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Dbl and Vav Mediate Transformation via Mitogen-Activated Protein Kinase Pathways That Are Distinct from Those Activated by Oncogenic Ras

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Vav and Dbl are members of a novel class of oncogene proteins that share significant sequence identity in a ~250-amino-acid domain, designated the Dbl homology domain. Although Dbl functions as a guanine nucleotide exchange factor (GEF) and activator of Rho family proteins, recent evidence has demonstrated that Vav functions as a GEF for Ras proteins. Thus, transformation by Vav and Dbl may be a consequence of constitutive activation of Ras and Rho proteins, respectively. To address this possibility, we have compared the transforming activities of Vav and Dbl with that of the Ras GEF, GRF/CDC25. As expected, GRF-transformed cells exhibited the same reduction in actin stress fibers and focal adhesions as Ras-transformed cells. In contrast, Vav- and Dbl-transformed cells showed the same well-developed stress fibers and focal adhesions observed in normal or RhoA(63L)-transformed NIH 3T3 cells. Furthermore, neither Vav- or Dbl-transformed cells exhibited the elevated levels of Ras-GTP (60%) observed with GRF-transformed cells. Finally, GRF, but not Vav or Dbl, induced transcriptional activation from Ras-responsive DNA elements (*ets*/AP-1, *fos* promoter, and κB). However, like Ras- and GRF-transformed cells, both Vav- and Dbl-transformed cells exhibited constitutively activated mitogen-activated protein kinases (MAPKs) (primarily p42^{MAPK}/ERK2). Since kinase-deficient forms of p42^{MAPK}/ERK2 and p44^{MAPK}/ERK1 inhibited Dbl transformation, MAPK activation may be an important component of its transforming activity. Taken together, our observations indicate that Vav and Dbl transformation is not a consequence of Ras activation and instead may involve the constitutive activation of MAPKs.

Ras proteins are members of a large superfamily of Ras-related proteins (>50 mammalian members) (7, 56). Like Ras, Ras-related proteins also function as GDP/GTP-regulated molecular switches that control diverse cellular functions (8, 27). The Ras proteins (H-, K-, and N-Ras) function as key intermediates in signal transduction pathways involving upstream receptor and nonreceptor tyrosine kinases and downstream serine/threonine kinases (e.g., the mitogen-activated protein kinases [MAPKs]; also referred to as extracellular signal-regulated kinases [ERKs]) (34, 41). However, the different Ras-related proteins play distinctly different roles in normal cell physiology (7, 56). For example, members of the Rho family are involved in controlling the organization of the actin cytoskeleton (28), whereas members of the Rab family are involved in intracellular transport processes (37), and Ran/TC4 is a regulator of both cell cycle progression and the nuclear-cytosolic trafficking of RNA and protein (7).

The activities of Ras and Ras-related proteins are controlled by regulated GDP/GTP cycles (6). Two distinct classes of regulatory proteins that control Ras GDP/GTP cycling have recently been identified. Whereas GTPase-activating proteins (GAPs; p120-GAP and NF1-GAP) inactivate Ras function by stimulating the intrinsic GTPase activity of Ras to produce the inactive GDP-bound form, guanine nucleotide exchange factors (GEFs; GRF/CDC25 and SOS1/2) activate Ras proteins by triggering formation of the active GTP-bound form. Other members of the Ras superfamily are regulated by unique GAPs and GEFs (6). For example, several GAPs have been identified for Rho family proteins (e.g., p190, n-chimerin, and RhoGAP), while proteins with homology to the Dbl proto-oncogene protein (e.g., Dbl, Vav, and Ect2) are believed to function as GEFs for Rho family proteins.

Structural mutations in Ras itself that activate Ras transforming potential either prevent GAP-stimulated GTP hydrolysis or accelerate GDP/GTP exchange (6, 8). The net consequence of either of the biochemical defects is to favor constitutively elevated levels of active, GTP-complexed Ras and consequently to trigger constitutive activation of the Ras signal transduction pathway. The loss of Ras GAP (3, 16) or constitutive activation of Ras GEFs (2, 12, 39) may also deregulate Ras GDP/GTP cycling, resulting in constitutive activation of the Ras signal transduction pathway.

While the deregulated function of Ras GEFs may lead to cellular transformation, there is evidence that the aberrant function of Rho GEFs may also deregulate and activate the transforming activities of Rho family proteins (6). The prototype for Rho GEFs is the Dbl oncogene protein (18). Dbl was first identified as a transforming protein in NIH 3T3 transfection assays. Subsequently, three additional transforming proteins, Vav, Ect2, and Tim, that share significant sequence identity (30%) with a ~250-amino-acid sequence in Dbl (Dbl homology [DH] domain) were identified (13, 33, 36). The

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recently identified Tiam-1 protein, which promotes T-cell invasion, also contains a DH domain (26). Other proteins with DH domains include the yeast CDC24 protein (GEF for the yeast CDC42 homolog), a Ras GEF, GRF/CDC25 (6), and the protein encoded by the breakpoint cluster region (BCR) gene which is involved in reciprocal translocations with the Abl tyrosine kinase locus in chronic myelogenous leukemias (35). Presently, no transforming activity has been described for DH domains of GRF or BCR. Further, despite their homology with Dbl, no Rho GEF activity has been identified for Vav, Ect2, GRF, or BCR. However, the ability of different DH domain-containing proteins to associate with certain Rho family proteins is consistent with the possibility that they function as GEFs for as yet unidentified Rho family proteins (30, 36).

Dbl has been shown to function as a GEF specifically for two members of the Rho family of proteins (CDC42Hs and RhoA) (29, 30). Consequently, DH domain-containing proteins are believed to function as GEFs for different members of the Rho family. The transforming functions of Dbl, Vav, and Ect2 are activated by N-terminal deletion of sequences upstream of the DH domain in each protein (32, 36, 49). Therefore, it has been proposed that these presumably deregulated GEF oncogene proteins can then trigger constitutive activation of Rho proteins. Since Rho family proteins regulate the actin cytoskeleton (45, 46), the chronic activation of Rho function may then perturb the organization of the actin cytoskeleton and cause alterations in cellular growth and morphology. However, the precise mechanism of transformation by DH domain-containing oncogene proteins is presently not known.

