Vav Transformation Requires Activation of Multiple GTPases and Regulation of Gene Expression

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Abstract

Although Vav can act as a guanine nucleotide exchange factor for RhoA, Rac1, and Cdc42, its transforming activity has been ascribed primarily to its ability to activate Rac1. However, because activated Vav, but not Rac-specific guanine nucleotide exchange factors, exhibits very potent focus-forming transforming activity when assayed in NIH 3T3 cells, Vav transforming activity must also involve activation of Rac-independent pathways. In this study, we determined the involvement of other Rho family proteins and their signaling pathways in Vav transformation. We found that RhoA, Rac1, and Cdc42 functions are all required for Vav transforming activity. Furthermore, we determined that Vav activation of nuclear factor-κB and the Jun NH_2-terminal kinase mitogen-activated protein kinase (MAPK) is necessary for full transformation by Vav, whereas p38 MAPK does not seem to play an important role. We also determined that Vav is a weak activator of Elk-1 via a Ras- and MAPK/extracellular signal-regulated kinase kinase–dependent pathway, and this activity was essential for Vav transformation. Thus, we conclude that full Vav transforming activation is mediated by the activation of multiple small GTPases and their subsequent activation of signaling pathways that regulate changes in gene expression. Because Vav is activated by the epidermal growth factor receptor and other tyrosine kinases involved in cancer development, defining the role of aberrant Vav signaling may identify activities of receptor tyrosine kinases important for human oncogenesis. (Mol Cancer Res 2004;2(12):702–11)

Introduction

Vav proteins (Vav, Vav2, and Vav3) are members of the Dbl family of proteins that function as guanine nucleotide exchange factors (GEF) and activators of Rho family small GTPases (reviewed in refs. 1-3). To date, 23 mammalian Rho family proteins have been identified of which Rac1, RhoA, and Cdc42 have been the best characterized and most intensely studied (reviewed in refs. 4, 5). Dbl family proteins promote formation of the active GTP-bound protein, and GTPase activating proteins stimulate the formation of the inactive GDP-bound protein. Whereas some Dbl family proteins serve as GEFs for a specific Rho family protein, others exhibit the ability to activate multiple Rho family proteins. For example, Fgd1 is an activator of Cdc42 (6), Lsc is an activator of RhoA (7), and Tiam1 is an activator of Rac (8). By contrast, Vav has been shown to function as a GEF for RhoA, RhoG, Rac1, and Cdc42 (9-12).

Vav and many other Dbl family proteins were identified initially as transforming proteins (1-3). Their transforming activities have been attributed to their ability to cause deregulated activation of Rho family proteins. Thus, like Rho family proteins, Dbl family proteins are regulators of actin cytoskeletal organization, gene expression, and cell cycle progression. Furthermore, constitutively activated mutants of RhoA, Rac1, and Cdc42 exhibit transforming potential (13). Vav is also activated by the epidermal growth factor receptor and other receptor tyrosine kinases (14). Because up-regulated expression and activation of the epidermal growth factor receptor is observed in a wide variety of human cancers (15, 16), Vav signaling may also contribute to epidermal growth factor receptor–mediated oncogenesis.

Although Vav has been shown to act as a GEF for RhoA, Rac1, and Cdc42 (9-12) and to regulate the actin cytoskeletal changes associated with these three GTPases (6), its transforming activity has been attributed to Rac1 but not to RhoA or Cdc42 activation (17). However, whereas Vav exhibits potent focus-forming activity (18, 19), Rac-specific GEFs do not (20, 21) when assayed in NIH 3T3 focus formation transformation assays. Furthermore, the signaling activities stimulated by Vav important for transformation have not been determined. Studies of effector domain mutants of Rac1, RhoA, and Cdc42 have attributed their transforming potential to their ability to alter gene expression and to promote cell cycle progression rather than to their ability to cause changes in actin cytoskeletal organization (22-25). Whether this is the case for Vav and whether Vav activates signaling pathways independent of those mediated by its Rho family protein substrates have not been determined. In this study, we show that Vav transforming activity is dependent on RhoA and Cdc42 as well as on Rac1. Furthermore, we determined that Vav activation of the nuclear factor-κB (NF-κB) and Jun transcription factors are important for transformation, indicating the importance of altered gene expression in promoting Vav function. Although both Jun NH_2-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) are activated by Vav, only JNK is important for

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Vav transformation. Finally, we implicate the importance of a Ras/MAPK/extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK pathway that is independent of Rho family proteins in promoting Vav transformation. When taken together, our observations support the importance of multiple GTPases and their activation of gene expression in promoting Vav transformation.

Results

RhoA, Rac1, and Cdc42 Are Required for Vav Transformation

Although Vav can act as a GEF for multiple Rho family proteins, Vav transforming activity has been ascribed primarily to its ability to activate Rac1 (10, 17). However, whereas activated Vav causes potent focus-forming activity when assayed in NIH 3T3 transformation assays (19, 26), neither activated Rac1 nor Rac-specific Dbl family proteins (e.g., Tiam1) cause significant focus-forming activity (20, 21, 27). Therefore, we speculated that Vav must cause transformation by activation of Rac-independent pathways. Because our previous studies showed that Vav proteins also functioned as a GEF for RhoA and Cdc42 as well as Rac1 (9, 11), we wanted to determine if Vav transformation also required the function of RhoA and Cdc42.

