transfections were performed using calcium phosphate co-precipitation in conjunction with glycerol shock as described previously (32). For each assay, cognate empty vector was used as a control. For focus formation analyses, transfected NIH 3T3 cells were maintained in growth medium for 12–14 days. The cultures were then stained with crystal violet (0.5%), and the number of foci of transformed cells was then quantitated. To select for NIH 3T3 cells stably expressing constitutively activated Vav2, pAX142 ΔN-191 Vav2 (1 µg) were co-transfected with the pZIP-NeoSV(x)1 empty plasmid (neomycin resistant) and maintained in growth medium supplemented with G418 (400 µg/ml). NIH 3T3 cells stably expressing constitutively activated Vav2 were transfected with 2 µg of cognate mammalian expression vector pAX142, pAX142 ΔN-186 Vav, or pAX142 ΔN-191 Vav2 using the LipofectAMINE Plus (Life Technologies, Inc.) reagent according to manufacturer's instructions. 24 h after transfection, cells were trypsinized and plated on 10 µg/ml fibronectin-coated glass coverslips (Carolina Biologicals) and allowed to attach and spread for 2 h. 

**Transient Expression Assays—NIH 3T3 Cells**

NIH 3T3 cells were transfected by calcium phosphate co-precipitation as described previously (32). 48 h after transfection, cells were starved for 15 h with Dulbecco's modified Eagle medium supplemented with 0.5% bovine calf serum to reduce the level of serum activation of c-Jun. Analyses of the cell lysates of the transiently transfected NIH 3T3 cells were performed using enhanced chemiluminescence reagents and a Monolight 2020 luminometer (Analytical Luminescence) as described previously (33, 34). The reporter constructs utilized were previously described; Gal4-Jun encodes the Gal4 DNA-binding domain fused to the NH2-terminal transactivation domain of c-Jun (35), and 5xGal4-Luc contains the luciferase gene under control of tandem copies of the Gal4 DNA-binding sequences and the minimal promoter of c-fos.

**Guanine Nucleotide Exchange Assays—DNA fragments encoding either the human Vav2 DH domain (residues 191–402) or the tandem DH/PH/CRD (residues 191–573) were generated by polymerase chain reaction and inserted into the NcolXhoI sites of the bacterial expression vector pET-28a (Novagen). The bacterial expression constructs were transformed into the Escherichia coli strain BL21 (DE3), and protein expression was induced with 1 mM IPTG at 25 °C. The recombinant His6-tagged Gal4 DNA-binding domain fusion proteins were purified from bacterial lysate on a nickel-nitrilotriacetic acid agarose column (Qiagen). The Vav2 DH protein was further purified on a S-200 column (Qiagen). The Vav2 DH protein was further purified on a S-200 column (Qiagen). The Vav2 DH protein was further purified on a S-200 column (Qiagen).
protein was kindly provided by J. Sondek. Bacterially expressed GST-RhoA(191–401), GST-Rac1(158–216) and GST-Cdc42(175–257) were expressed and purified essentially as described (36). Bacterially expressed Ha-Ras (1–166) was purified as described previously (37).

