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Distinct Role of Phosphatidylinositol 3-Kinase and Rho Family GTPases in Vav3-induced Cell Transformation, Cell Motility, and Morphological Changes*

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Vav3 is a member of the Vav family of guanine nucleotide exchange factors (GEFs) for the Rho family GTPases. Deleting the N-terminal calponin homology (CH) domain to generate Vav3-(5–10) or deleting both the CH and the acidic domain to generate Vav3-(6–10) results in activating the transforming potential of Vav3. Expression of either the full-length Vav3 or its truncation mutants led to activation of phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinase (MAPK), focal adhesion kinase (FAK), and Stat3. We investigated the requirement of these signaling molecules for Vav3-induced focus formation and found that PI3K and its downstream signaling molecules, Akt and p70 S6 kinase, are required, albeit to varying degrees. Inhibition of PI3K had a more dramatic effect than inhibition of MAPK on Vav3-(6–10)-induced focus formation. Activated PI3K enhanced the focus-forming activity of Vav3-(6–10). Wild type FAK but not Y397F mutant FAK enhanced Vav3-(6–10)-induced focus formation. Dominant negative (dn) mutant of Stat3 resulted in a 60% inhibition of the focus-forming activity of Vav3-(6–10). Moreover, Rac1, RhoA, and to a lesser extent, Cdc42, are important for Vav3-(6–10)-induced focus formation. Constitutively activated (ca) Rac synergizes with Vav3-(6–10) in focus formation. This synergy requires signaling via Rho-associated kinase (ROK) and p21-activated kinase (PAK), downstream effectors of Rac. Consistently, a ca PAK mutant enhanced, whereas a dn PAK mutant inhibited the focus-forming ability of Vav3-(6–10). Despite having potent focus-forming ability, Vav3-(6–10) has very weak colony-forming ability. This colony-forming ability of Vav3-(6–10) can be enhanced dramatically by co-expressing an activated PI3K and to some extent by co-expressing an activated PAK mutant or c-Myc. Interestingly, inhibition of PI3K and MAPK had no effect on the ability of either wild type or Vav3-(6–10) to induce cytoskeletal changes including formation of lamellipodia and filopodia in NIH 3T3 cells. Over expression of Vav3 or Vav3-(6–10) resulted in an enhancement of cell motility. This enhancement was dependent on PI3K, Rac1, and Cdc42 but not on Rho. Overall, our results show that signaling pathways of PI3K, MAPK, and Rho family GTPases are differentially required for Vav3-induced focus formation, colony formation, morphological changes, and cell motility.

Rho family GTPases are members of the Ras superfamily. Like all members of the Ras superfamily, Rho family GTPases function as molecular switches, cycling between an inactive GDP-bound state and an active GTP-bound state. They have been shown to play an important role in regulating cell cytoskeletal architecture, cell motility, activation of lipid and protein kinases, gene expression, and cell proliferation (reviewed in Refs. 1–4). The Rho family GTPases have also been associated with cell transformation and oncogenesis, either by conferring metastatic potential to transformed cells or by promoting transformation mediated by oncoproteins such as Ras and activated insulin-like growth factor-I receptor (3, 5–8). Although members of the Rho family GTPases have been found to be overexpressed in some tumors, there is no evidence of gain of function mutants of these proteins in human tumors (9). This suggests that deregulation of their upstream regulators plays an important role in their activation observed in tumorigenesis. The switch to the GTP-bound, active form of Rho family GTPases requires the participation of regulatory molecules known as guanosine nucleotide exchange factors (GEFs).[1] The Dbl homology domain-containing proteins are the largest family of GEFs specific for Rho family GTPases (reviewed in Refs. 10 and 11). Several members of these GEFs are highly transforming when overexpressed either as wild type or truncated proteins (10). This further supports the importance of the regulation of the GDP-GTP cycle of Rho family GTPases in the control of cell growth.

Vav family proteins are GEFs for the Rho family GTPases. The first member, Vav1, was originally identified as a transforming gene product in fibroblasts with a deletion of 67 amino acids in its N terminus as compared with the proto-oncogene product (12). Since then several additional members of this family have been identified including Vav2, Vav3, CelVav, a *Caenorhabditis elegans* homolog, and droVav, a *Drosophila* homolog (10, 13). All Vav proteins contain several characteristic domains including a calponin homology (CH) domain, an acidic domain (AD), Dbl homology and pleckstrin homology (PH) domains, a zinc finger domain, a short proline-rich region, and two SH3 domains flanking a single SH2 domain. Vav1 expression is restricted to hematopoietic cells, whereas Vav2 and Vav3 expression pattern is more ubiquitous in nature (14–16).

The enzymatic activity of Vav family proteins has been

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[1] The abbreviations used are: GEF, guanine nucleotide exchange factor; PI3K, phosphatidylinositol 3-kinase; MAPK, mitogen-activated protein kinase; FAK, focal adhesion kinase; dn, dominant negative; ca, constitutively activated; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; CH, calponin homology; AD, acidic domain; PH, pleckstrin homology; STAT, signal transducers and activators of transcription; HA, hemagglutinin; wt, wild type; ROK, Rho-associated kinase; PAK, p21-activated kinase; C3, *Clostridium botulinum* ADP-ribosyltransferase C3.

shown to be regulated by tyrosine phosphorylation (10, 17). Vav enzymatic activity was shown to be additionally modulated by phosphorylated forms of phosphatidylinositol (18). Phosphatidylinositol 3-kinases phosphorylate inositol lipids at the D-3 position of the inositol ring to generate the 3-phosphoinositides (19). Phosphoinositides serve as second messengers by binding and regulating proteins containing PH domains or FYVE fingers (reviewed in Refs. 20 and 21). Vav activity increases 2-fold in the presence of phosphatidylinositol 3,4,5-trisphosphate (PIP3), a product of phosphatidylinositol 3-kinase (PI3K), whereas it is completely inhibited in the presence of PI3K substrate, phosphatidylinositol 4,5-bisphosphate (PIP2) (18). The positive and negative regulation of Vav by phosphatidylinositol is mediated via the PH domain of Vav. The role of phospholipids may be to modulate Vav activity rather than serve as independent activators of Vav function since phospholipids cannot activate unphosphorylated Vav (reviewed in 10). The phospholipids may therefore play a more important role under conditions where Vav is not optimally activated for example by tyrosine phosphorylation.

We have previously shown that Vav3 expression leads to activation of Rho, Rac, and Cdc42, membrane ruffling, and filopodia formation in NIH 3T3 cells (16). Vav1 and Vav2 harbor transforming potential that is manifested upon deletion of the N-terminal negative regulatory sequences (12, 14, 22). Similarly, deletion of the N-terminal sequences of Vav3 leads to Vav3-induced cell transformation (16). Furthermore, we have previously shown that Vav3 gets recruited and phosphorylated by several receptor protein-tyrosine kinases including insulin-like growth factor I receptor, insulin receptor (IR), epidermal growth factor receptor, and Ros (16). Additionally, Vav3 also interacts with several molecules known to be involved in signaling downstream of receptor protein-tyrosine kinases such as Grb2, Shc, and the p85 subunit of PI3K (16).

Rho, Rac, and Cdc42 directly affect integrin function since they stimulate the formation of focal complexes that contain integrins as well as paxillin, vinculin, and focal adhesion kinase (FAK) (23, 24). Indeed, blocking the function of Rac or Rho even in the presence of extracellular matrix inhibits integrin clustering and focal complex formation, indicating that both binding of cells to the extracellular matrix and the function of Rac or Rho GTPases are necessary for the formation of focal complexes (24). Tyrosine phosphorylation of FAK, a crucial component of integrin signaling, was found to be a Rho-dependent process (25, 26). Analysis of results gathered from several studies suggests that the following sequence of events may occur upon integrin engagement (27). Initial integrin clustering results in rapid activation of Rac and Cdc42, and this activation of Rac and Cdc42 induces formation of lamellipodia and filopodia, leading to cell spreading and subsequent activation of Rho. Rho induces maturation of cell contacts to form well developed focal adhesions and actin stress fibers, which in turn may be required for full activation of FAK activity (27). Vav3 is a direct upstream activator of Rho family GTPases, and since FAK plays a crucial role in integrin signaling (reviewed in Ref. 28), it is likely that Vav3 may modulate FAK activation.

Stat3, a member of the STAT (signal transducers and activators of transcription) family of proteins, has been shown to play an important role in cell transformation (29–31). Vav1 and Rac1 have recently been implicated in the activation of Stat3 (32, 33). Because Stat3 is required for cell transformation mediated by various oncogenes including Src and an activated insulin-like growth factor I receptor (29–31), it is possible that Vav3-induced cell transformation may also be dependent on Stat3.

In this paper we have examined whether Vav3 expression has an effect on some of the signaling pathways that can be activated by the Vav3-interacting molecules mentioned above. These pathways include the Ras/Raf/MAPK pathway, which can be activated by Grb2 and Shc and the PI3K pathway as well as FAK and Stat3-mediated signaling. Subsequently, we examined the role of Rho family GTPases, PI3K, MAPK, Stat3, and FAK in Vav3-mediated biological processes including focus formation, cell morphological changes, and motility. We show that Rho family GTPases are required for Vav3-induced focus formation, morphological alterations, and cell motility induced by Vav3 in NIH 3T3 cells. On the other hand, PI3K and MAPK are differentially required for Vav3-mediated cellular functions, *i.e.* they are required for Vav3-mediated cell transformation and cell motility but not for Vav3-induced morphological alterations. Overall, our observations suggest a differential role for PI3K, MAPK, and Rho family GTPases in Vav3-induced cell transformation *versus* changes in cell morphology and cell motility.

EXPERIMENTAL PROCEDURES

Transfection, Focus Formation Assay, and Soft Agar Colony Formation Assay—NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% calf serum. NIH 3T3 cells were transfected by calcium phosphate precipitation for focus formation and colony assay as described previously (7). Each transfection for focus formation assay was performed in triplicate. After transfection, 1 plate was maintained in 5% calf serum for focus formation assay as described previously (7). The second plate was put under selection using Geneticin (G418) (Sigma), and the resistant colonies were scored to serve as a control for transfection efficiency and cell viability after co-transfections with various dominant negative constructs. The 3rd was extracted 48 h post-transfection to generate total cell lysates as described below in order to check the protein expression levels of the various transfected constructs.