For these analyses, we used various inhibitors of Rho family protein function. First, we employed the widely used dominant-negative mutants RhoA(19N), Rac1(17N), and Cdc42(17N) (27-29). By analogy to the equivalent dominant-negative Ras(17N), these mutants form nonproductive complexes with specific Dbl family proteins that function as a GEF for that particular GTPase (30). We and others have used these mutants to assess the contribution of specific Rho family proteins to the transforming action of other Dbl family proteins (29, 31, 32). Transfection of ΔN-186 Vav alone caused the appearance of >150 foci of transformed cells per dish (Fig. 1A). Cotransfection of dominant-negative RhoA(19N), Rac1(17N), or Cdc42(17N) caused a 55% to 85% reduction in focus-forming activity, indicating that all three GTPases are important for Vav transformation.

Next, we used two other approaches to specifically block RhoA function. Consistent with our observations with the dominant-negative RhoA(19N), we also found that coexpression of p190RhoGAP (33) caused a 80% reduction, whereas coexpression of C3 botulinum toxin (a specific inhibitor of RhoA, RhoB, and RhoC but not Rac1 or Cdc42 function; refs. 34, 35) caused essentially a complete inhibition of Vav focus-forming activity (Fig. 1B). Their different abilities to inhibit Vav focus formation are likely to reflect their different mechanisms, and hence efficiencies, of inhibiting RhoA function.

Finally, we used the expression of isolated Rho GTPase binding domains (RBD) of effectors as specific inhibitors of the activated, GTP-bound forms of RhoA, Rac, or Cdc42. The RBD fragment from Wiskott-Aldrich syndrome protein (WASP), a Cdc42-specific effector, has been used as an inhibitor of activated Cdc42 function (36-38). We and others previously used this construct to determine that the Fgd1 Dbl family protein required Cdc42 for its transforming activity (29, 39). Similarly, the isolated binding domain of Rhotekin has

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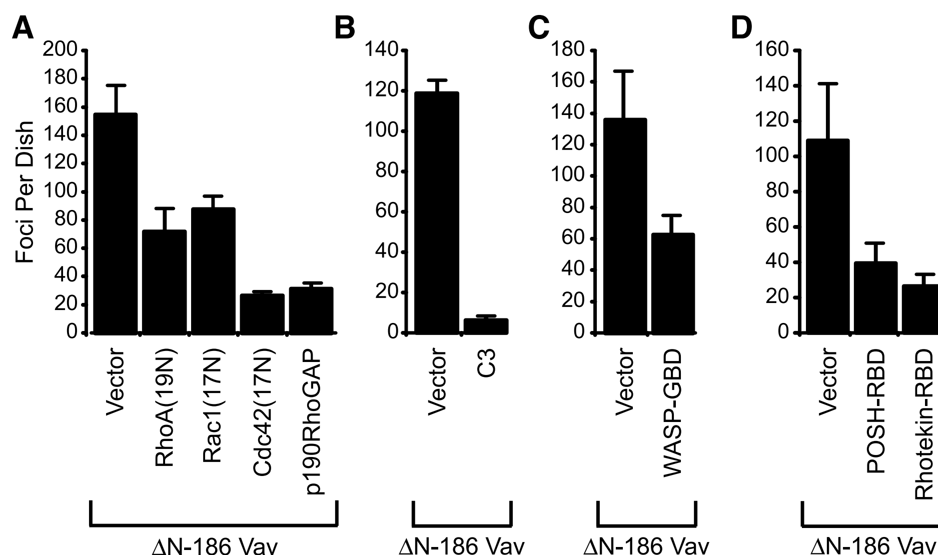

FIGURE 1. RhoA, Rac1, and Cdc42 are required for Vav transformation. **A.** Coexpression of dominant-negative RhoA(19N), Rac1(17N), or Cdc42(17N) blocked ΔN-186 Vav focus forming. Coexpression of p190RhoGAP also reduced Vav focus-forming activity. NIH 3T3 cells were cotransfected with the pAX142 expression vector encoding ΔN-186 Vav (100 ng/dish) either with 2 μg of the empty pZIP-NeoSV(x)1 expression vector or pZIP-NeoSV(x)1 expression vectors encoding dominant-negative mutants of RhoA, Rac1, Cdc42, or p190RhoGAP. **B.** Coexpression of the C3 botulinum toxin, an inhibitor of RhoA function, reduces Vav focus-forming activity. NIH 3T3 cells were cotransfected with pAX142 encoding ΔN-186 Vav (100 ng) and either 2 μg of the empty pcDNA3 vector or pcDNA3 encoding C3. **C.** Coexpression of the isolated Cdc42-binding fragment from WASP reduces Vav focus-forming activity. NIH 3T3 cells were cotransfected with pAX142 encoding ΔN-186 Vav either with 2 μg of the pyDF30 empty vector or pyDF30 encoding WASP-RBD. **D.** Coexpression of the isolated Rac-binding fragment from Posh or the isolated Rho-binding fragment from Rhotekin reduced Vav focus-forming activity. NIH 3T3 cells were cotransfected with pAX142 encoding ΔN-186 Vav (100 ng) and 2 μg of either the pRK5 empty vector or pRK5 encoding Posh-RBD or Rhotekin-RBD. *Columns,* averages of three dishes and representative of at least three independent assays.