Recently, Vav has been shown to function as an exchange factor for Ras proteins during T-cell activation (24). This observation was surprising since Vav does not contain the CDC25 homology domain found in all other mammalian or yeast Ras exchange factors (GRF, SOS1, SOS2, CDC25, and SDC25) and instead shares homology with the Rho GEF Dbl (20). In the studies presented here, we have compared the transforming activities of Vav and Dbl with those of oncogenic Ras and GRF to determine if Vav causes transformation via activation of Ras and the Ras signal transduction pathway. Our results indicate that GRF, but not Vav or Dbl, transformation is indistinguishable from oncogenic Ras transformation. Instead, the strong similarities between RhoA transformation and Vav and Dbl transformation are more consistent with the possibility that Dbl family oncogenes cause transformation via deregulated Rho protein function.

MATERIALS AND METHODS

Molecular constructs. The pZIP-*ras*H(WT) and pZIP-*ras*H (61L) retrovirus vectors encode wild-type and oncogenic human H-Ras proteins, respectively, and have been described previously (11). pZIP-*dbl* encodes a truncated (deletion of N-terminal residues 1 to 498) and transforming form of human Dbl (19). pMEX-*vav* (pJC12) encodes a truncated (deletion of N-terminal residues 1 to 577) and transforming version of mouse Vav (provided by Mariano Barbacid, Bristol-Myers Squibb) (15). pJ4Ω-GRF encodes human GRF/CDC25 (human homolog of the yeast CDC25 Ras GEF; provided by Larry Feig, Tufts University) (54). pZIP-*rho*A(63L) encodes a mutant human RhoA protein which contains a substitution analogous to the Gln-61-to-Leu mutation that activates Ras transforming activity (33a). pCMV expression constructs containing the ERK cDNA sequences encoding the kinase-deficient $p42^{MAPK}$/ERK2(K52R) and $p44^{MAPK}$/ERK1(K71R) mutants have been described previously and were provided by Melanie Cobb (University Texas Southwestern) (47, 58).

Cell culture and transformation assays. NIH 3T3 mouse fibroblasts were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum. DNA transfections were done by the calcium phosphate precipitation technique as described previously (11). For transformation studies, 50 ng of pZIP-*ras*H(61L), 100 ng of pJ4Ω-GRF, 100 ng of pZIP-*dbl*, 5 μg of pZIP-*rho*A(63L), and 2 μg of pMEX-*vav* were used in each 60-mm-diameter dish. Transfected cultures were maintained in growth medium, and the appearance of transformed foci was quantitated after 14 to 16 days. To establish cell lines that stably expressed exogenously introduced human GRF or *dbl* sequences, transfected cells were selected in growth medium that was supplemented with 400 μg of G418 (Geneticin; GIBCO BRL) per ml. Vav-expressing cells were isolated by cotransfection of pMEX-*vav* (Neo^s) plasmid DNA with pZIP-NeoSV(x)1 (Neo^r) plasmid DNA (50:1 ratio), followed by selection in G418-containing growth medium.

Immunofluorescence analyses. For visualization of stress fibers and focal adhesion components, cells were plated on coverslips in growth medium. After 16 to 24 h, the cells were fixed in 3.7% formaldehyde in phosphate-buffered saline for 7 min, washed in Tris-buffered saline (150 mM NaCl, 50 mM Tris-HCl [pH 7.6], 0.1% sodium azide), and permeabilized with 0.5% Triton X-100 in Tris-buffered saline for 6 min. Double labeling was performed as follows. Actin was stained with either tetramethyl rhodamine isothiocyanate-phalloidin (600 mU/ml) or fluorescein isothiocyanate-phalloidin (5 U/ml) (Molecular Probes, Inc., Eugene, Ore.); the focal adhesion protein vinculin was stained with antivinculin monoclonal antibody 7f9 (23) (kindly provided by Alexey Belkin), followed by fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.) or tetramethyl rhodamine isothiocyanate-conjugated goat anti-mouse immunoglobulin G (Chemicon International, Inc., Temecula, Calif.) (10).

Analysis of in vivo guanine nucleotide association. Determination of the guanine nucleotide bound to Ras in vivo was done as described previously (11, 38). Briefly, NIH 3T3 cells that were transformed with GRF, Dbl, or Vav were metabolically labeled overnight in phosphate-free growth medium supplemented with 1 mCi of $^{32}P_i$ per ml. The labeled cells were then solubilized in a 1% Triton X-100 detergent buffer (11) and immunoprecipitated with anti-Ras monoclonal antibody Y13-259 (22). The bound guanine nucleotides were eluted from the immunoprecipitated Ras proteins, then separated by thin-layer chromatography, and quantitated on an AMBIS beta scanner (38).

Activation of MAPKs. Activation of MAPK was determined by the differential mobility between the inactive and active (phosphorylated) forms of the $p42^{MAPK}$/ERK2 and $p44^{MAPK}$/ERK1, using rabbit anti-MAPK polyclonal antibody 691 (Santa Cruz Biotechnology) as described previously (58). Cell lysates were prepared from NIH 3T3 cells transformed by oncogenic Ras, GRF, Vav, and Dbl, then resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to immobilon (Millipore) blotting membranes. Western blot (immunoblot) analyses were performed with the enhanced chemiluminescence detection system as described by the manufacturer (Amersham). The activation of MAPK is observed as a change in the electrophoretic mobility (slower mobility) of $p42^{MAPK}$/ERK2 and $p44^{MAPK}$/ERK1. Finally, to directly measure the degree of MAPK activation, an in-gel kinase assay using myelin basic protein as a substrate for MAPK activity was performed, using procedures described previously (58).