Antibodies and Reagents—Generation of anti-Vav3 antibody has been described previously (16). Anti-phosphotyrosine RC20 conjugated to horseradish peroxidase, goat anti-Rabbit IgG-horseradish peroxidase, goat anti-mouse IgG-horseradish peroxidase, and anti-MEK-1 were purchased from Transduction Laboratories. phospho-Akt (Thr-308), and phospho-p42/p44 extracellular signal-regulated kinase, and phospho-Stat3 (Tyr-705) antibodies were obtained from Cell Signaling Technology. Anti-HA monoclonal antibody 12CA5 was purchased from the Mount Sinai Hybridoma Core facility. LY294002, PD98059, and rapamycin were purchased from Calbiochem.

Expression vectors wild type Vav3, Vav3-(5–10) (ΔCH), and Vav3-(6–10) (ΔCH + AD) have been described previously (16). Dominant negative (dn) and constitutively activated (ca), mutants of Rho have been described previously (7). Expression vectors for *Clostridium botulinum* ribosyltransferase C3 (C3 transferase) and effector site mutants of Rac and Cdc42 were obtained from Dr. Alan Hall (Medical Research Council Laboratory for Molecular Cell Biology, University College London), and the Rho effector site mutants were from Dr. Negishi (Kyoto University, Japan). Dbl expression vector was obtained from Dr. Andrew Chan (Derald H. Ruttenberg Cancer Center, Mount Sinai School of Medicine). The dn p85 (Δp85) mutant was obtained from Dr. Julian Downward (34), and ca PI3K (BD110), which has the p110 binding domain of human p85α attached to the N-terminal region of p110, was obtained from Dr. Yasu Fukui (35). The ca PAK and dn PAK constructs were obtained from Dr. Ed Manser (Institute of Molecular and Cellular Biology, The National University of Singapore). The dn MEK1 mutant was provided by Dr. Michael Weber (Department of Microbiology, University of Virginia Health Science Center). The wild type (wt) PTEN construct was obtained from Dr. Yamada (National Institutes of Health). dn Akt was obtained from Dr. Philip Tsichlis (Fox Chase Cancer Center). Wild type, dominant negative, and activated Stat3 constructs were obtained from Dr. James E. Darnell, Jr. (Laboratory of Molecular Cell Biology and Genetics, The Rockefeller University).

Protein Analysis—NIH 3T3 cells transfected using LipofectAMINE 2000 (Invitrogen) or calcium phosphate precipitation were lysed in radioimmune precipitation buffer and subjected to immunoprecipitation or Western blotting 24–48 h post-transfection, as described previously (16). 500 μg of total protein was used for immunoprecipitation, and 20 μg was used for direct Western analysis.

Immunofluorescence Staining—Parental NIH 3T3 cells were trans-

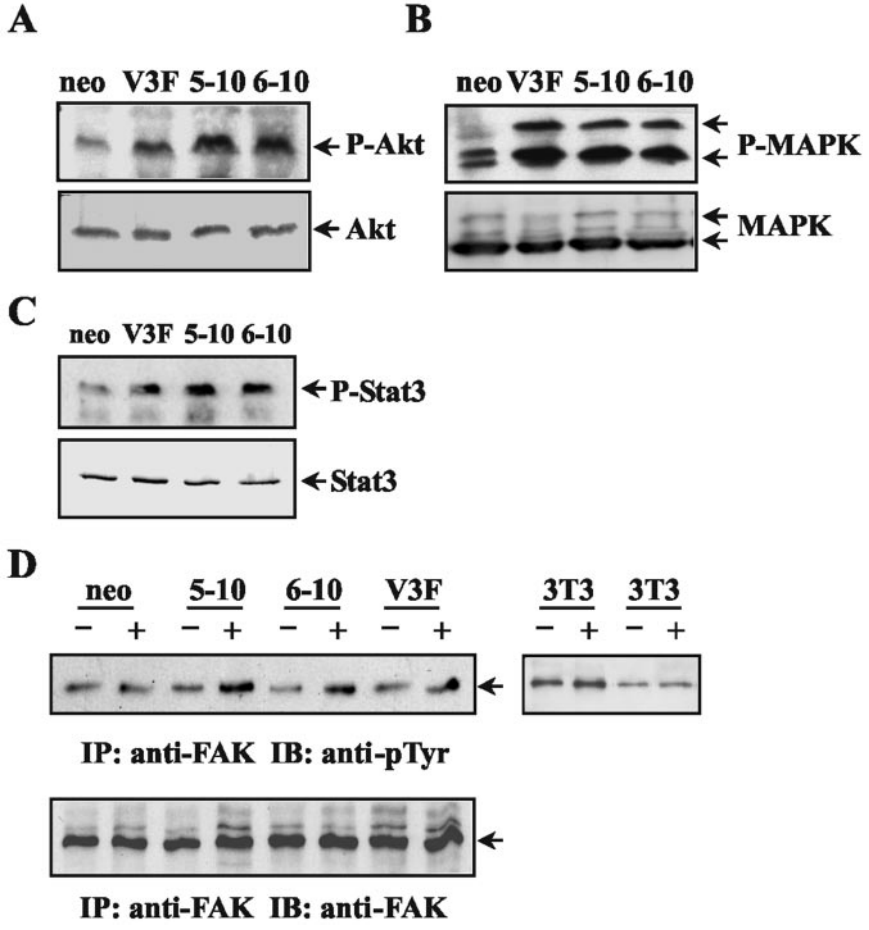

FIG. 1. **Expression of Vav3 and its mutants leads to an activation of Akt, MAPK, Stat3, and FAK.** *A*, *B*, and *C*, total cell lysates from phEF neo (control), full-length Vav3 (*V3F*), Vav3-(5–10) (ΔCH), or Vav3-(6–10) (ΔCH + AD) vector-transfected NIH 3T3 cells were prepared, and 25 μg of each lysate was analyzed in duplicate by Western blotting. One set of filters was probed with phospho-specific antibody against Akt (*P-Akt*) (*A*), p42/p44 MAPK (*P-MAPK*) (*B*), or Stat3 (P-Stat3) (*C*), and the second set of filters was blotted with antibodies against the respective proteins for protein levels of Akt, p42/p44MAPK, or Stat3. *D*, NIH 3T3 cells transfected with either control or Vav3 and various Vav3 mutant-transfected cells were serum-starved for 24 h followed by stimulation with 10% fetal calf serum for 15 min. Cells were extracted, and 1 mg of protein lysate was immunoprecipitated (*IP*) with anti-FAK antibody. The samples were divided in two equal parts and Western-blotted (*IB*) with anti-phosphotyrosine (*pTyr*; *top panel*) or anti-FAK antibody (*bottom panel*), respectively. Two independent NIH 3T3 cells were serum-starved and stimulated in parallel as described above to serve as control for FAK tyrosine phosphorylation upon serum stimulation.

fected using LipofectAMINE 2000 transfection reagent (Invitrogen) and subjected to immunofluorescence analysis as described previously (16). The cells were double-stained with anti-Vav3 followed by anti-rabbit secondary antibody coupled to fluorescein isothiocyanate along with either an appropriate secondary antibody coupled to rhodamine or rhodamine-labeled phalloidin for 1 h.

Cell Migration and Cell Movement—Cell migration was assayed in Boyden chambers (8.0-μm pore size polyethylene terephthalate membrane, FALCON cell culture insert (BD PharMingen)). NIH 3T3 cells were transfected using LipofectAMINE 2000 reagent. Cells were trypsinized 24–48 h later and counted. 200 μl of 5×10^4 cells in serum-free medium were added to the upper chamber. Inserts were incubated for 3–4 h at 37 °C with 500 μl of 5% calf serum in Dulbecco's modified Eagle's medium in the lower chamber. Cells on the inside of the inserts were removed with a cotton swab, and cells on the underside of the insert were fixed and stained with crystal violet. Photomicrographs of three random regions were taken, and the number of cells were counted to calculate the average number of cells that had transmigrated. The Scion Image 4.02 (Scion Corp.) software was used to count the migrated cells.

RESULTS

Vav3 Expression Leads to Activation of MAPK, PI3K, Stat3, and FAK—Vav3 and its deletion mutant, Vav3-(6–10) (ΔCH + AD) have been shown to act as a GEF for various members of the Rho family GTPases (16). To identify additional signaling pathways activated by Vav3, NIH 3T3 cells were transiently transfected with the control plasmid, full-length Vav3, Vav3-(5–10) (ΔCH), or Vav3-(6–10) (ΔCH+AD). Analysis of the total cell lysates generated from these transfected cells showed an increase in phospho-AKT level, which is indicative of increased PI3K activity, and an increase in MAPK, as measured by using an activation state-specific anti-phospho-MAPK antibody (Fig. 1, *A* and *B*). As mentioned above, Rho family GTPases have recently been shown to play a role in Stat3 activation (32, 33). We therefore investigated the effect of Vav3 and its mutants, 5–10 and 6–10, on the activation status of Stat3. We observed an increase in Stat3 tyrosine phosphorylation upon Vav3 expression, as detected by using a phospho-Stat3 (Tyr-705) antibody that specifically detects Stat3 phosphorylated at tyrosine 705, which is known to trigger the activation of Stat3 (Fig. 1*C*). Rho family GTPases have been shown to play an important role in the formation of focal complexes (23, 24). Tyrosine phosphorylation of FAK, a crucial component of integrin signaling, was found to be a Rho-dependent process (25, 26). Immunoprecipitation of FAK from cell lysates unstimulated or stimulated with serum showed that there was increased tyrosine phosphorylation of FAK in cells transfected with either full-length Vav3, Vav3-(5–10), or Vav3-(6–10) over the cells transfected with control plasmid (Fig. 1*D*). The low level of FAK tyrosine phosphorylation upon serum stimulation observed under our experimental conditions in control-transfected cells was confirmed using two additional independent NIH 3T3 cells (Fig. 1*D*). Overall, our data indicate that Vav3 expression results in increased activation of PI3K, MAPK, Stat3, and FAK.