The GDP dissociation assays were carried out by the filter binding method that was described previously (38). To prepare [3H]GDP-loaded GST-RhoA, GST-Rac1, or GST-Cdc42, solutions containing 10 mM HEPES, pH 7.5, 100 mM NaCl, 7.5 mM EDTA, 15 mM GDP, 5.5 mM [3H]GDP, and 12.5 mM of the GT-Pase were incubated for 25 min at 20 °C. The [3H]GDP-bound GT-Pases were then stabilized by supplementing the solution with 20 mM MgCl₂. Nucleotide exchange reactions were performed at 20 °C by diluting the [3H]GDP-loaded GST-RhoA, GST-Rac1, or GST-Cdc42 to 4 mM in reaction mixtures containing either 4 mM GEF (Vav2 DH, Dbs DH, Tiam1 DH/PH) or no GEF, 10 mM HEPES, pH 7.5, 5 mM MgCl₂, 100 mM NaCl, 1 mM dithiothreitol, 50 µg/ml bovine serum albumin, and 100 µM GTP (final concentrations). 30 µl of each reaction mixture were sampled at 0, 5, 10, and 20 min and quenched in 1 ml of ice-cold dilution buffer containing 20 mM Tris, pH 7.5, 100 mM NaCl, and 20 mM MgCl₂. The amount of [3H]GDP remaining bound to the GT-Pases was measured by filtering the quenched samples over nitrocellulose followed by scintillation counting. The percentage of [3H]GDP remaining bound at each time point for the GEF catalyzed and uncatalyzed remaining bound at each time point for the GEF catalyzed and uncatalyzed reactions were evaluated relative to time 0 of the uncatalyzed reaction. Dbs DH and Tiam1 DH/PH were included as positive controls for substrate specificity (2). Therefore, the recent report that Vav2 showed distinct GEF activities from Vav was unexpected (18).

To further evaluate the activity of the DH domain of Vav2, we constructed, expressed, and purified a bacterially expressed hexahistidine-tagged DH domain fragment of Vav2 (residues 191–401) and analyzed the effect of this protein on the incorporation of cold GTP into [3H]GDP-loaded RhoA, Rac1, or Cdc42 (Fig. 2). RhoA, Rac1, or Cdc42 alone did not exhibit significant intrinsic GDP dissociation. However, the Vav2 DH domain stimulated rapid and complete GDP dissociation from RhoA, Rac1, and Cdc42 within ~20 min. In fact, the reaction went to completion in 5 min with both Rac1 and Cdc42, whereas only 25% of the [3H]GDP was released from RhoA after the same time period. These assays were performed at both stoichiometric and substoichiometric conditions (data not shown). Furthermore, as controls, we examined the in vitro GEF exchange activities of Dbl family members Dbs (DH domain) and Tiam-1 (DH/PH domain) on their respective GT-Pases, Cdc42 and Rac1 (14, 20). We found that Vav2 had similar exchange activities on Rac1 and Cdc42, as did Tiam-1 and Dbs, respectively. Finally, unlike proto-Vav and onco-Vav (deletion of residues 1–67), which require phosphorylation for efficient GEF activity in vitro (15, 16), the catalytic activity of the isolated DH domain of Vav2 was not only more efficient than previously observed for Vav but was also phosphorylation-independent. Therefore, in our assays, we found that Vav2, like Vav, displayed in vitro substrate specificities toward RhoA, Rac1, and Cdc42. Moreover, we found that Vav2 displayed comparable activity for Rac1 and Cdc42 and lower activity for RhoA.

RESULTS

Vav2 Demonstrates GEF Exchange Activity on RhoA, Rac1, and Cdc42 in Vitro—The Vav and Vav2 DH domains share strong sequence identity (51%) (24, 25) (Fig. 1). Furthermore, our sequence alignment and dendrogram analyses of DH domains showed that related DH domains shared similar substrate specificity (2). Therefore, the recent report that Vav2 showed distinct GEF activities from Vav was unexpected (18).