Transcriptional activation of RREs. To determine the ability of Vav and Dbl to induce transcriptional activation from Ras-responsive elements (RREs) that are preferentially activated by oncogenic Ras proteins (31), promoters containing three different RREs were used to drive expression of the chloramphenicol acetyltransferase (CAT) gene. pB4X-CAT contains four tandem copies of the *ets*/AP-1 sequence from the polyomavirus enhancer (57), p*fos*-CAT contains the *fos* promoter (5), and MHC WT CAT contains three tandem copies of the NFκB binding sequence from the major histocompatibility complex I enhancer (4). Transcriptional activation assays were done in NIH 3T3 fibroblasts transiently cotransfected with each CAT reporter plasmid (1 μg of pB4X-CAT or 5 μg of p*fos*-CAT or MHC WT CAT) together with 100 ng of pZIP-*ras*H(61L) or 5 μg of pZIP-NeoSV(x)1 (empty vector control), pJ4Ω-GRF, pZIP-*dbl*, or pMEX-*vav*. Forty-eight hours after transfection, the cultures were lysed and CAT activity was measured as described previously (31). Briefly, the cells were harvested in 250 mM Tris-HCl (pH 7.8) and then subjected to three cycles of freeze-thawing. Lysates were then precleared by centrifugation at 10,000 rpm, and endogenous CAT was heat inactived. The cells were then incubated at 37°C for 45 min in a reaction mixture containing 250 mM Tris-HCl (pH 7.8), 0.1 μCi of [^{14}C]chloramphenicol (NEN), and 0.34 mM acetyl coenzyme A. After separation by thin-layer chromatography, the degree of conversion to acetylated forms was quantitated on an AMBIS beta scanner.

RESULTS

Vav- and Dbl-transformed cells exhibit a transformed morphology which is distinct from that of Ras- or GRF-transformed cells. Since GRF can cause transformation by activating endogenous Ras function, we anticipate that GRF- and oncogenic Ras(61L)-induced foci should be essentially indistinguishable. In contrast, RhoA-induced transformation of NIH 3T3 cells results in distinctly different transformed foci (40, 52). Thus, the appearance of Vav- and Dbl-induced foci may provide an indication of their mechanism of transformation. Therefore, we compared the morphological alterations caused by GRF, Vav, and Dbl transformation. Consistent with the role of GRF as an activator of Ras, GRF-transfected cells formed morphologically transformed foci which were similar to the swirling and spreading appearance of oncogenic Ras(61L)-induced foci (Fig. 1A). In contrast, both Vav- and Dbl-induced foci were similar to RhoA(63L)-induced foci and were very distinct from either Ras(61L)- or GRF-induced foci. These foci lacked the swirling and very refractile appearance of GRF-induced foci and contained rounded, nonrefractile, and densely packed cells (Fig. 1A).

We then compared the morphologies of individual GRF-, Vav-, and Dbl-transformed cells with those of Ras(61L)- and RhoA(63L)-transformed cells. GRF-transformed cells exhibited the same spindle-shaped, elongated, and very refractile appearance as Ras(61L)-transformed cells (Fig. 1B). In contrast, Vav- and Dbl-transformed cells were similar in appearance to RhoA(63L)-transformed cells and were more cuboidal and much less refractile than Ras-transformed cells (Fig. 1B). Furthermore, neither Ras- nor GRF-transformed cell populations contained the multinucleated giant cells that are observed in Vav-, Dbl-, and RhoA-transformed cells (18, 33). Thus, whereas the morphologic changes observed with GRF transformation were indistinguishable from those observed with Ras transformation, the morphologic changes induced by Vav and Dbl were very distinct.

Vav- and Dbl-transformed cells show increased formation of actin stress fibers and focal adhesions. Whereas Ras-transformed cells typically show decreased numbers of actin stress fibers, the transient expression of activated Rho proteins stimulates the formation of actin stress fibers and focal adhesions (45). Therefore, we compared the effects of GRF, Vav, and Dbl transformation on actin cytoskeletal organization. NIH 3T3 cells stably expressing each transfected gene were used for these analyses. Fixed cells were stained with fluorescent phalloidin to reveal actin stress fibers and with antibodies against vinculin to reveal focal adhesions. The actin cytoskeleton of NIH 3T3 cells was well developed and contained abundant stress fibers that extended across the cell (Fig. 2A). Vinculin staining of focal adhesions was prominent at the cell periphery but was also detected more centrally. The focal adhesions appeared to be large and to stain evenly. In contrast, both Ras- and GRF-transformed cells possessed sparse, less prominent focal adhesions, which were localized only at the margins of cells, where they anchored the few thin stress fibers that were present (Fig. 2A).

We then compared the cytoskeletal organization of Vav- and Dbl-transformed cells with that of RhoA(63L)-transformed cells (Fig. 2B). Dbl-transformed cells displayed a well-organized actin cytoskeleton, with numerous stress fibers that were anchored in elongated, feather-like focal adhesions. These focal adhesions were found both at the cell periphery and more centrally and appeared to be more prominent than those in untransformed cells. Vav-transformed cells showed a similar appearance of stress fiber numbers as untransformed cells. These stress fibers were relatively fine, especially where they extended into lamellipodia. The prominent lamellipodia are characteristic of migratory cells (9). Focal adhesions were increased in Vav-transformed cells, but they were generally smaller and finer than those in untransformed cells.

The relatively normal appearances of stress fibers and focal adhesions indicates that the cytoskeleton is not impaired in fibroblasts transformed with Vav or Dbl. Instead, the appearances of stress fibers and focal adhesions are similar to those seen in RhoA(63L)-transformed cells (Fig. 2B). Thus, the consequences of Vav and Dbl transformation on both stress fiber formation and focal adhesions contrasts sharply with Ras(61L) and GRF transformation and instead more closely resemble what was observed with RhoA(63L) transformation.