Signaling Pathways Required for Vav3-induced Focus Formation—The deletion of the CH and AD domains of Vav1 and Vav2 led to their constitutive activation and expression of these truncated mutants in NIH 3T3 cells induced foci formation (12, 14, 22). Similarly, Vav3 deletion mutants Vav3-(5–10) and Vav3-(6–10), particularly the latter, can cause cell transformation of NIH 3T3 cells as measured by focus formation assay (16). The N-terminal truncation mutants of Vav3 were shown to induce very compact and elevated foci composed of small, fusiform, and refractile cells (Fig. 2*A* and Ref. 16). We compared the foci induced by Vav3-(6–10), Dbl, ca Rho, and ca Ras (Fig. 2*A*). Vav3-(6–10)-induced foci are very different from the

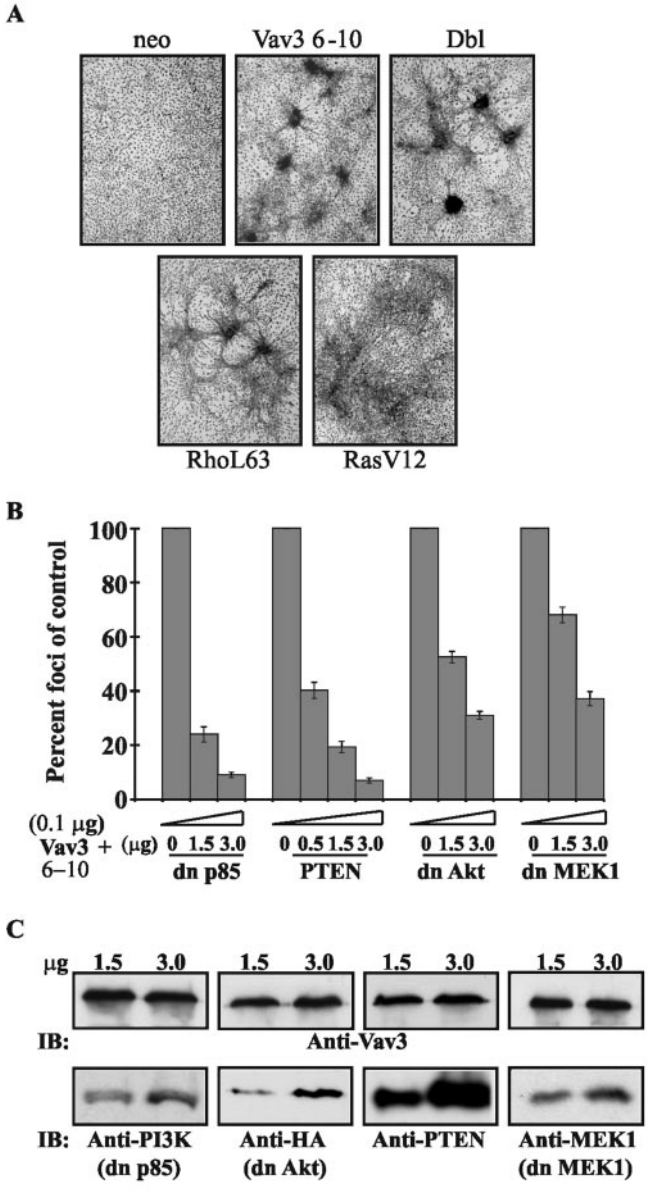

FIG. 2. **Vav3-induced focus formation requires PI3K and MAPK.** *A*, NIH 3T3 cells were transfected with 0.1 μg of phEF neo, Vav3-(6–10), Dbl, Rho L63, or Ras V12 vector, and a focus assay was performed as described under "Experimental Procedures." A representative photomicrograph of the foci induced by the agents described above is presented. *B*, 0.1 μg of phEF Vav3-(6–10) was co-transfected with either control vector, dn p85, PTEN, dn Akt, or dn MEK1 vector at the indicated concentrations, and the focus assay was carried out as described under "Experimental Procedures." Results from at least three independent experiments were compiled, calculated, and depicted in the form of histogram. *IB*, immunoblot. *C*, protein expression levels of various transfectants are shown from a typical experiment. 20 μg of total cell lysates were prepared and analyzed by Western blotting.

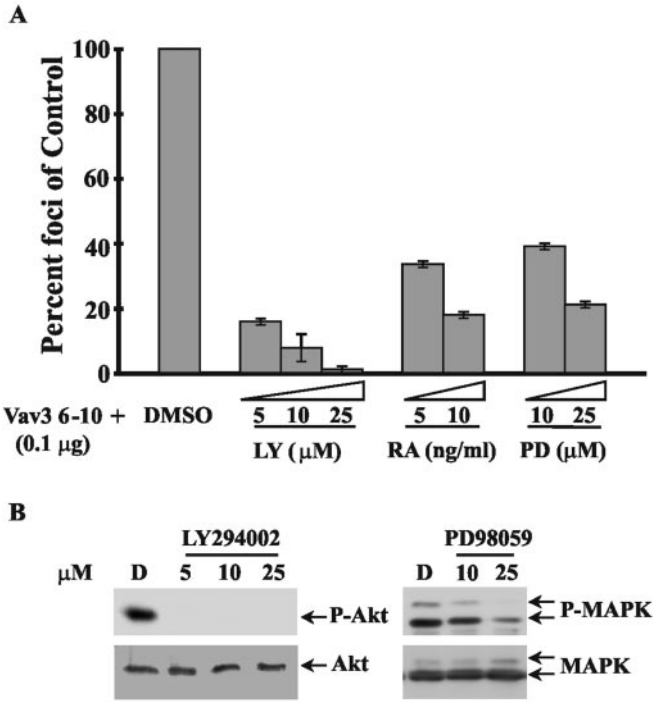

FIG. 3. **Effect of pharmacological inhibitors of PI3K, MAPK, and p70 S6 kinase on Vav3-induced cell transformation.** *A*, NIH 3T3 cells transfected with 0.1 μg of phEF Vav3-(6–10) were subjected to focus assay with or without the drugs, LY294002 (*LY*) (10 μM), rapamycin (*RA*) (10 ng/ml), or PD98059 (*PD*) (25 μM). Results from three independent experiments were calculated and presented. *B*, the inhibitory efficacy of LY294002 and PD98059 on PI3K and MAPK, respectively, was assessed by Western blotting using anti-P-Akt and anti-P-MAPK antibodies to detect the activation level of the respective kinases. A parallel filter was probed with anti-Akt and anti-MAPK, respectively, for protein controls. *DMSO*, Me_2SO; *D*, DMSO or Me_2SO.

oncogenic Ras-induced foci, which are composed of wide spreading, rounded cells, whereas the Vav3-(6–10)-induced foci closely resemble the foci induced by Dbl, a GEF for Rho and Cdc42. Additionally, Vav3-(6–10)-induced foci are morphologically more similar to the foci induced by ca Rho than the foci induced by RasV12, although ca Rho-induced foci are not as tight as those of Vav3-(6–10). This comparison of foci morphology suggests that Vav3 and Rho or other Rho family members may share common signaling pathways, leading to the distinct focus morphology observed here for Vav3-(6–10) and Rho.

We further investigated the role of the various pathways activated by Vav3 and its mutants in Vav3-induced focus formation. To determine the role of PI3K and MAPK in Vav3-induced cell transformation, we used genetic and pharmacological inhibitors of both pathways. Using increasing amounts of a dominant negative mutant of PI3K, Δp85, we showed dramatic inhibition of Vav3-(6–10)-induced focus formation (up to 90%) (Fig. 2*B*). Co-expression of PTEN, which is a lipid phosphatase and a negative regulator of PI3K signaling (36), also resulted in a dose-dependent inhibition. Co-transfection of increasing amounts of PTEN expression vector, 0.5, 1.5, and 3.0 μg, along with Vav3-(6–10) resulted in 60, 80, and 92% inhibition, respectively (Fig. 2*B*). Concomitant with the dn p85 results, inhibition of PI3K using a pharmacological inhibitor of PI3K, LY294002, resulted in almost complete inhibition of the focus formation (Fig. 3*A*). This indicates an important role of PI3K in Vav3-mediated focus formation. PI3K activation results in downstream activation of various signaling molecules, including Akt/protein kinase B. Using a kinase dead dn Akt we observed a 50–65% inhibition of Vav3-(6–10)-induced focus formation (Fig. 2*B*). The inhibition of p70 S6 kinase by rapamycin, which blocks p70 S6 kinase activation via mTOR, also resulted in a 60% inhibition of Vav3-(6–10)-induced focus formation (Fig. 3*A*). The inhibition observed with dn AKT and rapamycin is attenuated compared with inhibition of PI3K using Δp85 or LY294002. This suggests that multiple pathways downstream of PI3K, including Akt/protein kinase B pathway, may be required for Vav3-(6–10)-induced focus formation. Expression of the gene products of various constructs used are shown in Fig. 2*C*. The expression of Vav3-(6–10) protein was not affected by the co-expression of various dominant negative constructs (Fig. 2*C*).

Because PI3K seemed to play an important role in focus

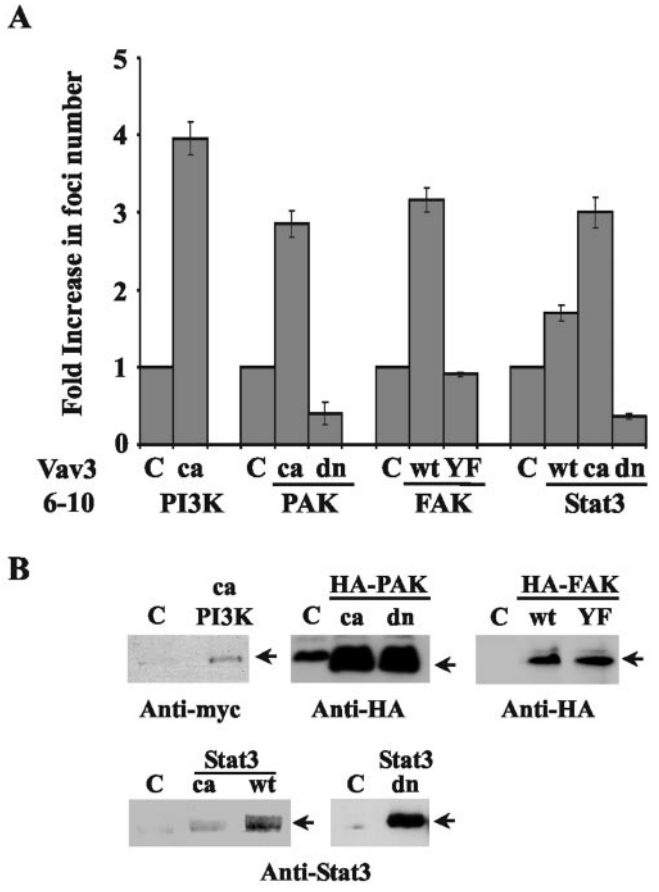

FIG. 4. **Vav3-induced focus formation is further enhanced using ca PI3K, ca PAK, and wt FAK.** *A*, 0.1 μg of phEF Vav3-(6–10) was co-transfected with 3 μg of either control vector, ca PI3K, ca HA-PAK, or dn HA-PAK, wt or Y397F (*YF*) mutant of HA-FAK, or wt, or caStat3, or dn Stat3, and focus formation assay was performed. *B*, 20 μg of total cell lysates were blotted with an anti-HA to detect HA-tagged PAK and FAK or anti-Myc antibody to detect myc-tagged PI3K or anti-Stat3 antibody to detect wt, caStat3, or dn Stat3 protein expression levels.

formation induced by Vav3-(6–10), we decided to examine whether an activated PI3K molecule, BD110, could further enhance the focus-forming ability of Vav3. BD110 has the p110 binding domain of human p85α attached to the N-terminal region of p110 and shown to function as a constitutively activated PI3K molecule (35). Co-expression of Vav3-(6–10) along with activated PI3K resulted in enhancing the focus-forming ability of Vav3 by 4-fold (Fig. 4*A*). The activated PI3K by itself did not induce significant focus formation (data not shown).