To further evaluate the activity of the DH domain of Vav2, we constructed, expressed, and purified a bacterially expressed hexahistidine-tagged DH domain fragment of Vav2 (residues 191–401) and analyzed the effect of this protein on the incorporation of cold GTP into [3H]GDP-loaded RhoA, Rac1, or Cdc42 (Fig. 2). RhoA, Rac1, or Cdc42 alone did not exhibit significant intrinsic GDP dissociation. However, the Vav2 DH domain stimulated rapid and complete GDP dissociation from RhoA, Rac1, and Cdc42 within ~20 min. In fact, the reaction went to completion in 5 min with both Rac1 and Cdc42, whereas only 25% of the [3H]GDP was released from RhoA after the same time period. These assays were performed at both stoichiometric and substoichiometric conditions (data not shown). Furthermore, as controls, we examined the in vitro GEF exchange activities of Dbl family members Dbs (DH domain) and Tiam-1 (DH/PH domain) on their respective GT-Pases, Cdc42 and Rac1 (14, 20). We found that Vav2 had similar exchange activities on Rac1 and Cdc42, as did Tiam-1 and Dbs, respectively. Finally, unlike proto-Vav and onco-Vav (deletion of residues 1–67), which require phosphorylation for efficient GEF activity in vitro (15, 16), the catalytic activity of the isolated DH domain of Vav2 was not only more efficient than previously observed for Vav but was also phosphorylation-independent. Therefore, in our assays, we found that Vav2, like Vav, displayed in vitro substrate specificities toward RhoA, Rac1, and Cdc42. Moreover, we found that Vav2 displayed comparable activity for Rac1 and Cdc42 and lower activity for RhoA.

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Because sequences flanking the DH domain may influence DH domain substrate specificity, we also evaluated the activity of a bacterially expressed fragment of Vav2 that contains the DH domain together with the COOH-terminal PH and cysteine-rich domains (designated DH/PH/CRD). We have found that the equivalent version of Vav (DH/PH/CRD) is functional \textit{in vivo} for signaling and transforming activity (48). For these analyses we employed a fluorescence spectroscopic analysis of mant-GDP incorporation into GDP-preloaded Rac1, Cdc42, RhoA, and Ha-Ras bacterially expressed proteins (Fig. 3). Similar to our observations with the DH domain alone, we found that the DH/PH/CRD polypeptide displayed comparable activity for Rac1 and Cdc42, lower activity for RhoA, and no activity for Ha-Ras. Thus, the addition of flanking COOH-terminal sequences required for Vav DH domain activity \textit{in vivo} did not alter the GTPase specificity of the Vav2 DH domain.

Vav and Vav2 Cause Indistinguishable Transformed Phenotypes in NIH 3T3 Cells—In contrast to previous analyses (18), our \textit{in vitro} analyses showed that Vav2 does act as a GEF for Rac1 and Cdc42, as well as RhoA, \textit{in vitro}. The previous study also found that Vav and Vav2 caused distinct transformed phenotypes when expressed in NIH 3T3 cells (18, 25). For example, whereas Vav caused the appearance of foci of transformed cells that consisted of piled up, nonrefractile cells, Vav2 was reported to cause the appearance of foci that contained multinucleated cells that included rounded and refractile cells. This was consistent with their observed differences in Vav and Vav2 substrate specificity. However, because our analyses showed that Vav and Vav2 displayed the same substrate specificity, we next determined whether Vav and Vav2 caused similar or distinct transformation phenotypes.

For these analyses, we used an NH$_2$-terminal truncated mutant of mouse Vav, \( \Delta N-186 \) Vav, which lacks NH$_2$-terminal residues 1–186 (Fig. 1). We recently showed that this mutant of Vav is several 100-fold more potent in focus-forming activity when compared with the originally cloned onco-Vav (deletion of residues 1–67) (27). We also generated an equivalent version of human Vav2 by deleting residues 1–191 from its NH$_2$ terminus (designated \( \Delta N-191 \) Vav2) (Fig. 1). Thus, both these mutants lack the entire NH$_2$-terminal sequences upstream of the DH domain. A similar truncation mutant of Vav2 lacking residues 1–184 was shown previously to transform NIH 3T3 cells (25).

Like \( \Delta N-186 \) Vav, \( \Delta N-191 \) Vav2 showed potent focus-forming activity when transfected into NIH 3T3 cells (\(-40 \) foci/\( \mu \)g of DNA). The transformed foci caused by \( \Delta N-191 \) Vav2 were indistinguishable in appearance from those caused by \( \Delta N-186 \) Vav and consisted of dense clusters of nonrefractile cells (Fig. 4A). These foci were similar to those caused by activated forms of RhoA or Rac1 but very distinct from those caused by activated Ras or by activated serine/threonine or tyrosine kinases (not shown). We observed no multi-nucleated giant cells for either the Vav- or Vav2-induced foci.