Levels of Ras-GTP are not elevated in Vav- or Dbl-transformed cells. The observation that Vav functions as an activator of Ras in hematopoietic cells (24) suggested that Vav transformation may be a consequence of constitutive Ras activation. Cells transformed by Ras GEFs, which share homology with the yeast CDC25 Ras GEF (e.g., yeast SDC25 and mammalian GRF/CDC25), displayed elevated levels of Ras-GTP (12, 44). Therefore, we evaluated whether Vav- and Dbl-transformed cells also exhibit increased levels of activated, Ras-GTP. Whereas normal Ras proteins exist primarily in the inactive, GDP-complexed form in unstimulated cells, oncogenic Ras proteins show greatly elevated levels of Ras-GTP (60 to 80%) (38). In agreement with previous observations, we observed low levels of Ras-GTP (<20%) in untransformed NIH 3T3 cells, whereas GRF-transformed cells showed significantly increased levels of Ras-GTP (60%) (Fig. 3). However, we did not observe any significant elevations in Ras-GTP levels in either Vav- or Dbl-transformed cells. Thus, in contrast with GRF-transformed cells, no constitutive activation of wild-type Ras function is observed in NIH 3T3 cells transformed with these two DH-containing oncogene proteins.

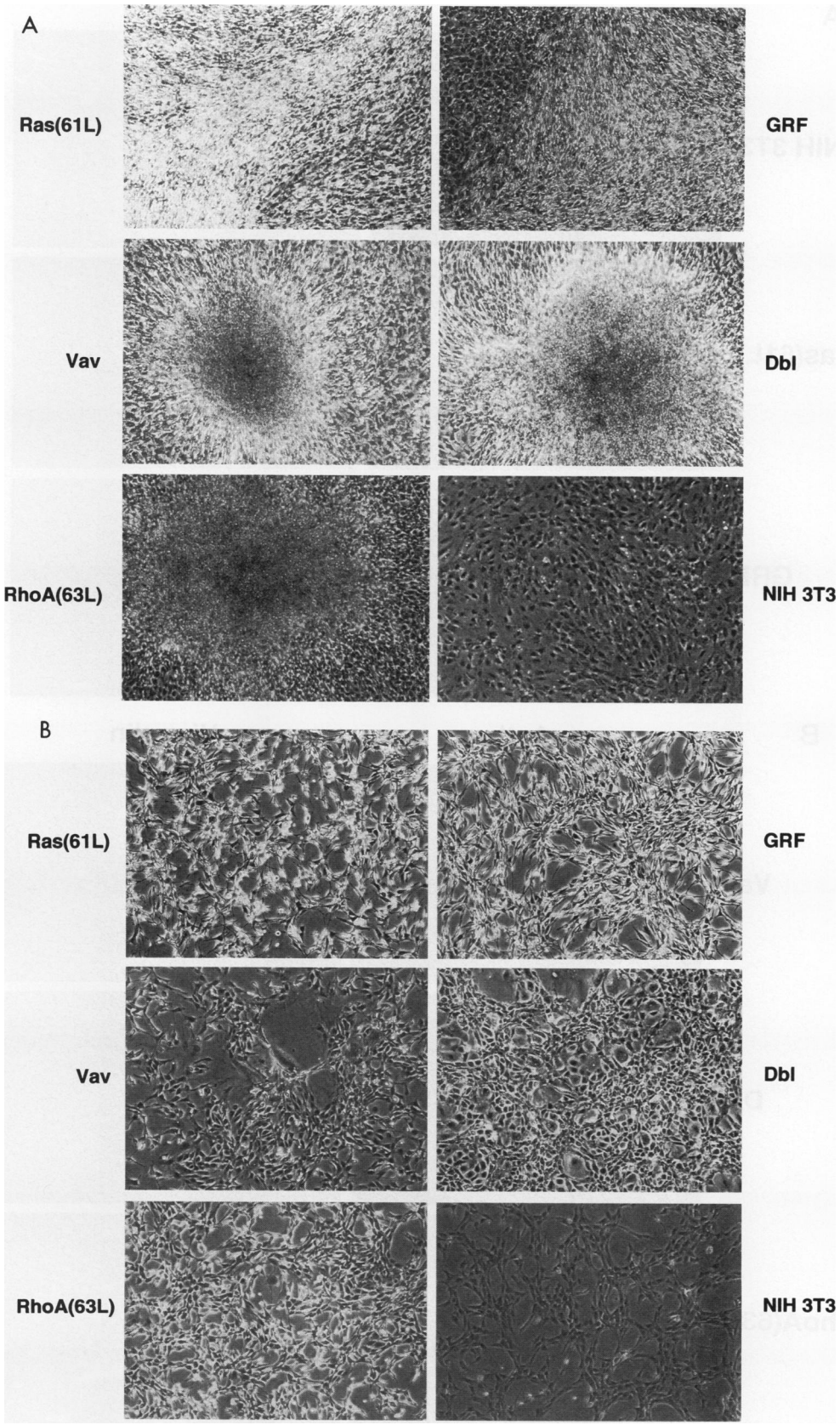

FIG. 1. Oncogenic Ras(61L) and GRF induce transformed foci (A) and morphologies (B) which are distinct from those induced by Vav, Dbl, or RhoA(63L).