Consistent with enhanced FAK activation upon Vav3 expression described above, we observed that wt FAK synergized with Vav3-(6–10) in focus formation (Fig. 4*A*). Likewise, the wt FAK did not induce foci by itself (data not shown). This result suggests that FAK and its downstream signaling pathways can contribute to Vav3-induced focus formation. Tyr-397 is a major autophosphorylation site on FAK and serves as the recruitment site for PI3K and Src (37). The Y397F FAK mutant was unable to synergize with Vav3-(6–10) in focus formation. This is suggestive of a role for PI3K and/or Src downstream of FAK in Vav3-induced focus-formation (Fig. 4*A*). Stat3 has been shown to play an important role in cell transformation (29–31). We found that Vav3 expression led to an increase in Stat3 activation (Fig. 1*C*). Co-expression of a dominant negative mutant in the DNA binding domain of Stat3 (31) and Vav3-(6–10) resulted in a 60% inhibition of the focus-forming ability of Vav3-(6–10) (Fig. 4*A*). Additionally, we observed that an activated Stat3 and to some extent, wild type Stat3 increased the ability of Vav3-(6–10) to induce foci (Fig. 4*A*). The caStat3 by itself did not induce foci but was quite potent in enhancing the Vav3-(6–10)-induced focus formation since, despite its relatively low expression level, a nearly 3-fold enhancement was observed. These results suggest that Stat3 and Stat3-dependent functions are required for Vav3-induced focus formation. The expression of the various constructs from a typical experiment are shown in Fig. 4*B*.

Constitutive activation of the MAPK/extracellular signal-regulated kinase pathway has been associated with promotion and/or maintenance of the transforming and tumorigenic phenotype (38, 39). Inhibition of MAPK using a dominant negative MEK-1 resulted in a 30–60% inhibition of Vav3-mediated cell transformation (Fig. 2*B*). PD98059 treatment of 3T3 cells transfected with Vav3-(6–10) resulted in a 60–80% decrease in Vav3-(6–10)-induced foci (Fig. 3*A*). The results from the genetic and pharmacological inhibitors described above suggest that the MAPK pathway is important for Vav3-(6–10)-induced focus formation. However, when compared with the effect of inhibiting PI3K, inhibition of the MAPK pathway had a relatively milder effect on Vav3-(6–10)-induced focus formation. As shown in Fig. 3*B*, LY294002 and PD98059 effectively inhibited the activation of PI3K and MAPK, respectively, at the concentrations used.

Vav3-(6–10) has been shown to increase the GTP-bound form of RhoA and Rac-1 (16, 40). We used dominant negative mutants of Rho/Rac/Cdc42 to investigate the role of Rho family GTPases in Vav3-(6–10)-induced cell transformation (Fig. 5*A*). We observed a greater decrease in Vav3-(6–10)-induced focus formation in the presence of increasing concentrations of dn Rho and dn Rac as compared with dn Cdc42. Dominant negative Rho and dn Rac inhibited Vav3-(6–10)-induced focus formation by 90% at the highest concentration used *versus* 65% inhibition with dn Cdc42 (Fig. 5*A*). The expression level of the various constructs at the higher concentration is shown from a representative experiment (Fig. 5*B*). Again, the expression level of Vav3-(6–10) protein remains constant, showing that the various co-transfected molecules did not affect the Vav3 expression (Fig. 5*B*). The requirement of Rho was independently confirmed using a potent inhibitor of Rho, C3 transferase. C3 transferase ADP ribosylates Rho at Asn-41 and inactivates it. Vav3-induced focus formation was almost completely blocked in the presence of the highest concentration of C3 transferase. Although only RhoA and Rac-1 have been shown to be activated by Vav3-(6–10) (16), a dominant negative mutant of Cdc42 was able to inhibit Vav3-(6–10)-induced focus formation. This suggests that basal level activity of Cdc42 is required for Vav3-induced focus formation. Furthermore, among the constitutively activated mutants of Rho, Rac, and Cdc42, ca Rac was most efficient in synergizing with Vav3 in focus formation (Fig. 6*A*). Co-expression of Vav3-(6–10) with ca RhoA or its effector site mutants did not result in any significant increase in focus formation. Therefore, constitutive activation of Rac-1 and, thereby, activation of Rac-1-mediated signaling can further enhance Vav3-induced focus formation. We further dissected Rho/Rac/Cdc42-mediated signaling pathways by employing effector domain mutants of Rho/Rac/Cdc42, which selectively activate downstream effector molecules (Fig. 6). RacL61A37 is negative for ROK binding but positive for PAK signaling ($ROK^{-}PAK^{+}$), whereas RacL61C40 is $ROK^{+}PAK^{-}$ (41). Both effector site mutants were incapable of synergizing with Vav3-(6–10) in focus formation (Fig. 6*A*). This suggests that at least both ROK and PAK signaling components downstream of Rac are required in the enhancement of Vav3-induced focus formation. Rho, which is shown to activate ROK

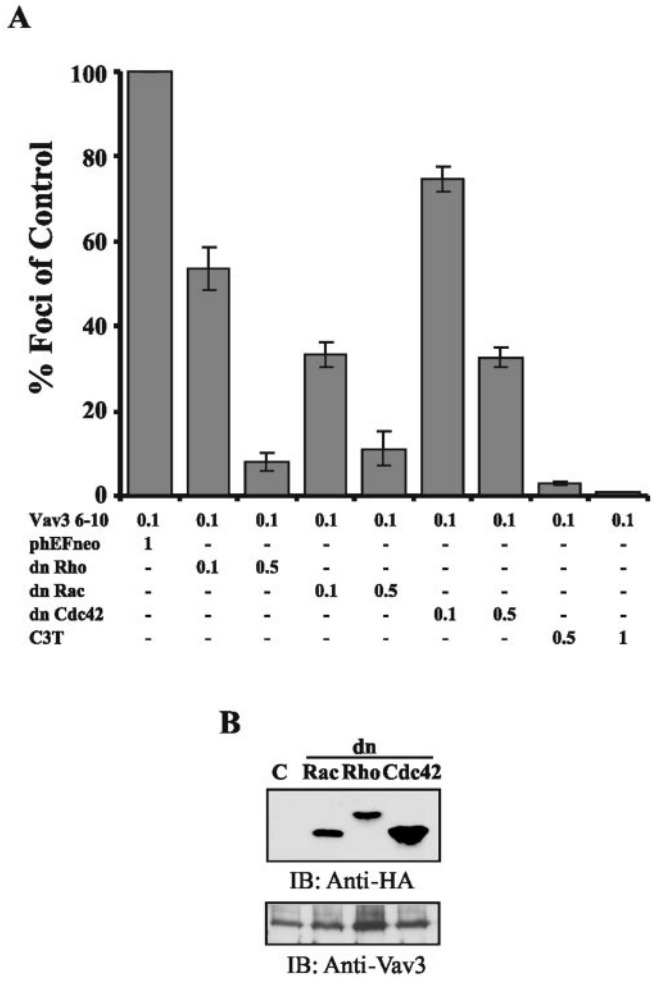

FIG. 5. **Rho family GTPases are required for Vav3-(6–10)-induced focus formation in NIH 3T3 cells.** phEF Vav3-(6–10) (0.1 μg) was co-transfected with increasing amounts of either control phEF neo, HA-dn Rho/Rac/Cdc42, or C3 transferase (*C3T*) vector for focus formation assays as described under "Experimental Procedures." The total amount of DNA transfected was kept constant using control vector. *A*, average results from at least three independent experiments were calculated and presented. *B*, the expression level of dn Rac/Rho/Cdc42 at the higher concentration (0.5 μg) and Vav3-(6–10) was detected by Western analysis (*IB*) of 20 μg of the respective cell lysate using anti-HA antibody and anti-Vav3 antibody, respectively. The results from a typical experiment are shown here.

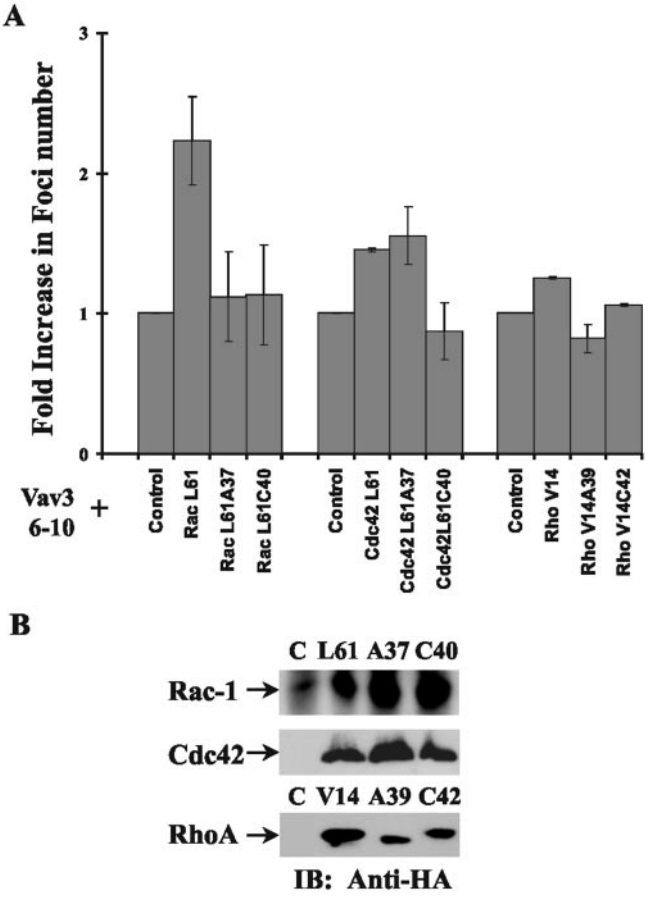

FIG. 6. **Effect of various effector site mutants of ca Rho/Rac/Cdc42 on Vav3-(6–10)-induced focus formation.** phEF Vav3-(6–10) (0.1 μg) was co-transfected with 1 μg of either ca or effector site mutants of HA-tagged Rho/Rac/Cdc42. *A*, average results from three independent experiments are shown. *B*, the expression levels of the gene products of the various constructs were determined by Western analysis (*IB*) using anti-HA antibody.

but not PAK, did not enhance Vav3-induced foci. The moderate increase in Vav3-induced focus formation observed with Cdc42L61 was lost when an effector site mutant of Cdc42, Cdc42L61C40, which is unable to interact with PAK, whereas it was maintained in the effector site mutant Cdc42L61A37 which retains the ability to interact with PAK (Fig. 6A). These observations point to the importance of PAK-mediated signaling in Vav3-(6–10)-induced focus formation (see below and Fig. 4). The expression levels of the various effector site mutants of Rho, Rac, and Cd42 from a typical experiment are shown in Fig. 6*B*.