We then compared the morphology of NIH 3T3 cells stably expressing activated Vav or Vav2. Both caused equivalent consequences on cellular morphology (Fig. 4B). \( \Delta N-186 \) Vav- and \( \Delta N-191 \) Vav2-transformed cells exhibited a nonrefractile, strongly adherent appearance that was slightly more elongated than the vector-transfected, untransformed NIH 3T3 cells. We also plated NIH 3T3 cells that stably expressed Vav or Vav2 onto soft agar and examined their ability to grow in an anchorage-independent manner (Fig. 4C). We found that similar to cells expressing activated Vav activated Vav2-expressing cells formed colonies in soft agar. Thus, consistent with our data showing similar GEF activities, we observed that activated Vav and Vav2 caused qualitatively and quantitatively similar transformed phenotypes when expressed in NIH 3T3.

Vav2 Transforming Activity Requires RhoA, Rac1, and Cdc42—We recently showed that Vav transformation of NIH 3T3 cells required the activity of RhoA, Rac1, and Cdc42 (27). Therefore, to assess the importance of RhoA, Rac1, and Cdc42 function in Vav2 transforming activity, we performed similar experiments using \( \Delta N-191 \) Vav2 using various approaches to inhibit the function of Rho family GTPases.

We co-transfected \( \Delta N-191 \) Vav2 with expression vectors encoding dominant negative mutants RhoA(19N), Rac1(17N), or Cdc42(17N). By analogy to the Ras(17N) dominant negative protein, these dominant negative mutants have been proposed to inhibit Rho family protein function by forming nonproductive interactions with Dbl family members that associate with the Rho GTPase substrate (41–43). We and others have used these mutants to characterize the involvement of specific Rho family GTPases in transformation by various Dbl family proteins (12, 20, 44). Co-expression of dominant negative RhoA(19N), Rac1(17N), or Cdc42(17N) caused significant reductions, ranging from 65 to 90\%, in Vav2 focus-forming activity (Fig. 5A).

We also used other approaches to support the inhibition of Vav2 transformation that we observed with the dominant negative Rho GTPase mutants. We co-transfected \( \Delta N-191 \) Vav2 with p190 Rho GAP, a protein that stimulates the intrinsic GTP hydrolysis rate of RhoA (45), as a second approach to evaluate an involvement of RhoA in Vav2 transformation. We also co-transfected \( \Delta N-191 \) Vav2 with a plasmid encoding the \( C3 \) \textit{botulinum} toxin, which specifically inhibits RhoA, RhoB, and RhoC (but not Rac1 or Cdc42) function (Fig. 5B), or with a plasmid encoding the isolated Cdc42 binding domain of WASP.
a Cdc42-specific effector (Fig. 5C). This WASP fragment has been utilized as a specific inhibitor of Cdc42 function (10, 30, 31). Co-expression of p190 Rho GAP or C3 decreased Vav2 transforming activity by 90% (Fig. 5, A and B), whereas cotransfection with the WASP fragment decreased Vav2 transforming activity by 50% (Fig. 4C). Taken together with the data from the use of dominant negative GTPases, our observations support a role for RhoA, Rac1, and Cdc42 in mediating Vav2 transforming activity.

Vav2 Stimulates c-Jun Transcriptional Activity—Vav has been shown to be a strong activator of the JNK mitogen-activated protein kinase and, consequently, the c-Jun transcription factor (17, 46). In most cell types, JNK activity is stimulated by activated Rac and Cdc42, but not RhoA (6, 7). Therefore, we examined the ability of ΔN-191 Vav2 to stimulate c-Jun transcriptional activity. For these studies, we performed transient transcriptional assays in NIH 3T3 cells. We transfected cognate empty vector or vectors encoding constitutively activated RhoA(63L), Rac1(61L), or ΔN-191 Vav2 and assessed c-Jun transcriptional activation (Fig. 6). In our assays, we found that constitutively activated RhoA(63L) did not cause activation of c-Jun, whereas both Rac(61L) and Vav2 caused over 3-fold stimulation of c-Jun activity.