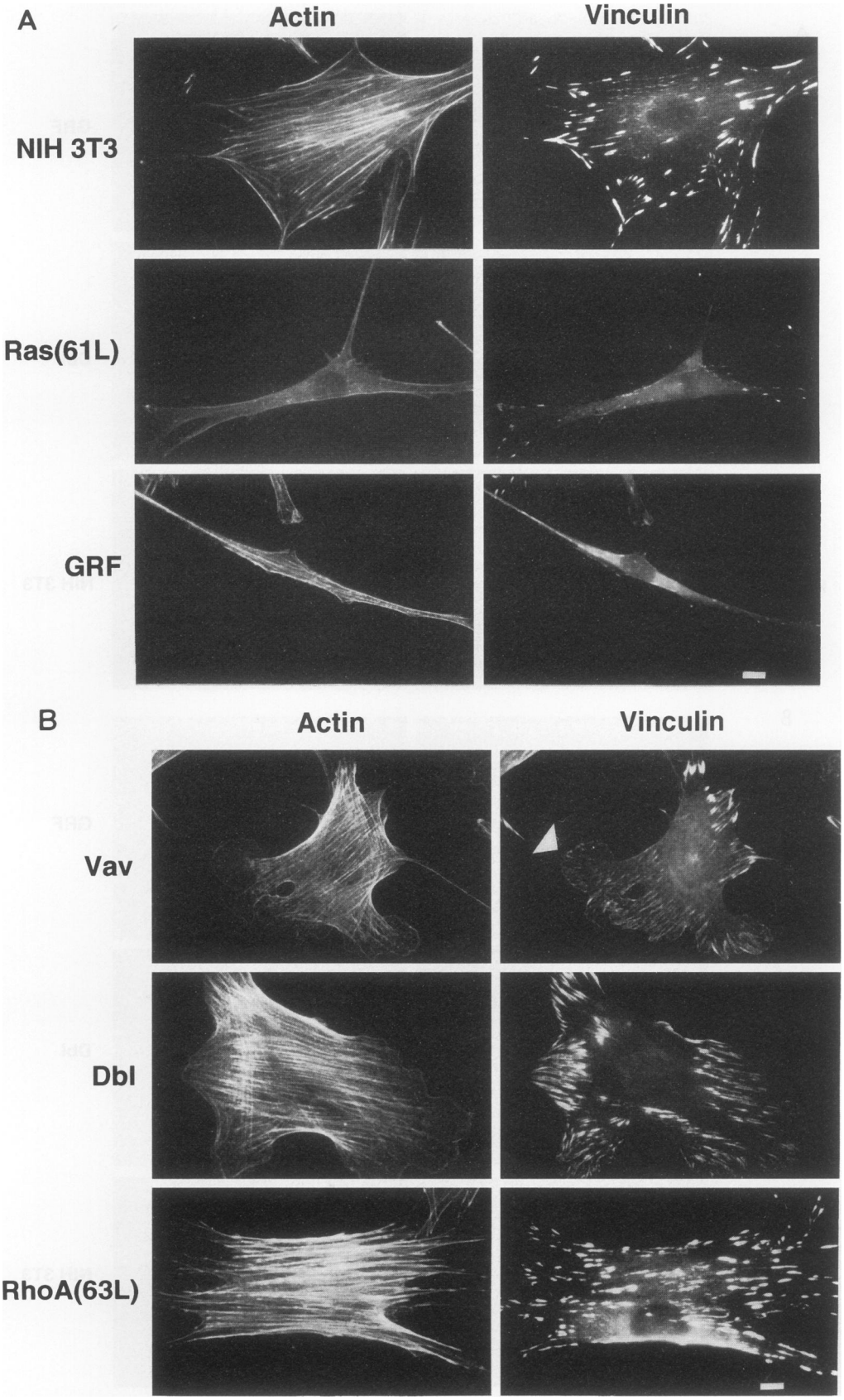

FIG. 2. NIH 3T3 cells transformed by Ras(61L) or GRF (A) or Vav, Dbl, or RhoA(63L) (B) were analyzed by indirect immunofluorescence analysis for actin cytoskeletal organization and focal adhesions with phalloidin and antivinculin antibodies, respectively. The arrow indicates a lamellipodium. Bar = 10 μm.

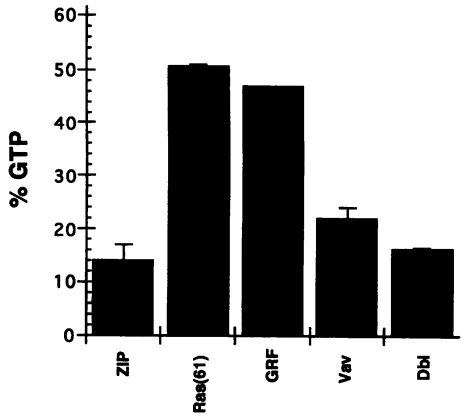

FIG. 3. Determination of the in vivo GDP/GTP association of Ras in NIH 3T3 cells transformed by Ras(61L), GRF, Vav, and Dbl. Values represent the percentage of GTP associated with Ras {[cpm of GTP/(cpm of GDP + cpm of GTP)] × 100} after normalizing for the molar difference between GTP and GDP.

The Ras(17N) dominant inhibitory mutant blocks Vav and Dbl transformation. The Ras(17N) dominant inhibitory mutant protein blocks endogenous Ras function by forming inactive complexes with Ras GEFs (14, 21). Consequently, overexpression of Ras GEFs (GRF and SOS1) has been shown to overcome Ras(17N) inhibitory action (14, 42, 43, 51). To determine whether Vav or Dbl transformation could also overcome Ras(17N)-mediated loss of Ras GEF function, we evaluated whether Ras(17N) could inhibit their transforming activities. Ras(61L), GRF, Vav, and Dbl focus formation assays were done in the presence or absence of cotransfected Ras(17N). As shown previously, oncogenic Ras(61L) focus-forming activity, which does not require Ras GEF stimulation, was not altered by cotransfection with Ras(17N) (Fig. 4A). Similarly, GRF focus-forming activity was not significantly reduced by cotransfection with Ras(17N). This result is consistent with other studies which show that exogenous Ras GEF activity can overcome the inhibitory action of Ras(17N) (14, 42, 43, 51). In contrast, we observed that Ras(17N) significantly reduced both Vav and Dbl focus-forming activities. Thus, Vav or Dbl transforming activity does not overcome Ras(17N) antagonism of Ras GEF function and loss of endogenous Ras function.

We also compared the abilities of GRF, Vav, and Dbl to overcome Ras(17N) inhibition of wild-type Ras stimulation of transcriptional activation from reporter plasmids which contain the CAT gene and upstream promoter sequences that contain the *ets*/AP-1 RRE. Oncogenic, but not normal, Ras proteins can stimulate (10- to 20-fold) transcriptional activation from RREs (31). However, Ras GEFs (GRF, SOS1, CDC25, and SDC25) can synergistically activate normal Ras stimulation of transcription from RREs (14, 42–44). The Ras(17N) dominant inhibitory mutant is a potent inhibitor of this activity; increasing the amount of cotransfected Ras GEF will readily overcome this inhibition. We observed that cotransfection of increased GRF, but not Vav or Dbl, could readily reverse Ras(17N) inhibition of transcription (Fig. 4B). These results suggest that neither Vav nor Dbl could overcome the loss of Ras GEF activity.