Collectively, effector site mutants that are compromised in their ability to interact with and activate PAK seem to be unable to enhance the focus-forming ability of Vav3. This dissection of the signaling pathways activated by Rho/Rac/Cdc42 suggests that PAK-mediated signaling may play an important role in Vav3-induced focus formation. To further examine this possibility we studied the role of an activated PAK molecule on Vav3-induced focus formation. We observed a 2–3-fold enhancement of the Vav3-(6–10) focus-forming ability by a membrane-bound constitutively activated PAK, PAK-C*AAX* and, conversely, a 3–4-fold inhibition by a dn PAK mutant (kinase dead) (Fig. 4*A*). The expression levels of respective mutants of PAK (Fig. 4*B*) and the GTPases (Fig. 5*B* and 6*B*) are shown.

Vav3-induced Colony-forming Ability Is Enhanced by an Activated PI3K Mutant—Vav3-(6–10) is able to potently induce focus formation but is very weak in promoting anchorage-independent growth (16). Several signaling molecules known to play a role in anchorage-independent growth were screened for their ability to enhance the colony-forming ability of Vav3-(6–10). They include constitutively activated Stat3, cyclin D1, Myc, caCdc42, myr-Akt, ca PAK (PAK-C*AAX*), and ca PI3K (BD110). From the screening we observed that activated PI3K was able to dramatically enhance the colony-forming ability of Vav3-(6–10) (Fig. 7*A*). As shown in Fig. 7*B*, Vav3-(6–10) or ca PI3K had a very low basal level of colony-forming ability in soft agar. When Vav3-(6–10) and ca PI3K were co-expressed, there was a dramatic increase in size and number of the colonies formed (Fig. 7*B*). Because PAK played a crucial role in Vav3-mediated focus formation, we analyzed the effect of an active, membrane-targeted PAK, ca PAK, on Vav3-induced anchorage-independent growth and found that it was able to significantly enhance the colony-promoting activity of Vav3-(6–10) (Fig. 7*A*). In addition, co-expression of c-Myc was also able to significantly enhance the colony-forming activity of Vav3-(6–10) (Fig. 7*A*). This is consistent with the notion that c-Myc is a target of activated Stat3 (42), which we have shown here to be activated

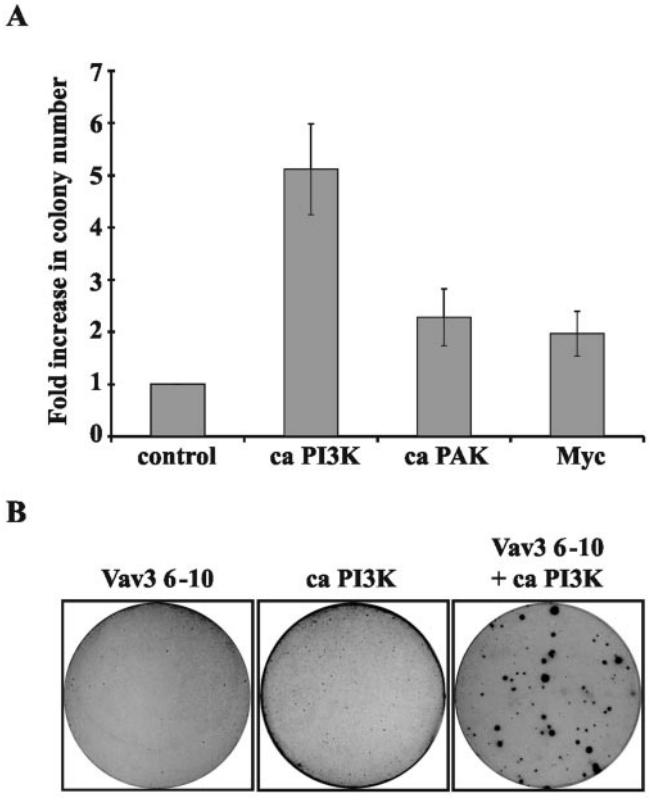

FIG. 7. **Activated PI3K can enhance the colony-forming ability of Vav3-(6–10).** 0.1 μg of phEF Vav3-(6–10) was co-transfected with 3.0 μg of either control, ca PI3K, ca PAK, or wt Myc expression vector. *A*, colony assay was performed as stated under "Experimental Procedures," and average results from three independent experiments are represented. *B*, enhancement in both the size and colony number of Vav3-(6–10)-induced colonies in the presence of ca PI3K is shown in this representative experiment.

upon Vav3 expression and is important for Vav3-induced focus formation. Activated mutants of Rac1/Cdc42/RhoA had no significant enhancing activity (data not shown).

Vav3-mediated Morphological Changes Are Resistant to the Inhibition of PI3K or MAP Kinase—Full-length Vav3 and its deletion mutants, Vav3-(5–10) and Vav3-(6–10), have been shown to induce cell morphological changes, such as membrane ruffling and filopodia formation (16, 40). The data presented above demonstrate the importance of PI3K and MAPK in Vav3-induced focus formation and the involvement of PI3K in Vav3-induced anchorage-independent growth. We next investigated the roles of these pathways in Vav3-mediated morphological changes. Treatment of the Vav3 (Fig. 8*A*, *a–c*), Vav3-(6–10) (Fig. 8*A*, *d–i*), or control plasmid (Fig. 8*A*, *j–l*) transfected cells with Me_2SO (a solvent control), LY294002, or PD98059 did not have any effect on Vav3-induced cell morphological changes. As shown in Fig. 3*B*, 10 μM LY294002 and 25 μM PD98059 substantially inhibited the PI3K and MAPK activation, respectively. Vav3-(6–10) induced two slightly different morphological changes (16). Vav3-(6–10) expression can either lead to extensive membrane ruffling (Fig. 8*A*, *d–f*) or a combination of membrane ruffling and filopodia formation (Fig. 8*A*, *g–i*). Neither of the Vav3-(6–10)-induced cytoskeletal changes was inhibited by LY294002 or PD98059. This result was reproduced under various conditions, *e.g.* cells kept in serum-free media before and during treatment, and extended treatment with the drugs for up to 72 h. Co-expression of a dominant negative Stat3 mutant also did not inhibit Vav3-(6–10)-induced cytoskeletal changes (Fig. 8*B*, *c* and *d*). These data indicate that Vav3-induced morphological alterations are refractory to inhibition of PI3K, MAPK, and Stat3, a result very different from that for cell transformation described above. As expected, the dominant negative mutant of Rac was able to inhibit the morphological alterations induced by Vav3 (Fig. 8*B*, *a* and *b*).

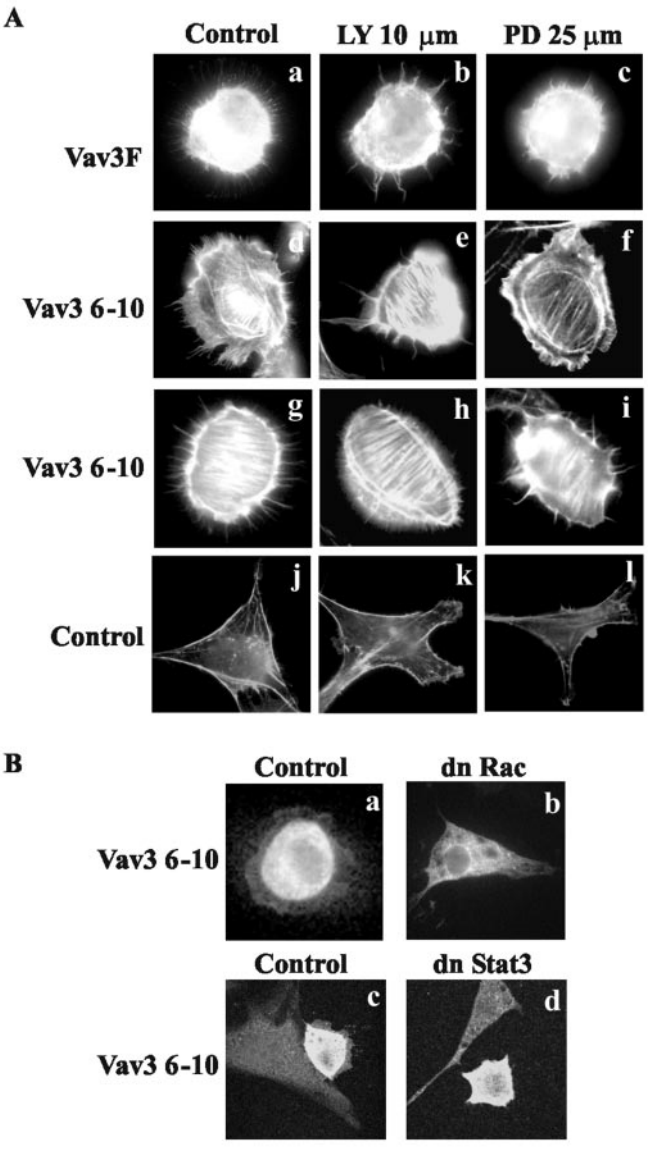

FIG. 8. **Full-length Vav3- and Vav3-(6–10)-mediated cytoskeletal rearrangements are insensitive to PI3K and MAPK inhibitors.** *A*, NIH 3T3 cells transfected with 0.5 μg of phEF Vav3F (*a–c*), phEF Vav3-(6–10) (*d–i*), or phEF neo (control) (*j–l*) vector-transfected cells were plated on coverslips and treated overnight with either control (Me_2SO), LY294002, or PD98059 and double-stained using anti-Vav3 antibody and phalloidin conjugated with rhodamine as described under "Experimental Procedures." *B*, 0.5 μg of phEF Vav3-(6–10) was co-transfected with 1.5 μg of phEF HA-dn Rac (*a* and *b*) or pRcCMV FLAG-tagged dn Stat3 (*c* and *d*), and cells were double-stained with anti-Vav3 antibody and anti-HA or anti-FLAG antibody as described under "Experimental Procedures."