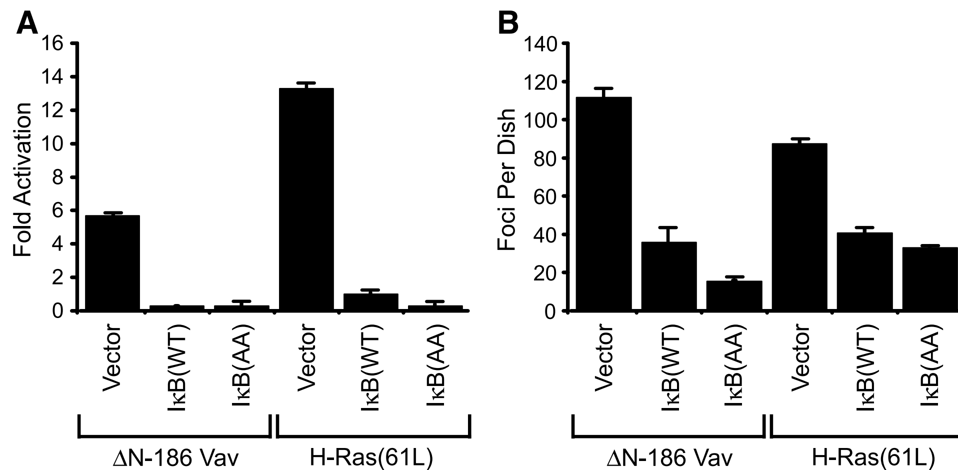

FIGURE 2. Inhibition of NF-κB blocks Vav signaling and transforming activity. **A.** Coexpression of WT and AA IκB blocks ΔN-186 Vav activation of Elk-1. NIH 3T3 cells were transiently transfected with pAX142 encoding ΔN-186 Vav either with the pAX142 empty vector or pAX142 encoding WT or AA IκB (500 ng) together with a NF-κB luciferase reporter plasmid (2.5 μg). Activity is normalized to fold activation above the activity for the empty vector control. **B.** Inhibition of NF-κB impairs Vav focus formation. Coexpression of WT or AA IκB impaired ΔN-186 Vav focus-forming activity in NIH 3T3 cells. Similar concentrations of the plasmid DNA expression vectors were used as described in **A**. *Columns,* averages of three dishes and representative of at least three independent assays.

been used to show that Dbs transforming activity is dependent on RhoA but not Cdc42 activation (40). We also generated a fragment containing the RBD of Posh (41), a Rac-specific effector, as a specific inhibitor of activated Rac (42). We observed that coexpression of the WASP-RBD, Posh-RBD, and Rhotekin-RBD fragments also reduced Vav focus formation by ~50% to 75% (Fig. 1C and D). Taken together with the ability of other approaches to block RhoA, Rac, and Cdc42 function, these results support the contribution of all three GTPases in mediating Vav transforming activity.

NF-κB and JNK Activation Are Required for Vav Transforming Activity

We next determined what downstream signaling activity caused by Vav activation of GTPases contributes to transformation of NIH 3T3 cells. Previous studies showed that Rac1, RhoA, and Cdc42 are activators of the NF-κB transcription factor (43). Because we determined previously that Ras transformation requires the activation of NF-κB (44, 45) and because Ras activation of NF-κB is mediated in part by activation of Rac (46), we determined if Vav transformation is also dependent on activation of NF-κB.

For these analyses, we used expression vectors encoding wild-type (WT) or mutant (AA) versions of IκB to inhibit NF-κB activation. IκB forms a complex with NF-κB in the cytoplasm and prevents the nuclear translocation and activation of NF-κB (47). IκB phosphorylation promotes its degradation, thus releasing and activating NF-κB. The IκB(AA) mutant contains serine-to-alanine substitutions at the two key sites of phosphorylation, thus making it insensitive to degradation and release from NF-κB. We showed previously that coexpression of WT and AA IκB both can block NF-κB activation by Ras, Dbl, and Dbs, causing the inhibition of their transforming activities (32, 44). Therefore, we included activated H-Ras(61L) as a control for these assays. As shown in Fig. 2, coexpression of either WT or AA IκB greatly inhibited H-Ras(61L) and Vav activation of NF-κB in transient expression analyses in NIH 3T3 cells. Similarly, coexpression of WT or AA IκB also blocked H-Ras(61L) and Vav transforming activity in NIH 3T3 focus formation assays (67% and 88%, respectively) for Vav. Thus, NF-κB activation is important for Vav transforming activity.

Previous studies have shown that Rac1 and Cdc42, but not RhoA, causes transient activation of the JNK and p38 MAPKs in NIH 3T3 cells (48, 49). Although JNK and p38 activation is typically associated with apoptotic responses (50, 51), we and others have shown that JNK activation is required for the transforming activity of Ras, Dbl family proteins, and other oncoproteins (13, 52). By contrast, p38 activation has been found to antagonize Ras transformation (52-54). Therefore, we determined if JNK and p38 are activated persistently in Vav-transformed cells and contribute to transformation.

We determined that Vav-transformed NIH 3T3 cells showed persistent activation and phosphorylation of JNK (Fig. 3A). Next, we used two approaches to block JNK activity. First, we used a kinase-dead mutant of SEK1, the upstream activator of JNK, as a reagent to block Vav activation of JNK (55). We showed previously that this mutant protein could block JNK activation by the Dbl and Dbs Dbl family proteins and that SEK1(AL) could block their ability to cause transformation of NIH 3T3 cells (32). As a control for these experiments, we included activated H-Ras(61L) (Fig. 3B). As we have shown previously, coexpression of WT or dominant-negative SEK1 effectively blocked H-Ras(61L) focus-forming activity. Similarly, coexpression of WT or AA versions of SEK1 also caused a significant reduction (~80%) in Vav focus-forming activity. The ability of WT SEK1 to function as a dominant negative is likely to be due to its overexpression and titration of the upstream activators of SEK1.