Vav and Dbl transformation cause MAPK activation but not transcriptional activation from RREs. If Vav transforming activity is a consequence of Ras activation, then Vav-transformed cells should exhibit activation of downstream events associated with oncogenic Ras transformation. Two downstream events that have been shown to correlate strongly with Ras transforming activity are the constitutive activation of $p42^{MAPK}$/ERK2 and $p44^{MAPK}$/ERK1 (41, 48) and the stimulation of transcriptional activity from RREs (50). Therefore,

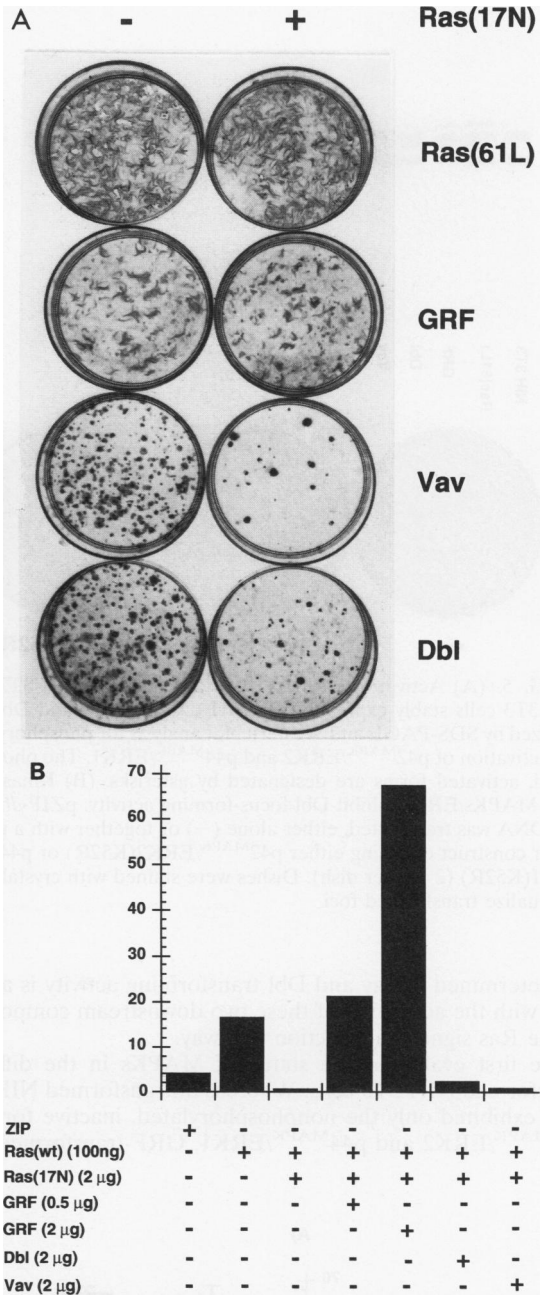

FIG. 4. (A) The Ras(17N) dominant negative protein inhibits Vav and Dbl, but not Ras(61L), focus-forming activity. NIH 3T3 cells were transfected with pZIP-*ras*(61L), pJ4Ω-GRF, pMEX-*vav*, or pZIP-*dbl* plasmid DNA, either alone (−) or together (+) with pZIP-*ras*(17N) plasmid DNA (2 μg per dish). Dishes were stained with crystal violet to visualize transformed foci. (B) GRF, but not Vav or Dbl, overcomes Ras(17N) inhibition of endogenous Ras-GEF activity, as indicated by transcription activation from the Ras-responsive pB4X-CAT reporter plasmid. Ras(wt), wild-type Ras.

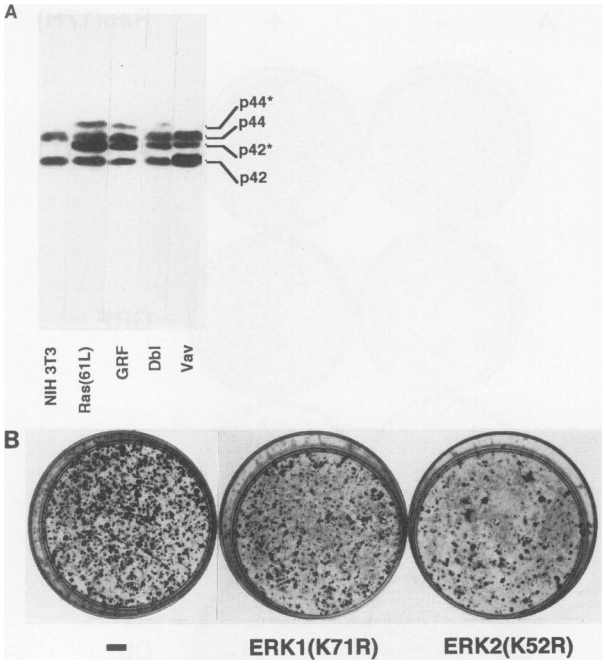

FIG. 5. (A) Activation of MAPKs in transformed NIH 3T3 cells. NIH 3T3 cells stably expressing Ras(61L), GRF, Vav, and Dbl were analyzed by SDS-PAGE and Western blot analysis for phosphorylation and activation of $p42^{MAPK}$/ERK2 and $p44^{MAPK}$/ERK1. The phosphorylated, activated forms are designated by asterisks. (B) Kinase-deficient MAPKs/ERKs inhibit Dbl focus-forming activity. pZIP-*dbl* plasmid DNA was transfected, either alone (−) or together with a pCMV vector construct encoding either $p42^{MAPK}$/ERK2(K52R) or $p44^{MAPK}$/ERK1(K52R) (2 μg per dish). Dishes were stained with crystal violet to visualize transformed foci.

we determined if Vav and Dbl transforming activity is associated with the activation of these two downstream components of the Ras signal transduction pathway.