Vav3 Expression Leads to an Increase in Cell Motility—The effect of Vav3 on the morphology of NIH 3T3 cells prompted us to examine the role of Vav3 in cell motility. We found that expression of full-length or Vav3-(6–10) (Fig. 9) but not the 3′ SH3-SH2-SH3 Vav3 fragment (Vav3 SH) (data not shown) was able to enhance cell motility of NIH 3T3 cells. Based on the potent ability of Vav3-(6–10) to enhance cell migration, we used this mutant (Vav3-(6–10)) to further dissect the signaling pathways that may play a role in Vav3-mediated cell migration. We found that Vav3-(6–10)-induced enhancement of cell

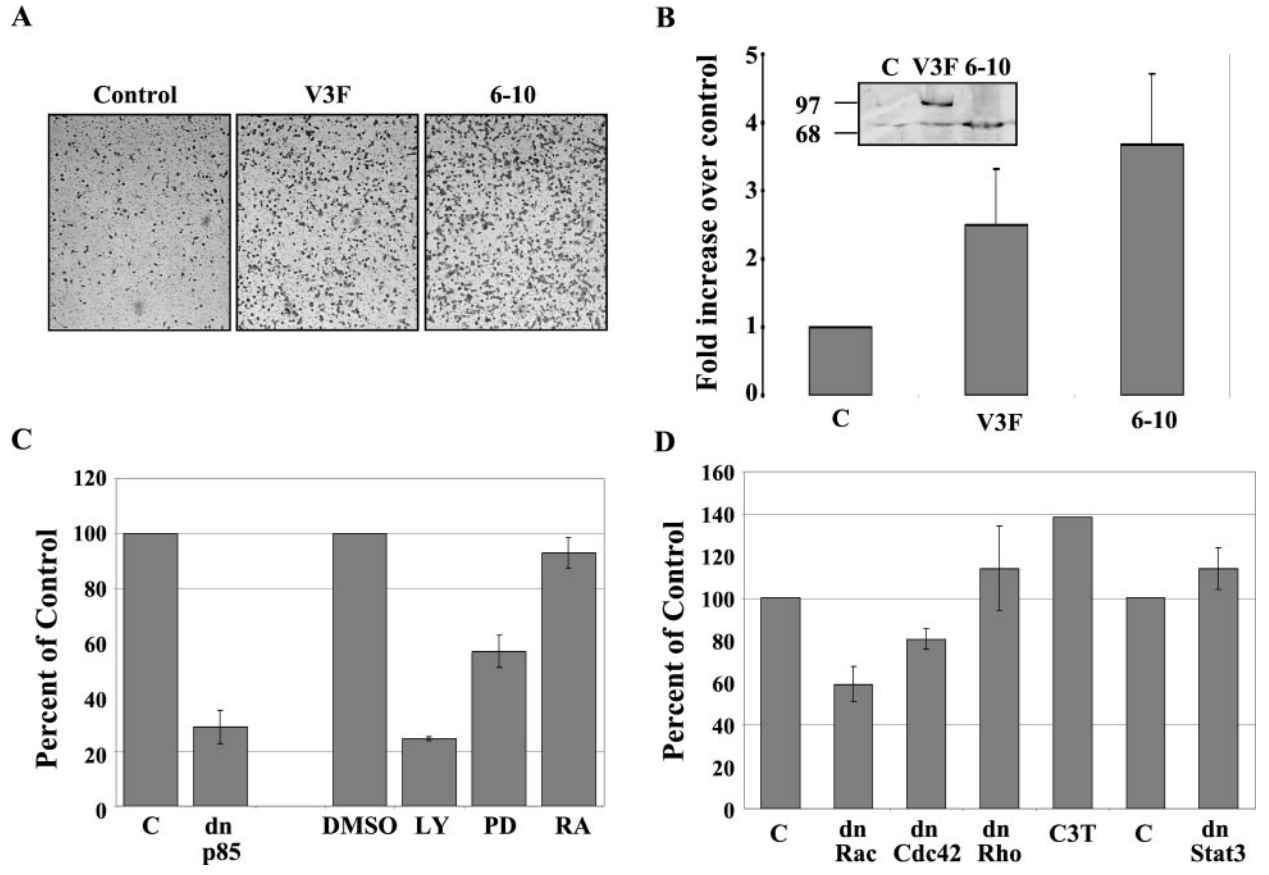

FIG. 9. **Vav3-mediated enhancement of cell motility requires Rho family GTPases and PI3K.** NIH 3T3 cells transfected with either control phEF neo, Vav3F, or Vav3-(6–10) were used in a cell motility assay as stated under "Experimental Procedures." *A*, the photomicrographs of migrated cells are shown. *B*, the results from three independent experiments were calculated and represented. The *inset* shows the expression levels of Vav3F and Vav3-(6–10) from a representative experiment. *C*, NIH 3T3 cells were transfected with 2 μg of phEF Vav3-(6–10) and 24 h later were treated with LY294002 (10 μM), PD98059 (25 μM), or rapamycin (10 ng/ml) for 6 h and subjected to cell motility assay. NIH 3T3 cells were co-transfected with 0.5 μg Vav3-(6–10) and 1.5 μg of either phEF neo (control (*C*)) or dn p85 (*C*) or dn Rac, dn Rho, dn Cdc42, C3 transferase, or dn Stat3 (*D*). The transfected cells were subjected to cell motility assay 24 h later. The average results from three independent experiments were calculated and represented. The migrated cells were counted using Scion Image 4.02 (Scion Corp.).

motility requires PI3K since expression of a dn PI3K or treatment with LY294002 resulted in dramatic inhibition of the Vav3-(6–10)-mediated cell migration (Fig. 9*C*). By contrast, the enhancement in cell motility was not inhibited by rapamycin, suggesting that p70 S6 kinase-mediated signaling is dispensable for this activity (Fig. 9*C*). In addition, inhibition of MAPK by PD98059 also resulted in partial inhibition of Vav3-(6–10)-induced cell motility (Fig. 9*C*). Analysis of the role of Rho family GTPases revealed that dn Rac and to some extent dn Cdc42 were able to inhibit Vav3-(6–10)-mediated enhancement of cell motility (Fig. 9*D*). The dn Rho and C3 transferase, on the other hand, could not inhibit Vav3-(6–10)-induced cell motility. A dominant negative Stat3 mutant had no significant effect on Vav3-(6–10)-induced cell motility (Fig. 9*D*). These results suggest that the PI3K, MAPK, and Rac/Cdc42 signaling, but not p70 S6 kinase Rho or Stat3 signaling, are involved in Vav3-(6–10)-induced cell motility.

DISCUSSION

Vav3 is a member of the Vav family of GEFs specific for Rho family GTPases. We have shown that like Vav1 and Vav2, N-terminal deletion of Vav3 leads to its oncogenic activation (16). Deletion of the AD domain in addition to the CH domain was required for full activation of the transforming ability of Vav3 (16). Vav3 has been shown to become rapidly tyrosine-phosphorylated and associate with several receptor protein-tyrosine kinases and downstream signaling molecules (16, 40, 43). In the present study we show that Vav3 expression results in an increased activation of PI3K and MAPK and tyrosine phosphorylation of FAK and Stat3. PI3K and MAPK are necessary for Vav3-induced cell transformation and cell motility, whereas their activities seem to be dispensable for Vav3-induced cell morphological changes. Rho, Rac, and Cdc42 are required for Vav3-induced cell transformation and cell morphology, whereas inhibition of Rac and Cdc42 but not Rho inhibited Vav3-induced cell motility.

Our results suggest that, like Vav1 and Vav2, N-terminal truncation of Vav3 results in the removal of a negative regulatory element and activation of its transforming potential. This was demonstrated by the ability of the N-terminal truncated Vav3 to induce foci in NIH 3T3 cells. The Vav3 deletion mutants induce foci in a dose-dependent manner. The experiment to study the focus-forming ability of Vav3 was repeated with several NIH 3T3 cell lines derived from different laboratories and yielded similar results. In direct contrast, Movilla and Bustelo (40) report that wild type Vav3, Vav3-(Δ1–144), or Vav3-(Δ1–184) in the presence or absence of activated Lck (Y505F) failed to induce cellular transformation in NIH 3T3 cells. The reason for this discrepancy is unclear at the moment. Different lines of NIH 3T3 cells used could account for the difference. However, we have used 3 independently obtained stocks of NIH 3T3 cells and observed the focus-forming activity of Vav3-(6–10) in all of them (16). There also seems to be some controversy over the morphological appearance of foci induced by the various oncogenic Vavs. One group reported that onco-

genic Vav2 induces foci with distinct morphology compared with oncogenic Vav1 (44), whereas another group reported that both Vav1 and Vav2 induced foci with indistinguishable morphology (14). Other than differences in the cell line and expression systems used by various groups, the reasons for these conflicting reports are unclear at the moment.

Vav1, Vav2, and Vav3 are the only known Rho GEFs that have SH2 domains and exhibit regulation of GEF activity by tyrosine phosphorylation. The unique structure of Vav, with a Dbl homology-PH module and an SH3-SH2-SH3 cassette suggests that it may play a role in coupling GTPases to certain types of receptors. It is likely that recruitment of Vav to the receptor via the membrane-targeting property of its PH domain and SH2/SH3 protein-protein interaction domains may provide a nucleation site for multiple signaling complexes (45). In line with this view we and others have previously shown that Vav3 is rapidly tyrosine-phosphorylated and associates with several receptor protein-tyrosine kinases and signaling molecules, such as Grb2, p85, Shc, and phospholipase C-γ1 (16, 40, 43). In this study, we observed that overexpression of Vav3 resulted in increased activation of PI3K and the MAPK and tyrosine phosphorylation of FAK and Stat3. Each of these signaling molecules has been linked to the function of Vav1 and/or Vav2, although until now they have not been linked to that of Vav3. For example, Vav1 has been shown to bind to the p85 subunit of PI3K, and this binding results in an increased Vav-associated PI3K activity (46).