Second, we used the SP600125 pharmacologic inhibitor of JNK activity (56), which we used previously to determine that JNK activity was required for Ras transformation (52). NIH 3T3

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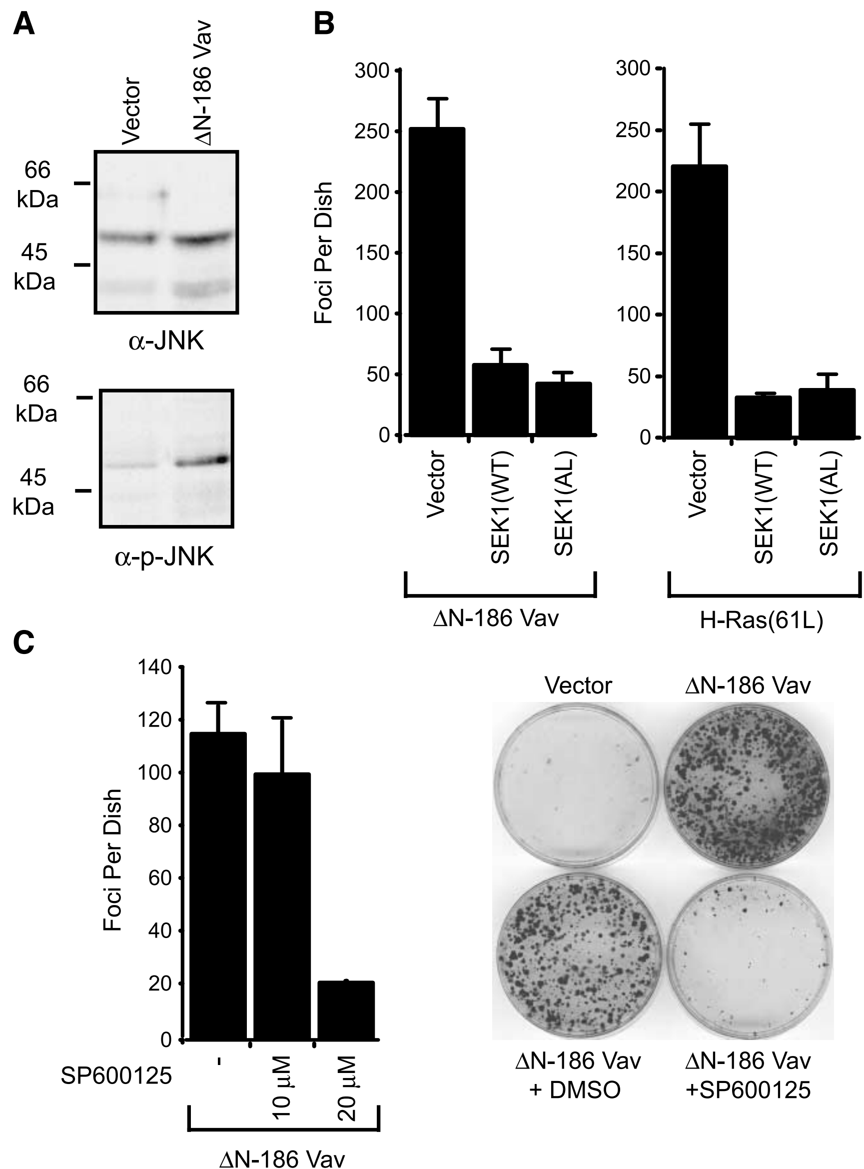

FIGURE 3. Inhibition of JNK activation impairs Vav transforming activity. **A.** Western blotting of total JNK and phospho-JNK in control (*Vector*) and Vav-transformed NIH 3T3 cells revealed increased phospho-JNK levels. **B.** Coexpression of kinase-deficient SEK1(AL) impaired ΔN-186 Vav focus-forming activity. Activated H-Ras(61L) was included as a positive control for these assays. NIH 3T3 cells were cotransfected with pAX142 encoding ΔN-186 Vav (100 ng) or H-Ras(61L) (50 ng) either with the empty pAX142 vector or pAX142 encoding SEK1(WT) or kinase-dead SEK1(AL) (1.5 μg). **C.** Inhibition of JNK activity with the specific inhibitor, SP600125, reduced ΔN-186 Vav focus-forming activity. NIH 3T3 cells were transfected with pAX142 empty vector or pAX142 encoding ΔN-186 Vav (100 ng) and supplemented with either 10 or 20 μmol/L SP600125. The appearance of foci of transformed cells was determined after 14 days. *Columns,* averages of three dishes and representative of at least three independent assays.

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focus formation assays were done with the JNK inhibitor at two concentrations (10 and 20 μmol/L). At the higher concentration, the inhibitor caused a near complete block of ΔN-186 Vav transformation (Fig. 3C). JNK phosphorylation was reduced when NIH 3T3 cells expressing ΔN-186 Vav were treated with the JNK inhibitor for the time course of a focus formation assay (data not shown). We conclude that Vav activation of JNK is required for full transformation of NIH 3T3 cells.

p38 Activation Is Not Required for Vav Transforming Activity

The importance of p38 activation in Vav transformation was assessed with a pharmacologic inhibitor that specifically blocks p38 activity. First, Western blotting of cell lysates from stable NIH 3T3 cell lines revealed that activated, phosphorylated p38 was increased in ΔN-186 Vav cells over vector cells (Fig. 4A). However, when the SB203580 pharmacologic inhibitor was used at two concentrations, it did not significantly affect ΔN-186 Vav focus-forming activity (Fig. 4B). H-Ras (61L) was used as a control in this experiment to confirm that the inhibitor was working (data not shown). The SB203580 inhibitor increased H-Ras focus formation as was shown previously (52). In addition, we saw a modest decrease in phospho-p38 in NIH 3T3 cells expressing ΔN-186 Vav that were treated with the p38 inhibitor for a period of time equal to a focus formation assay (data not shown). These data suggest that, although p38 activity is stably up-regulated in Vav-transformed cells, it is not necessary for the transforming activity of Vav.