We first evaluated the status of MAPKs in the different transformed NIH 3T3 cells. Whereas untransformed NIH 3T3 cells exhibited only the nonphosphorylated, inactive forms of $p42^{MAPK}$/ERK2 and $p44^{MAPK}$/ERK1, GRF-transformed cells showed significant levels of the phosphorylated, activated forms of MAPKs with slower electrophoretic mobility of MAPKs that are observed in oncogenic Ras-transformed cells (Fig. 5A). Increased levels of MAPK activation were also observed in Vav- and Dbl-transformed cells (primarily $p42^{MAPK}$/ERK2) but to a lesser extent than that seen with either Ras- or GRF-transformed cells. Increased MAPK activity was found to correspond to the presence of these activated forms of MAPKs (data not shown).

We recently observed that kinase-deficient (but not wild-type) forms of MAPKs could block oncogenic Ras-induced transcriptional activation (58) and focus-forming activity (54a). Thus, MAPK activation is essential for Ras transformation. To determine if MAPK activation in Vav- and Dbl-transformed cells is important for transformation, Dbl focus formation assays were done in the presence or absence of a cotransfected pCMV plasmid construct that encodes the kinase-deficient form of $p42^{MAPK}$/ERK2 or $p44^{MAPK}$/ERK1. Dbl focus-forming activity was markedly reduced in cultures cotransfected with $p42^{MAPK}$/ERK2(K52R) and reduced to a lesser extent in cultures cotransfected with $p44^{MAPK}$/ERK1(K71R) (Fig. 5B). Thus, like oncogenic Ras transformation, Dbl transformation may require MAPK activation.

Since the stimulation of transcriptional activation from RRE-containing promoters strongly correlates with normal and oncogenic Ras biological activity (50, 58), we next compared the abilities of GRF, Vav, and Dbl to stimulate transcriptional activation from CAT reporter plasmids which are regulated by upstream promoter sequences that contain three different RREs (*ets*/AP-1, *fos* promoter, and NFκB). Whereas transient transfection of pJ4Ω-GRF caused significant (5- to 10-fold) activation, neither pZIP-*dbl*- nor pMEX-*vav*-transfected cells showed any activity from the three reporter plasmids (Fig. 6; data for NFκB not shown). Since the activation of these RREs is strongly correlated with activation of the Ras signal transduction pathway (50, 58), these results suggest that Vav- and Dbl-mediated transformation occurs via mechanisms which are distinct from Ras transformation.

DISCUSSION

The Vav oncogene protein shares significant sequence identity with the catalytic domain of the Dbl oncogene protein (1). Dbl functions as a guanine nucleotide exchange factor and

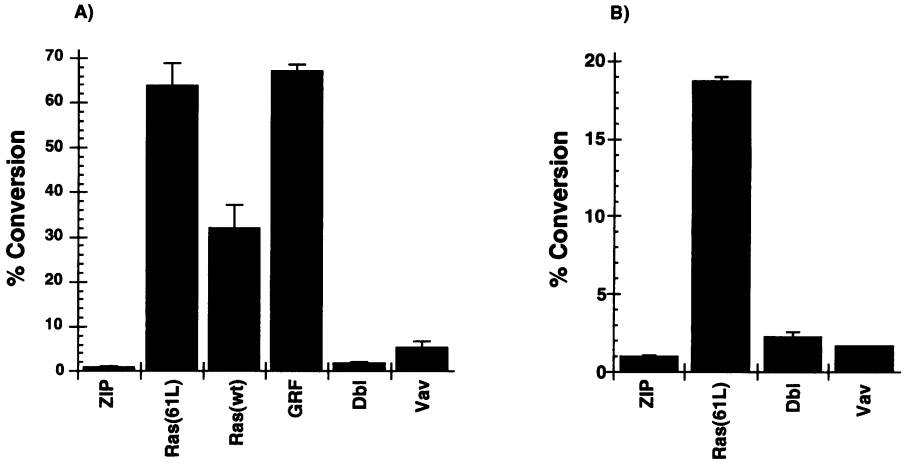

FIG. 6. Vav and Dbl do not stimulate transcriptional activation from CAT reporter plasmids that contain the *ets*/AP-1 (pB4X-CAT) RRE from polyomavirus enhancer (A) or the *fos* promoter (p*fos*-CAT) (B). Ras(wt), wild-type Ras.

activator of two Rho family proteins, CDC42Hs and RhoA (29, 30), and Dbl transforming activity is directly correlated with this catalytic function (30). Consequently, it has been suggested that the transforming activities of Dbl and other DH-containing oncogene proteins (Vav and Ect2) may be due to their constitutive activation of specific Rho family proteins (6). However, the recent observation that Vav functions as a guanine nucleotide exchange factor and activator of Ras proteins (24) suggested that Vav transformation may occur via constitutive activation of Ras function.

To determine if Vav transformation is due to its function as a Ras, rather than a Rho, GEF, we compared the transformed phenotype induced by Vav and Dbl with the transformed phenotype of NIH 3T3 cells transformed by the Ras GEF, GRF/CDC25 (12). Our results demonstrate that the transformed phenotype triggered by GRF is indistinguishable from that induced by oncogenic Ras, whereas transformation by Vav and Dbl more closely resembles transformation by activated RhoA. Thus, the ability of Vav to function as a Ras GEF is apparently not the basis for Vav transformation. Consequently, whereas GRF-mediated transformation appears to be due to activation of Ras and the Ras signal transduction pathway, Vav and Dbl transformation is apparently mediated by a different mechanism. Instead, since Vav- and Dbl-transformed cells possess many of the transformed properties associated with RhoA-transformed cells (focus and cellular morphology and retention of actin stress fibers and focal adhesions), we speculate that DH domain-containing oncogene proteins may trigger transformation via constitutive activation of Rho family proteins. Final demonstration of such a mechanism will require determination that Rho GTP/GDP ratios are elevated in Vav- or Dbl-transformed cells.