We observed that the oncogenic N-terminal-truncated mutant of Vav3, Vav3-(6–10)-induced focus formation was dependent on PI3K activity since a dn PI3K, PTEN, a negative regulator of PI3K, and LY294002, a pharmacological inhibitor of PI3K, could efficiently inhibit Vav3-(6–10)-induced focus formation. Additionally, Vav3-(6–10)-induced focus formation was further enhanced in the presence of an activated PI3K. These data suggest a requirement of PI3K and its signaling for Vav3-induced focus formation. PI3K has been shown to play a regulatory role in enhancing tyrosine-phosphorylated Vav1 function since inositol 1,4,5-trisphosphate, a product of PI3K, can stimulate the nucleotide exchange activity of Vav1 by 2–3-fold (18). It is possible that in addition to serving as an upstream regulator of Vav function, PI3K may also serve as a downstream effector of Vav3. Several lines of evidence gathered so far with reference to Vav1 support our hypothesis. Tyrosine-phosphorylated Vav1 was shown to co-immunoprecipitate with p85, the regulatory subunit of PI3K, via the SH2 domain of p85, and this binding resulted in an increased Vav-associated PI3K activity (46). Furthermore, bone marrow-derived mast cells from $Vav1^{-/-}$ showed a more transient production of inositol 1,4,5-trisphosphate and activation of Akt/protein kinase B, an indicator of PI3K activity, as compared with the $Vav1^{+/+}$ mast cells (47), thus confirming a role of Vav1 in sustained activation of PI3K and Akt. Additionally, we observed an increase in PI3K activity as reflected by increased Akt phosphorylation in the presence of exogenous Vav3. These observations suggest that in addition to playing a regulatory role in Vav activation, PI3K activity may itself be regulated by Vav. Moreover, Rac and Cdc42, targets of Vav1, have been shown to directly couple to the p85 subunit of PI3K in their GTP-bound form, suggesting that PI3K can be an effector of Rac and Cdc42 (48–50). Interaction of Rac-GTP with PI3K *in vitro* stimulated the activity of the enzyme by 2–9-fold (48). There is further evidence suggesting that in mast cells, Vav and Rac may lie either upstream or parallel of PI3K/protein kinase B in FCεRI signaling (51).

In the present study we found that Vav3 expression results in increased MAPK activation. Additionally, inhibition of the MAPK pathway using either dn MEK1 or PD098059 resulted in decreased Vav3-(6–10)-induced cell transformation. This result suggests a requirement of MAPK in Vav3-induced focus formation, although comparatively, the inhibition of PI3K had a more adverse effect on Vav3-induced focus formation. We have previously shown that Vav3 can interact with Grb2 in a ligand-dependent manner (16). Vav1 has been shown to associate with Grb2, and this association was mediated via the N-SH3 domain of Vav1 and the C-SH3 domain of Grb2 (52, 53). A recent study further characterized the interaction between Grb2 and Vav1 (54). The association of Vav with Grb2 could thereby potentially engage the Grb2/Sos complex, leading to Ras/MEK/extracellular signal-regulated kinase activation. In support of this hypothesis it has been shown that T-cells derived from $Vav\text{-}1^{-/-}$ mice were defective in various T-cell receptor-mediated signaling pathways including activation of MAPK (55).

We found that Vav3 expression results in increasing the tyrosine phosphorylation of FAK, an important cytoplasmic protein-tyrosine kinase in integrin signaling. Additionally, the focus-forming activity of Vav3-(6–10) was further enhanced in the presence of wild type FAK. These data suggest that Vav3 can modulate the integrin signaling pathway by enhancing the activity of FAK. Our data show that FAK-mediated signaling plays a positive role in Vav3-induced focus formation. Vav3 was shown to activate Rho (16), and tyrosine phosphorylation of FAK was found to be Rho-dependent (25, 26). Rho has been shown to be required for modulating integrin clustering and formation of large integrin clusters, which leads to further enhancement of FAK function (23, 24). Thus, expression of Vav3, a Rho-GEF, enhances Rho activation, which can increase FAK tyrosine phosphorylation. FAK plays an important role downstream of integrins in cell survival, cell migration, and cell transformation (reviewed in Ref. 28). Autophosphorylation of FAK on tyrosine 397 creates a docking site for PI3K and Src. We found that mutation of Tyr-397 to phenylalanine resulted in abolishing the enhancement of Vav-induced focus formation by FAK. This suggests that at least FAK-mediated Src and/or PI3K signaling is required for this effect.

The ability of cells to grow in the absence of adhesion to extracellular matrix or anchorage-independent growth is one of the hallmarks of transformed cells. It is also the best *in vitro* correlate of tumorigenicity (56). Vav3-(6–10) can potently induce loss of contact inhibition but is virtually unable to induce anchorage-independent growth. We found that activated PI3K and to some extent activated PAK and Myc could enhance Vav3-induced anchorage-independent growth, as measured by colony formation in soft agar. It is curious that ca Rac, which is an upstream activator of PAK, was unable to enhance the colony-forming ability of Vav3. It was recently shown that an activated Rac is unable to cause PAK activation in non-adherent cells (57). Rather, cell adhesion promoted membrane targeting of the active Rac, which is required for PAK activation (57). This may explain why activated PAK, but not ca Rac, is able to enhance the Vav3-(6–10) colony-forming activity.

In contrast to the important role of PI3K, MAPK, and Stat3 in Vav3-induced focus formation, our results show that PI3K, MAPK, and Stat3 are dispensable for Vav3-induced morphological changes. Vav3-(6–10)-induced cell morphological changes are resistant to inhibition by PI3K and MAPK inhibitors and expression of a dominant negative Stat3 mutant. This suggests that Vav3-induced cell transformation and morphological changes may be mediated by separate signaling pathways. There is some evidence suggesting that cell transformation and modulation of cell morphology, two distinct properties of Rho family GTPases, can be separated (58). Additionally,

there is evidence that membrane ruffling induced by RacV12 (ca Rac) is insensitive to PI3K inhibition (59). Our data suggest that the overexpression of Vav3 may be sufficient to cause membrane ruffling and filopodia formation irrespective of the PI3K activation status. Contrary to the finding reported by Movilla and Bustelo (40) that full-length Vav3 is unable to induce morphological changes unless co-expressed with $LckY^{505F}$, we observed that just overexpression of the wild type, full-length Vav3 was sufficient to cause morphological alterations of NIH 3T3 cells. A similar situation has been reported with respect to Vav2. Vav2 overexpression was shown to have no effect on actin by Schuebel *et al.* (14), whereas Marignani and Carpenter (60) show that overexpression of Vav2 induced extensive lamellipodia. Because wild type Vav3-induced morphological changes are also insensitive to PI3K inhibition, apparently the overexpressed Vav3 can be activated and signal to induce morphological changes via a pathway independent of PI3K, for example by direct binding to receptor protein-tyrosine kinases and becoming tyrosine-phosphorylated and activated. However, we cannot completely rule out the possibility that in the presence of elevated levels of Vav3, the reduced level of inositol 1,4,5-trisphosphate, as a result of PI3K inhibition, could still interact with a fraction of Vav3 and lead to its activation.

Vav family members have been implicated in playing a role in cell motility. For example, Vav2 induces prominent membrane ruffling and enhanced stress fiber formation and leads to increased rates of cell migration (61). This activity of Vav2 requires Rac1 and Cdc42. Rho family GTPases have been shown by various groups to be important mediators of cell movement (2, 6, 61, 62). It has been hypothesized that Rac plays a role in generating protrusive membrane structures driving cell movement. Cdc42, on the other hand, is believed to have a major role in controlling cell polarity for directed cell movement (2). Vav3-mediated enhancement of NIH 3T3 cell motility was inhibited by PI3K inhibitor, LY294002, MAPK inhibitor, PD98059, and dominant negative mutants of Rac1 and Cdc42. On the other hand, inhibition of p70 S6 kinase using rapamycin and inhibition of Rho and Stat3 using dn Rho or dn Stat3 failed to inhibit Vav3-mediated cell migration. Rac- and Cdc42-mediated enhancement of cell migration and invasion was shown to be dependent on PI3K (62). Additionally, $PTEN^{-/-}$ cells are more motile and contain higher levels of activated Rac and Cdc42 than wild type cells. The enhanced motility of $PTEN^{-/-}$ cells can be suppressed by dominant negative Rac and Cdc42 (63). Vav-2-mediated cell migration is also dependent on Rac and Cdc42 (61). The role of Rho in cell motility is more complicated. Recent evidence suggests that Rac activation down-regulates Rho activity and that the reciprocal balance between Rac and Rho activity determines cell morphology and migration (64, 65). Rottner *et al.* (64) show that inhibition of the Rho pathway caused dissolution of focal contacts and stimulated membrane ruffling and formation of new focal complexes, which were associated with increased cell motility. Therefore, even though our data suggest that Rho is not required in Vav3-(6–10)-induced cell motility, the detailed mechanism could actually be more complicated. The balance of Rac and Rho activity is probably required for the appropriate cell morphology and cell migration. However, Vav3-(6–10) is able to potently activate Rho, and we cannot rule out the possibility that there was residual Rho activity in the presence of dn Rho and C3 transferase used in our experiments.

Taken together, our results show the requirement of Rho family GTPases in Vav3-mediated cell transformation and cell motility. Rac1-mediated signaling can further accentuate activated Vav3-induced cell transformation. Downstream effectors of Rac1, ROK, and PAK are required for the synergistic transforming activity observed with activated Vav3 and activated Rac1. Stat3-mediated signaling is important for Vav3-induced cell transformation but not for Vav3-mediated cell cytoskeletal changes and cell motility. PI3K is more critical than MAPK for Vav3-induced cell transformation and cell motility. Activated PI3K can further enhance Vav3-induced focus formation and dramatically increase the ability of Vav3-(6–10) to induce anchorage-independent growth. PI3K activity appears to be dispensable for Vav3-mediated membrane ruffling and filopodia formation but is required for Vav3-induced cell transformation and cell motility. Vav3 apparently signals via pathways independent of PI3K and MAPK to induce morphological changes.