Inhibition of the ERK/Elk-1 Pathway Blocks Vav Transforming Activity

Although Vav is not a GEF for Ras, there is evidence that Vav may weakly activate Ras and the Raf/MEK/ERK pathway.

The mechanism by which Vav may promote this modest degree of Ras activation is not known. For example, we observed previously that Vav-transformed cells showed a modest up-regulation of Ras-GTP and activated ERKs (57). Furthermore, we found that dominant-negative mutants of Ras and ERK could block Vav transforming activity. Despite these observations, it was concluded that Vav transformation was mediated primarily through its ability to act as a GEF for Rho family proteins. However, in light of observations that coexpression of activated components of the Raf/MEK/ERK pathway together with activated Rho family GTPases causes a highly synergistic enhancement of their transforming activity (27, 29, 38, 58, 59), we have reassessed the contribution of a Raf/MEK/ERK pathway to Vav transformation.

For these analyses, we used dominant-negative MEK1(2A) (27, 60, 61) or a pharmacologic inhibitor of MEK1/2 (U0126; refs. 62, 63) to determine if MEK activation was required for Vav transforming activity. First, we compared the ability of Vav and H-Ras to activate Elk-1, a downstream target of the Raf/MEK/ERK pathway (64). Although activated H-Ras(61L) is a potent activator of Elk-1 in transient expression analyses in NIH 3T3 cells (120-fold activation), Vav also caused a weak (5-fold) but reproducible activation of Elk-1 (Fig. 5A). Coexpression of dominant-negative, but not WT, MEK1 essentially abolished the ability of activated Vav and Ras to activate Elk-1 (Fig. 5B). Furthermore, dominant-negative MEK caused a partial reduction in Vav and Ras focus-forming activity (Fig. 5C). Vav focus formation was reduced by >70%. Similarly, analyses with U0126 also showed that this MEK-specific inhibitor blocked Vav and H-Ras activation of Elk-1 in transient expression analyses (Fig. 6A) and Vav and H-Ras focus-forming activity when assayed in NIH 3T3 transformation assays (Fig. 6B). Vav focus formation was reduced by >90%, whereas only a partial reduction was seen for H-Ras and the Ras foci that appeared displayed an altered morphologic appearance similar to those caused by Vav (32). Thus, although Vav is a weak activator of a MEK/ERK/Elk-1 signaling pathway, possibly via Ras activation, these results suggest that this activation contributes to full Vav focus-forming activity. Alternatively, it may suggest that basal ERK activity is important for Vav transformation.

These analyses extend our previous observations (57) and suggest that Vav activation of Ras and the Raf/MEK/ERK pathway can contribute to Vav transformation. However, whether activation of Elk-1 is mediated through this kinase cascade, or instead is mediated by activation of Rho family GTPases, remains possible. Although our previous analyses have determined that in our assay conditions Rac1, RhoA, and Cdc42 are not activators of Elk-1, other studies have shown that these small GTPases can activate Elk-1 (65, 66). To distinguish between these two possibilities, we determined if Ras activation was required for activation of Elk-1. Because Vav activation of Rac1 and Cdc42, and consequently of JNK, should mediate Vav activation of the Jun transcription factor, Ras should not be required for this Vav activity.

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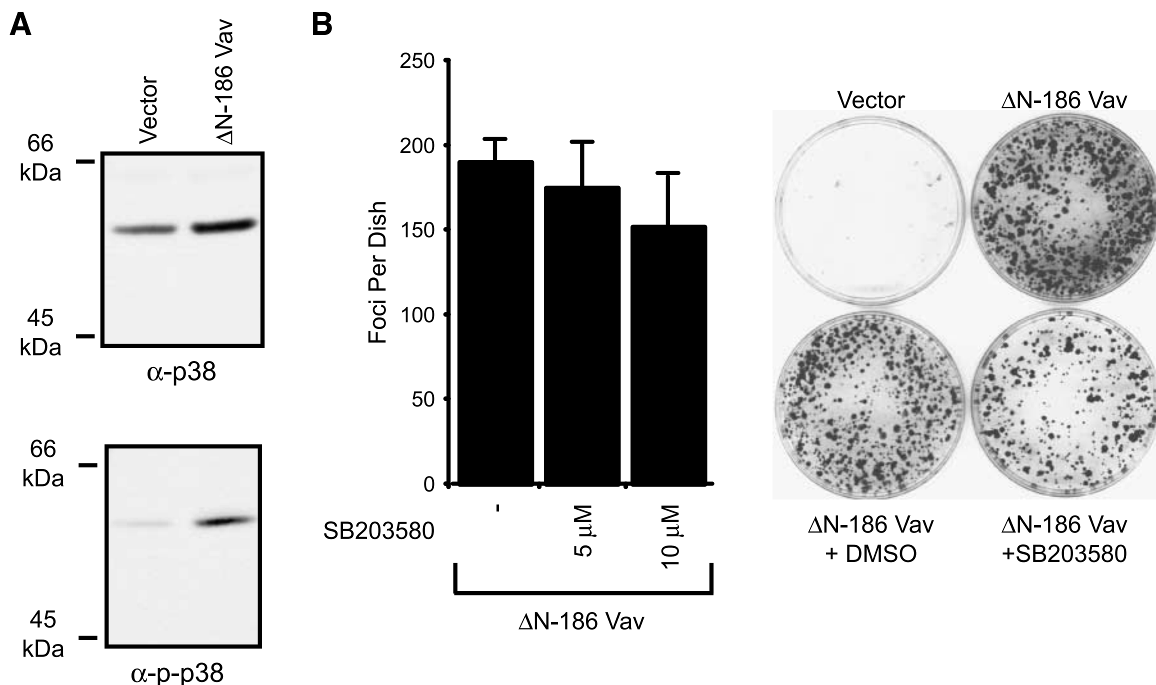