Vav2 Induces the Formation of Lamellipodia and Membrane Ruffles—Previous studies found that Vav and Vav2 caused distinct consequences on actin cytoskeletal organization when stably expressed in NIH 3T3 cells (18). Whereas Vav caused a disruption of stress fibers and a preferential localization of actin to peripheral membrane structures, Vav2 induced the formation of abundant stress fibers. In contrast, their analyses of NIH 3T3 cells transiently expressing Vav or Vav2 showed equivalent consequences, with both causing an induction of lamellipodia and membrane ruffling and no obvious formation of filopodia or stress fibers. However, this activity was distinct from the significantly larger lamellipodia caused by activated Rac1.

For our analyses, we transiently transfected NIH 3T3 cells with ΔN-186 Vav, ΔN-191 Vav2, or their respective cognate vectors. Cells expressing activated Vav or Vav2 displayed the same consequences on actin organization (Fig. 7) as activated Rac1 (not shown). When compared with empty vector-transfected NIH 3T3 cells, cells expressing activated Vav or Vav2 were more rounded and displayed lamellipodia and membrane ruffling, indicating that both are activators of Rac1 in vivo. In contrast to previous observations (18), we did not see a disruption of actin stress fibers by activated Vav. Instead, both activated Vav- and Vav2-expressing cells retained well developed actin stress fibers that were similar to those seen in untransformed NIH 3T3 cells.

Our observations that Vav2 caused the induction of lamellipodia and membrane ruffling and that dominant negative Rac1 could block Vav2 transforming activity supported the possibility that Vav2 transformation is associated with constitutive up-regulation of Rac1 function. To address this possibility, we utilized a GST fusion protein containing the Cdc42/Rac binding domain of PAK1 (residues 67–150) as an affinity reagent in a pull-down assay to determine the level of GTP-bound Rac in Vav2-transformed NIH 3T3 cells (47–49). The PAK serine/threonine kinase is an effector of both Rac and Cdc42 and binds preferentially to the active, GTP-bound forms of these two GTPases. When compared with untransformed NIH 3T3 cells, we found a 2.26-fold increase in Rac-GTP levels in Vav2-transformed cells (Fig. 8). Thus, consistent with our in vitro GEF analyses, Vav2 causes constitutive up-regulation of Rac activity.