Members of the Rho family have been implicated as regulators of actin cytoskeletal organization (45, 46). Microinjection of activated RhoA proteins into Swiss 3T3 cells causes the development of actin stress fibers and the appearance of focal adhesions (45). Therefore, while oncogenic Ras induces the loss of actin stress fibers and focal adhesions, one might expect that constitutive RhoA function would cause an increase in both stress fibers and focal adhesions. Consistent with the role of GRF as a Ras GEF (54), GRF-transformed cells also exhibited significant reductions in actin stress fibers and focal adhesions. However, as with RhoA(63L)-transformed cells, we observed no reductions (and possibly increases) in actin stress fibers and focal adhesions in Vav- and Dbl-transformed cells. Vav-transformed cells also showed formation of lamellipodia, suggesting an increased motility of these cells (55). Taken together, these findings strongly suggest the involvement of Rho family proteins, rather than Ras, in cellular transformation by Vav and Dbl.

If Vav transformation is a consequence of its function as a Ras GEF, then Vav-transformed cells should exhibit constitutively elevated Ras-GTP levels. Whereas we and others have observed elevated Ras-GTP levels in GRF-transformed cells (12, 44), neither Vav- nor Dbl-transformed cells showed any significant increase in Ras-GTP levels. In contrast to our observations, a recent study reported that Ras-GTP levels were elevated in Vav-transformed cells (25). The basis for this difference from our results is not clear. However, their observed elevation (35%) was not as high as the level typically seen with oncogenic Ras proteins (60 to 90%) or the level that we have observed for GRF-transformed cells (60%). Thus, whether their observed elevation is sufficient to account for full Vav transforming activity is not clear. Nevertheless, while our results do not eliminate the possibility that Vav possesses Ras GEF activity, our observations suggest that Vav transformation is distinct from GRF transformation and is not mediated by activation of endogenous Ras activity.

The growth-inhibitory phenotype of Ras(17N) is a consequence of its formation of an inactive complex with Ras GEFs (e.g., GRF/CDC25 and SOS), thus leading to the inactivation of endogenous Ras function (21). Consistent with this mechanism, we and others have shown that excess Ras GEF activity (GRF/CDC25 and SOS1) can overcome Ras(17N) inhibitory activity (14, 42, 43, 51). In contrast, we observed that Vav or Dbl, but not GRF, transforming activity was greatly impaired by Ras(17N). Furthermore, GRF, but not Vav or Dbl, could overcome Ras(17N) inhibition of transcriptional activation from a Ras-responsive reporter plasmid. Taken together, these data show that Vav and Dbl exhibit properties that are clearly distinct from those of GRF.

We can envision at least two possible interpretations of our Ras(17N) results. First, since Ras(17N) inhibition is a consequence of its ability to block endogenous Ras function, these results suggest that Ras function is required for Vav and Dbl transforming activity. This result would be expected if Vav and Dbl caused transformation by activating endogenous Ras function. Alternatively, a second interpretation of these data is that the loss of endogenous Ras function cannot be overcome by Vav or Dbl transformation. This result contrasts with the ability of other oncogene proteins that function downstream of Ras (e.g., Raf-1) to overcome Ras(17N) inhibition (21). We presently cannot distinguish between these two possibilities. However, since many of the other data presented in this study are inconsistent with Vav transformation via activation of endogenous Ras function, we favor the second possibility and suggest that Vav and Dbl do not possess activities which would be expected for a Ras GEF.

While our observation that Vav and Dbl fail to stimulate transcriptional activation from RREs argues against their causing transformation by activating Ras function, we did observe that MAPKs were constitutively activated in both Vav- and Dbl-transformed cells. MAPK activation has been reported recently in Vav-transformed cells (25). MAPK activation is also observed in Ras-transformed cells (34, 48), and our recent observations that kinase-deficient forms of MAPKs function as dominant inhibitory proteins and can specifically inhibit oncogenic Ras transformation (54a) and transcriptional activation (58) support an important role for MAPK activation in Ras transformation. In the present study, we show that these MAPK mutants can also block Dbl transforming activity. Thus, the activity of MAPKs are essential for Ras, as well as Dbl, transforming activity.

Our observations that Vav and Dbl did not cause transcriptional activation from plasmid reporter constructs that contain RREs provided further support that these proteins induce transformation via mechanisms distinct from oncogenic Ras. However, this absence of activation was surprising in light of our detection of activated MAPKs in Vav- and Dbl-transformed cells. MAPKs are believed to directly phosphorylate and activate the specific transcription factors (e.g., Jun and Ets family proteins), which in turn stimulate transcriptional activation from certain RREs (53). One possible explanation for these results may be that they merely reflect the differences between transiently transfected (CAT assays) and stably transfected (MAPK assays) cells. Alternatively, a second interpretation of these results is that $p42^{MAPK}$/ERK2 and $p44^{MAPK}$/ERK1 do not directly cause activation of these transcription factors. Consistent with this possibility, we have recently shown that these two isoforms of MAPK are not directly responsible for transcriptional activation of Jun, and instead, Jun is activated by a distinct Jun kinase which is also activated by

oncogenic Ras (17, 58). Thus, oncogenic Ras may induce the activation of additional downstream kinases which are not activated in Vav- and Dbl-transformed cells.

In summary, the studies presented here indicate that Vav and Dbl transformation occurs via mechanisms which are distinct from Ras activation. Thus, while Vav may function as a Ras GEF, this activity is not responsible for Vav-mediated transformation. However, Vav and Dbl did induce a Ras-independent activation of MAPKs that may be important for Vav and Dbl transformation. Altogether, our observed similarities between the transformed phenotype of Vav-, Dbl-, and RhoA-transformed cells support the possibility that DH domain-containing oncogene proteins mediate their transforming activity via deregulation of Rho family proteins. Which specific members of the Rho family are responsible and whether their activity is indeed chronically activated in Vav- or Dbl-transformed cells remain to be determined.

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