FIGURE 4. p38 kinase activity is not required for Vav transformation. **A.** Western blot analyses for activated, phosphorylated levels of p38 in NIH 3T3 cells stably transfected with the pCTV3 empty vector or pCTV3 encoding ΔN-186 Vav showed an increased phospho-p38 level in cells expressing Vav. **B.** Pharmacologic inhibition of p38 by SB203580 did not significantly impair Vav focus-forming activity. NIH 3T3 cells were transfected with pAX142 empty vector or pAX142 encoding ΔN-186 Vav (100 ng) and supplemented with either 5 or 10 μmol/L SB203580. The appearance of foci of transformed cells was determined after 14 days. *Columns,* averages of three dishes and representative of at least three independent assays.

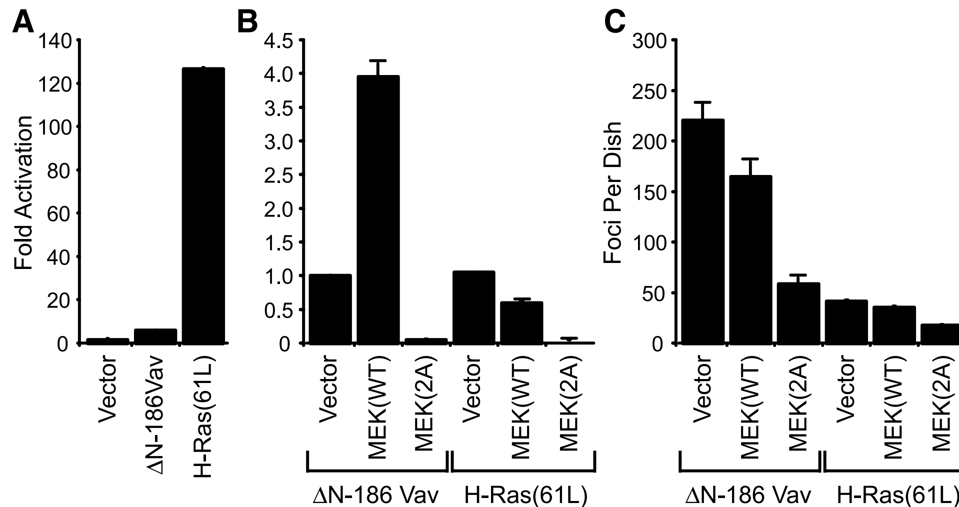

FIGURE 5. Dominant-negative MEK blocks Vav signaling and transforming activity. **A.** Vav is a weak activator of Elk-1. NIH 3T3 cells were transfected with pAX142 encoding either ΔN-186 Vav (500 ng) or H-Ras(61L) (50 ng) together with Gal4-Elk-1 (250 ng) and Gal4-Luc (2.5 μg) per 30-mm dish. Fold activation was determined by the number of relative luciferase units relative to the number of units seen with the empty pAX142 vector transfected cells. **B.** Dominant-negative MEK blocks Vav activation of Elk-1. NIH 3T3 cells were transfected with pAX142 encoding ΔN-186 Vav (100 ng) or H-Ras(61L) (50 ng) together with the empty pAX142 vector or pAX142 encoding WT or kinase-dead MEK1(2A) (500 ng) and Gal4-Elk-1 and Gal4-Luc reporter plasmids. Relative inhibition was determined by the activity relative to that seen with each empty vector transfected culture. **C.** Dominant-negative MEK blocks Vav focus-forming activity. Analyses were done on NIH 3T3 cells using the concentrations described in **B**. The appearance of foci of transformed cells was quantitated after 14 days. *Columns,* averages of three dishes and representative of at least three independent assays.

We found that dominant-negative Ras(17N) caused a 50% inhibition of Vav activation of Elk-1 but no inhibition of Jun activation (Fig. 7). This result is consistent with a mechanism where Vav activates Elk-1 via activation of Ras and the Raf/MEK/ERK pathway.

Discussion

Although Vav proteins can function as a GEF for Rac1, RhoA, and Cdc42, previous studies have determined that Vav transforming activity is mediated through activation of Rac (10, 17). In contrast, our analyses support the importance of all three GTPase targets for Vav transformation. Furthermore, we show that Vav transformation is dependent on multiple signaling pathways that cause activation of various transcription factors. We found that Vav transformation was dependent on activation of NF-κB, the JNK MAPK cascade (activators of the Jun and ATF-2 transcription factors), and Ras-dependent activation of Elk-1. Vav activation of Elk-1 seems to be independent of its ability to serve as a GEF for Rho family GTPases. Thus, we conclude that Vav activation of multiple small GTPases, to cause changes in gene expression, is important for Vav-mediated transformation.