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REFERENCES

1. Bishop, A. L., and Hall, A. (2000) *Biochem. J.* **348,** 241–255
2. Nobes, C. D., and Hall, A. (1999) *J. Cell Biol.* **144,** 1235–1244
3. Symons, M. (1996) *Trends Biochem. Sci* **21,** 178–181
4. Kjoller, L., and Hall, A. (1999) *Exp. Cell. Res.* **253,** 166–179
5. Clark, E. A., Golub, T. R., Lander, E. S., and Hynes, R. O. (2000) *Nature* **406,** 532–535
6. Michiels, F., Habets, G. G., Stam, J. C., van der Kammen, R. A., and Collard, J. G. (1995) *Nature* **375,** 338–340
7. Sachdev, P., Jiang, Y. X., Li, W., Miki, T., Maruta, H., Nur, E. K. M. S., and Wang, L. H. (2001) *J. Biol. Chem.* **276,** 26461–26471
8. Van Aelst, L., and D'Souza-Schorey, C. (1997) *Genes Dev.* **11,** 2295–2322
9. Fritz, G., Just, I., and Kaina, B. (1999) *Int. J. Cancer* **81,** 682–687
10. Bustelo, X. R. (2000) *Mol. Cell. Biol.* **20,** 1461–1477
11. Collins, T. L., Deckert, M., and Altman, A. (1997) *Immunol. Today* **18,** 221–225
12. Katzav, S., Cleveland, J. L., Heslop, H. E., and Pulido, D. (1991) *Mol. Cell. Biol.* **11,** 1912–1920
13. Dekel, I., Russek, N., Jones, T., Mortin, M. A., and Katzav, S. (2000) *FEBS Lett.* **472,** 99–104
14. Schuebel, K. E., Movilla, N., Rosa, J. L., and Bustelo, X. R. (1998) *EMBO J.* **17,** 6608–6621
15. Trenkle, T., McClelland, M., Adlkofer, K., and Welsh, J. (2000) *Gene* **245,** 139–149
16. Zeng, L., Sachdev, P., Yan, L., Chan, J. L., Trenkle, T., McClelland, M., Welsh, J., and Wang, L. H. (2000) *Mol. Cell. Biol.* **20,** 9212–9224
17. Crespo, P., Schuebel, K. E., Ostrom, A. A., Gutkind, J. S., and Bustelo, X. R. (1997) *Nature* **385,** 169–172
18. Han, J., Luby-Phelps, K., Das, B., Shu, X., Xia, Y., Mosteller, R. D., Krishna, U. M., Falck, J. R., White, M. A., and Broek, D. (1998) *Science* **279,** 558–560
19. Hawkins, P. T., Jackson, T. R., and Stephens, L. R. (1992) *Nature* **358,** 157–159
20. Leevers, S. J., Vanhaesebroeck, B., and Waterfield, M. D. (1999) *Curr. Opin. Cell Biol.* **11,** 219–225
21. Rameh, L. E., Arvidsson, A., Carraway, K. L., III, Couvillon, A. D., Rathbun, G., Crompton, A., VanRenterghem, B., Czech, M. P., Ravichandran, K. S., Burakoff, S. J., Wang, D. S., Chen, C. S., and Cantley, L. C. (1997) *J. Biol. Chem.* **272,** 22059–22066
22. Abe, K., Whitehead, I. P., O'Bryan, J. P., and Der, C. J. (1999) *J. Biol. Chem.* **274,** 30410–30418
23. Ridley, A. J., and Hall, A. (1992) *Cell* **70,** 389–399
24. Hotchin, N. A., and Hall, A. (1995) *J. Cell Biol.* **131,** 1857–1865
25. Barry, S. T., Flinn, H. M., Humphries, M. J., Critchley, D. R., and Ridley, A. J. (1997) *Cell Adhes. Commun.* **4,** 387–398
26. Clark, E. A., King, W. G., Brugge, J. S., Symons, M., and Hynes, R. O. (1998) *J. Cell Biol.* **142,** 573–586
27. Schwartz, M. A., and Shattil, S. J. (2000) *Trends Biochem. Sci* **25,** 388–391
28. Giancotti, F. G., and Ruoslahti, E. (1999) *Science* **285,** 1028–1032
29. Bromberg, J. F., Wrzeszczynska, M. H., Devgan, G., Zhao, Y., Pestell, R. G., Albanese, C., and Darnell, J. E., Jr. (1999) *Cell* **98,** 295–303
30. Turkson, J., Bowman, T., Garcia, R., Caldenhoven, E., De Groot, R. P., and Jove, R. (1998) *Mol. Cell. Biol.* **18,** 2545–2552
31. Zong, C. S., Zeng, L., Jiang, Y., Sadowski, H. B., and Wang, L. H. (1998) *J. Biol. Chem.* **273,** 28065–28072
32. Schuringa, J. J., Jonk, L. J., Dokter, W. H., Vellenga, E., and Kruijer, W. (2000) *Biochem. J.* **347,** 89–96
33. Simon, A. R., Vikis, H. G., Stewart, S., Fanburg, B. L., Cochran, B. H., and Guan, K. L. (2000) *Science* **290,** 144–147
34. Dhand, R., Hara, K., Hiles, I., Bax, B., Gout, I., Panayotou, G., Fry, M. J., Yonezawa, K., Kasuga, M., and Waterfield, M. D. (1994) *EMBO J.* **13,** 511–521
35. Kobayashi, M., Nagata, S., Kita, Y., Nakatsu, N., Ihara, S., Kaibuchi, K., Kuroda, S., Ui, M., Iba, H., Konishi, H., Kikkawa, U., Saitoh, I., and Fukui, Y. (1997) *J. Biol. Chem.* **272,** 16089–16092
36. Dahia, P. L. (2000) *Endocr. Relat. Cancer* **7,** 115–129
37. Chen, H. C., Appeddu, P. A., Isoda, H., and Guan, J. L. (1996) *J. Biol. Chem.* **271,** 26329–26334

38. Mansour, S. J., Matten, W. T., Hermann, A. S., Candia, J. M., Rong, S., Fukasawa, K., Vande Woude, G. F., and Ahn, N. G. (1994) *Science* **265,** 966–970
39. Cowley, S., Paterson, H., Kemp, P., and Marshall, C. J. (1994) *Cell* **77,** 841–852
40. Movilla, N., and Bustelo, X. R. (1999) *Mol. Cell. Biol.* **19,** 7870–7885
41. Lamarche, N., Tapon, N., Stowers, L., Burbelo, P. D., Aspenstrom, P., Bridges, T., Chant, J., and Hall, A. (1996) *Cell* **87,** 519–529
42. Bowman, T., Broome, M. A., Sinibaldi, D., Wharton, W., Pledger, W. J., Sedivy, J. M., Irby, R., Yeatman, T., Courtneidge, S. A., and Jove, R. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 7319–7324
43. Moores, S. L., Selfors, L. M., Fredericks, J., Breit, T., Fujikawa, K., Alt, F. W., Brugge, J. S., and Swat, W. (2000) *Mol. Cell. Biol.* **20,** 6364–6373
44. Abe, K., Rossman, K. L., Liu, B., Ritola, K. D., Chiang, D., Campbell, S. L., Burridge, K., and Der, C. J. (2000) *J. Biol. Chem.* **275,** 10141–10149
45. DeFranco, A. L. (2001) *Nat. Immunol.* **2,** 482–484
46. Shigematsu, H., Iwasaki, H., Otsuka, T., Ohno, Y., Arima, F., and Niho, Y. (1997) *J. Biol. Chem.* **272,** 14334–14340
47. Manetz, T. S., Gonzalez-Espinosa, C., Arudchandran, R., Xirasagar, S., Tybulewicz, V., and Rivera, J. (2001) *Mol. Cell. Biol.* **21,** 3763–3774
48. Bokoch, G. M., Vlahos, C. J., Wang, Y., Knaus, U. G., and Traynor-Kaplan, A. E. (1996) *Biochem. J.* **315,** 775–779
49. Tolias, K. F., Cantley, L. C., and Carpenter, C. L. (1995) *J. Biol. Chem.* **270,** 17656–17659
50. Zheng, Y., Bagrodia, S., and Cerione, R. A. (1994) *J. Biol. Chem.* **269,** 18727–18730
51. Djouder, N., Schmidt, G., Frings, M., Cavalie, A., Thelen, M., and Aktories, K. (2001) *J. Immunol.* **166,** 1627–1634
52. Ramos-Morales, F., Romero, F., Schweighoffer, F., Bismuth, G., Camonis, J., Tortolero, M., and Fischer, S. (1995) *Oncogene* **11,** 1665–1669
53. Ye, Z. S., and Baltimore, D. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 12629–12633
54. Nishida, M., Nagata, K., Hachimori, Y., Horiuchi, M., Ogura, K., Mandiyan, V., Schlessinger, J., and Inagaki, F. (2001) *EMBO J.* **20,** 2995–3007
55. Costello, P. S., Walters, A. E., Mee, P. J., Turner, M., Reynolds, L. F., Prisco, A., Sarner, N., Zamoyska, R., and Tybulewicz, V. L. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 3035–3040
56. Freedman, V. H., and Shin, S. I. (1974) *Cell* **3,** 355–359
57. del Pozo, M. A., Price, L. S., Alderson, N. B., Ren, X. D., and Schwartz, M. A. (2000) *EMBO J.* **19,** 2008–2014
58. Karnoub, A. E., Der, C. J., and Campbell, S. L. (2001) *Mol. Cell. Biol.* **21,** 2847–2857
59. Rodriguez-Viciana, P., Warne, P. H., Khwaja, A., Marte, B. M., Pappin, D., Das, P., Waterfield, M. D., Ridley, A., and Downward, J. (1997) *Cell* **89,** 457–467
60. Marignani, P. A., and Carpenter, C. L. (2001) *J. Cell Biol.* **154,** 177–186
61. Liu, B. P., and Burridge, K. (2000) *Mol. Cell. Biol.* **20,** 7160–7169
62. Keely, P. J., Westwick, J. K., Whitehead, I. P., Der, C. J., and Parise, L. V. (1997) *Nature* **390,** 632–636
63. Liliental, J., Moon, S. Y., Lesche, R., Mamillapalli, R., Li, D., Zheng, Y., Sun, H., and Wu, H. (2000) *Curr. Biol.* **10,** 401–404
64. Rottner, K., Hall, A., and Small, J. V. (1999) *Curr. Biol.* **9,** 640–648
65. Sander, E. E., ten Klooster, J. P., van Delft, S., van der Kammen, R. A., and Collard, J. G. (1999) *J. Cell Biol.* **147,** 1009–1022