DISCUSSION

Although the precise sequence requirements that dictate the GTPase specificity of DH domains remains to be delineated, our sequence alignment analyses of DH domain sequences showed a general relationship between sequence similarity and substrate utilization (2). Therefore, it was unexpected that Vav and Vav2, which share strong sequence identity in their DH domains, would possess GEF activity for distinct GTPase targets. Furthermore, it was found that activated Vav2 caused changes in cell morphology and actin cytoskeletal organization and a transformed phenotype that were distinct from those caused by activated Vav (18, 25). In contrast to these observations, we found that Vav and Vav2 share overlapping activities and that both can act as GEFs for RhoA, Rac1, and Cdc42.
FIG. 4. Vav and Vav2 cause similar transformed phenotypes in NIH 3T3 cells. A, foci of transformed cells induced by ΔN-186 Vav and ΔN-191 Vav2. NIH 3T3 cells were transfected using the pAX142 mammalian expression plasmid (1 μg) or pAX142 encoding ΔN-186 Vav (30 ng) and ΔN-191 Vav2 (1 μg). Photographs of foci were taken 14 days after transfection. B, morphology of cells stably expressing ΔN-186 Vav or ΔN-191 Vav2. NIH 3T3 cells stably transfected cells expressing activated Vav or Vav2 were established by pooling together multiple drug-resistant colonies (>100) after growth in medium supplemented with hygromycin or geneticin selection. C, Vav- and Vav2-transformed cells proliferate in the absence of anchorage. NIH 3T3 cells stably expressing activated Vav or Vav2 selected cells were seeded at 2 × 10⁵ cells/60-mm dish in growth medium containing 0.3% agar (over a 0.6% agar base layer) for 10 days. Photographs were taken at 10 days.
First, we found that bacterially expressed fragments of the isolated DH or DH/PH/CRD domains of Vav2 exhibited GEF activity for RhoA, Rac1, and Cdc42 in vitro. Second, NH2-terminal truncated Vav2 caused the appearance of foci of transformed cells that were similar to those induced by NH2-terminal truncated and activated Vav. Vav- and Vav2-transformed NIH 3T3 cells were similar in morphologic appearance on plastic, and both displayed similar abilities to proliferate in soft agar. Third, we showed that Vav2 transforming activity was reduced by co-expression of various inhibitors of Rac1, RhoA, and Cdc42. Fourth, like Vav, Vav2 also activated c-Jun, a downstream target of the JNK mitogen-activated protein kinase. Rac1 and Cdc42, but not RhoA, are activators of JNK in NIH 3T3 cells. Fifth, like Rac1, we also found that Vav2 could induce the formation of lamellipodia and membrane ruffles. Finally, we determined that Rac-GTP levels were constitutively up-regulated in Vav2-transformed cells. Thus, taken together, our data strongly suggest that Vav and Vav2 are activators of multiple, shared Rho family proteins.

Because Vav and Vav2 showed the most related sequence relationship with each other in their DH domain sequences (2), we had anticipated that Vav and Vav2 would share similar, if not identical, GTPase targets for their GEF functions. During the course of our studies, Bustelo and colleagues (18) showed that Vav2 was an activator of RhoA, RhoB, and RhoG but not Rac1 or Cdc42 in vitro. In contrast, we found that Vav2 exhibited strong GEF activity for Rac1 and Cdc42, as well as for RhoA. Possible explanations for the different results between our group and Bustelo and colleagues may be the distinct types of Vav2 constructs utilized in the assays as well as the fact that we used human rather than mouse Vav2. For their in vitro GEF exchange assays, Bustelo and colleagues used insect cell-expressed, poly-histidine-tagged versions of either full-length or NH2-terminal truncated and activated Vav2. In contrast, we employed bacterially expressed versions of the isolated DH or DH/PH/CRD domains of Vav2. Thus, it is possible that COOH-terminal sequences, such as the SH3 and SH2 domains, may influence the GTPase substrate specificity of the DH domain.

**Fig. 5.** Dominant negative RhoA, Rac1, and Cdc42 block Vav2 focus-forming activity. A, NIH 3T3 cells were transiently transfected both with the pAX142 and the pZIP-NeoSV(x1) mammalian expression vectors and with the pAX-ΔN-191 Vav2 (2 μg) together with pZIP-NeoSV(x1)(2 μg) or pZIP-NeoSV(α1)(2 μg). B, the C3 exoenzyme inhibits Vav2 transforming activity. NIH 3T3 cells were transfected with pcDNA3 (2 μg) and pAX142 vectors and with ΔN-191 Vav2 (2 μg) together with pCMV-C3 (2 μg). C, the WASP-GBD blocks Vav2 transforming activity. NIH 3T3 cells were transfected with the pDF30 (2 μg) and pAX142 vectors and ΔN-191 Vav2 (2 μg) with pDF30 (2 μg) or pDF30 WASP-GBD (2 μg). Data shown are representative of at least three independent assays.

**Fig. 6.** Vav2 activates c-Jun transcriptional activity. NIH 3T3 cells were transiently transfected with the cognate empty pAX142 mammalian expression vector and pAX142 plasmids encoding the constitutively activated Rho(63L) (500 ng/dish), Rac1(61L), and ΔN-191 Vav2, together with Gal4-Jun (250 ng/dish) and Gal4-Luc (2.5 μg/dish). Data shown are representative of three independent assays.