Broek and colleagues used a variety of assays to show that Vav can act as a GEF for Rac1, Cdc42, and RhoA (9). Additionally, Hall and colleagues found that microinjection of Vav into Swiss 3T3 cells caused the stimulation of actin cytoskeletal changes characteristic of activated RhoA, Rac1, and Cdc42 (6). Therefore, it was unexpected that Vav transformation was described to be dependent primarily on Rac activation (10, 17). Furthermore, the much stronger focus-forming activity seen with activated Vav, when compared with activated Rac-specific Dbl family proteins (20, 21), also suggests that Vav transformation is mediated by Rac-independent events. This prompted our interest in reevaluating the importance of other Rho family proteins in mediating Vav transformation. We found that Vav transformation did require the activities of Rac1, as well as Cdc42 and RhoA, for its transforming activity. Thus, the potent focus-forming activity of Vav may be accounted for in part by its ability to cause the coordinate activation of multiple Rho family proteins. These results are consistent with a previous study that showed that coexpression of the activated versions of multiple Rho family proteins can cause highly synergistic focus-forming activities (67). Similarly, it was concluded that Dbl transforming activity also required the activation of all its GTPase substrates (68). The basis for the different conclusions of our study and those described previously (10, 17) is not known. One possibility is that the approaches used in their assays to block RhoA or Cdc42 were not effective to block Vav activation of these other small GTPases. A second possibility may be the use of different strains of NIH 3T3 cells in our respective analyses. We have found that different strains of NIH 3T3 cells can exhibit very distinct abilities to be transformed by effector domain mutants of Ras (69).

Our previous studies that showed that inhibition of JNK or NF-κB activation could block oncogenic Ras transformation (45, 70) suggested the possibility that Vav transformation may also require the activation of these two signaling pathways. Ras activation of JNK and NF-κB is dependent on Rac function. Therefore, because Vav transformation is also dependent on Rac function, it is consistent with our observation that JNK and NF-κB activation are both required for Vav transformation. Thus, although JNK activation is most commonly associated with apoptosis-inducing stimuli, our observations extend the importance of JNK activation for growth promotion by various oncoproteins such as Ras, Abl, or Met (13). Finally, in our

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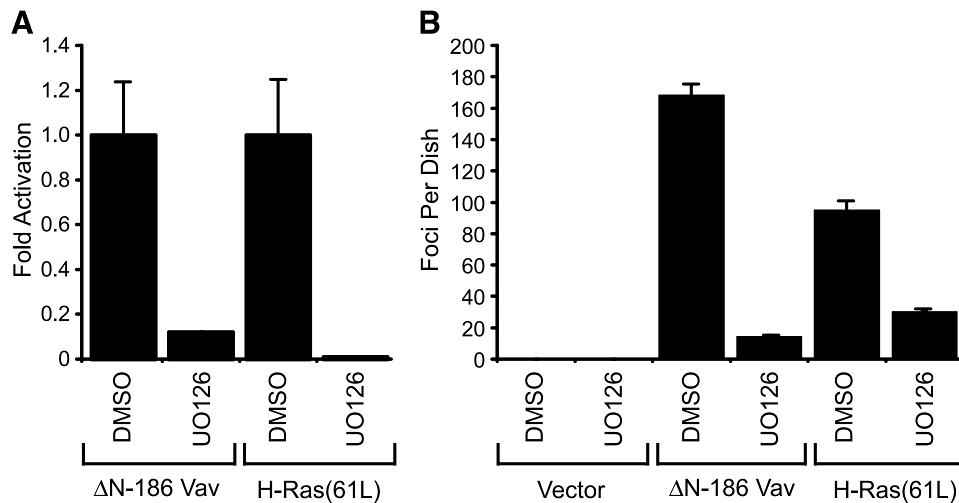

FIGURE 6. The U0126 MEK inhibitor blocks Vav signaling and transforming activity. **A.** U0126 blocks Vav activation of Elk-1. NIH 3T3 cells were transfected with pAX142 encoding either ΔN-186 Vav (500 ng) or H-Ras(61L) (50 ng) together with Gal4-Elk-1 (250 ng) and Gal4-Luc (2.5 μg) per 30-mm dish and either DMSO or U0126 dissolved in DMSO (30 nm final concentration). **B.** U0126 blocks Vav focus-forming activity. NIH 3T3 cells were transfected with pAX142 encoding ΔN-186 Vav (100 ng) or H-Ras(61L) (50 ng) and incubated with growth medium supplemented with DMSO or U0126 dissolved with DMSO (50 nmol/L final concentration). *Columns,* averages of three dishes and representative of at least three independent assays.

analyses of Ras transformation, we determined that NF-κB activation served an antiapoptotic function, because inhibition of NF-κB caused Ras-transformed rodent fibroblasts to undergo apoptosis (45). Whether NF-κB serves such a role for Vav transformation would be interesting to determine. The NF-κB requirement for Vav transformation that we observed may be due to protection from apoptosis in the focus formation assays. However, our preliminary observation that NF-κB inhibition could block the growth of Rac-transformed NIH 3T3 cells in soft agar, but not on plastic, suggests that NF-κB plays a different role for Vav versus Ras transformation.[2]

In contrast to JNK, the related p38 MAPK has been found to antagonize Ras transformation (52-54). Similarly, although p38 activity is stably up-regulated in Vav-transformed cells, we found that this activity is not required for Vav transformation. Whether Vav is an activator of Ras and the Raf/MEK/ERK pathway has been a subject of considerable controversy. Earlier studies by Altman and colleagues argued, rather unexpectedly, that Vav could function as a GEF for Ras (71, 72). Vav does not contain any sequences that are related to the Cdc25 homology domains required for Ras GEF activity (73, 74). Subsequently, two studies showed that Vav transformation was more consistent with its ability to activate Rho family proteins (57, 75). However, although the possibility that Vav also possessed GEF activity for Ras seemed unlikely, there remained a possibility that Vav may still cause activation of Ras by another mechanism. For example, it has been determined that the Grb2 adaptor protein can associate with the SH2 domain of Vav, providing a possible link to SOS and activation of Ras (76, 77). Raf has also been determined to interact with Vav, providing another possible mechanism for Vav activation of the Raf/MEK/ERK pathway (78). Our results showing that inhibition of MEK, by using either dominant-negative MEK or the U0126 MEK inhibitor, could effectively block Vav transformation extend our previous observations and support the involvement of such a pathway in Vav function. Furthermore, we found that inhibition of Ras impaired Vav activation of Elk-1, a downstream target of this kinase cascade, but not Vav activation of Jun. These data support a model where Vav can activate Ras and the Raf/MEK/ERK/Elk-1 pathway by a mechanism that