**Fig. 7.** Both Vav and Vav2 induce the formation of lamellipodia and membrane ruffling in NIH 3T3 cells. NIH 3T3 cells were transfected with ΔN-186 Vav or ΔN-191 Vav2 and were analyzed by indirect immunofluorescence for changes in the actin cytoskeleton. An anti-Vav antibody (Santa Cruz Biotech) and an anti-Vav2 antibody were used to detect Vav or Vav2 expression. Lamellipodia induction and membrane ruffling were visualized using Texas Red-labeled phalloidin antibodies. Data shown are representative of three independent experiments.
However, Bustelo and colleagues, as well as our unpublished observations, showed that the SH domains were dispensable for Vav function \textit{in vivo}. This would argue that the COOH-terminal SH2/SH3 domains do not play a significant role in dictating the substrate specificity of Vav and Vav2. Finally, changes in Rho GTPase substrate specificity may arise as a result of the different methods of protein expression (\textit{E. coli versus SF9} cells) utilized that may result in distinct protein modifications.

In addition to differences in GTPase substrates, Bustelo and colleagues also found that Vav and Vav2 caused distinct transformed phenotypes, as well distinct changes in cellular morphology and actin cytoskeletal organization, when expressed in NIH 3T3 cells. However, in our transformation, signaling, and actin organization analyses, we found that NH2-terminal truncated versions of Vav and Vav2 caused essentially indistinguishable consequences. Furthermore, we found that like Vav,3 Vav2 transforming activity was also dependent on the function of Rac1, RhoA, and Cdc42. Our signaling and actin cytoskeletal analyses also supported the possibility that Vav2 was an activator of Rac1 and/or Cdc42 \textit{in vivo}. Like activated Rac1, Vav2 induced membrane ruffling and activation of JNK. Our cell-based analyses employed an activated version of Vav2 that induced membrane ruffling and activation of JNK. Our signaling and actin cytoskeletal organization analyses, we found that NH2-terminal truncation of the calponin homology domain (Fig. 1). This region (residues 769–811) is not present in Vav and Vav2 transformation. Vav and Vav2 do diverge in their COOH termini. Our sequence analyses identified a serine-rich region located within the Vav2 COOH terminus, after the NH2 domain (Fig. 1). This region (residues 769–811) is not present in Vav and comprises over 30% serine residues. This region also contains a consensus tyrosine phosphorylation site. Thus, further studies need to be performed to determine whether this region endows Vav2 with functional differences from those ascribed to Vav.

In summary, we have determined that like Vav, Vav2 is an activator of Rac1, RhoA, and Cdc42. Whether these two highly related DbI family proteins serve the same functions in different cell types will require further elucidation of the extracellular signals that cause Vav2 activation. Additionally, although it is clear that Vav and Vav2 are activators of common small GTPases, whether they are distinct in their abilities to activate other Rho family proteins will be important to establish.

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**Fig. 8. Elevation of Rac-GTP levels in Vav2-transformed NIH 3T3 cells.** The amount of activated Rac-GTP was determined in cell lysates of untransformed (empty vector-transfected) or Vav2-transformed NIH 3T3 cells by using bacterially expressed GST-PAK (residues 67–150) (47–49). The amount of Rac associated with GST-PAK was visualized by separation by 15% SDS-polyacrylamide gel electrophoresis and Western blot analysis with anti-Rac antibody (Signal Transduction Lab). Quantitation was done by densitometry scanning of the autoradiogram and the level of Rac-GTP for untransformed NIH 3T3 cells was normalized to one. Western blot analysis of the total cell lysate was also done to verify equivalent levels of endogenous Rac expressing in both cell populations (not shown). Data shown are representative of two independent assays.
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