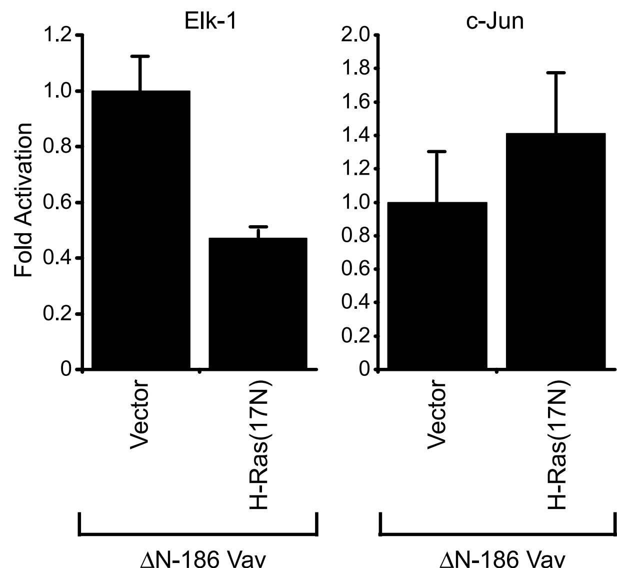

FIGURE 7. Vav activation of Elk-1, but not c-Jun, is dependent on Ras activation. Coexpression of the Ras(17N) dominant-negative impairs ΔN-186 Vav activation of Elk-1 but not c-Jun. NIH 3T3 cells were transiently cotransfected with pAX142 encoding ΔN-186 Vav (500 ng) either alone or together with pZIP-NeoSV(x)1 encoding the H-Ras(17N) dominant-negative protein (500 ng) together with Gal4-Elk-1 (250 ng) or Gal4-Jun (250 ng) and Gal4-Luc (2.5 μg). *Columns,* averages of three dishes and representative of at least three independent assays.

[2] A.E. Karnoub, G.W. Reuther, and C.J. Der, unpublished data.

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remains to be elucidated but is distinct from how Vav activates Rho family GTPases and the JNK/Jun pathway. We and others have shown that activated Raf can cooperate with activated Rho family proteins to cause potent synergistic focus-forming activity (79). Thus, we suggest that Vav activation of a Ras-mediated pathway provides an additional explanation for the very potent transforming activity of Vav when compared with the activity seen with even coordinate expression of multiple activated Rho family proteins. However, we emphasize that these results do not support the controversial notion that Vav is a Ras GEF.

A perplexing question regarding Dbl family proteins has been that several members of this family, including Vav, exhibit focus-forming activities that cannot be mimicked by simply coexpressing activated versions of their known Rho family targets. Our studies provide two partial explanations for why Vav focus-forming activity is so potent. First, Vav caused the coordinate activation of multiple Rho family proteins, and it is possible that Vav activates additional as yet to be identified Rho family proteins. Second, Vav can weakly activate Ras and the Raf/MEK/ERK pathway. Although this activity alone does not account for Vav transformation, it may cooperate with the signals activated by Rho family GTPases to further enhance Vav transforming potential. In summary, we propose that Vav can function as a GEF to stimulate the activation of multiple Rho family proteins, together with a distinct mechanism to activate Ras, to cause potent transformation of NIH 3T3 cells via changes in gene expression. The identification of the gene targets of NF-κB, Jun, and Elk-1 important for Vav transformation constitutes an important future direction for the dissection of how Vav regulates cellular proliferation.

Materials and Methods

Molecular Constructs

The pAX142 mammalian expression vector encoding the activated and highly transforming mouse ΔN-186 Vav has been described previously (19). pZIP-NeoSV(x)1 mammalian expression vectors encoding RhoA(19N), Rac1(17N), and Cdc42(17N) are dominant negatives of their respective GTPases and were described previously (29, 69). The pZIP-NeoSV(x)1 mammalian expression vector encoding p190Rho-GAP (33) and the pCDNA3 mammalian expression vector encoding C3 transferase were the generous gift of J. Settleman (Massachusetts General Hospital Cancer Center and Harvard Medical School, Boston, MA). pyDF30-WASP encodes the RBD from the Cdc42-specific effector WASP (37) and was provided by M. Symons (Institute for Medical Research at North Shore-LIJ, Manhasset, NY). The construction of pAX142 plasmids encoding IκB(WT), IκB(AA), SEK1(WT), SEK1(AL), MEK1(WT), and MEK1(2A) were described previously (32). The U0126 MEK inhibitor was provided by J. Trazskos (Dupont, Wilmington, DE). Expression vectors encoding the GTP-dependent binding domains of the Rac-specific effector, Posh, or the RhoA-specific effector, Rhotekin, were generated by PCR. The GTP-bound RhoA binding domain of Rhotekin (amino acids 18-89) was amplified by PCR from a glutathione *S*-transferase construct containing Rhotekin-RBD (kindly provided by L. Petch, University of North Carolina at Chapel Hill). A 5′ *Bam*HI and a 3′ *Eco*RI restriction sites were incorporated and the PCR product was subcloned into pRK5 mammalian expression vector. The cDNA sequence encoding the activated Rac-binding domain (RBD) of the Rac effector, Posh (amino acids 291-363), was constructed by oligonucleotide annealing of three complementary primer pairs (A:B, C:D, and E:F) followed by PCR of the annealed product. Briefly, oligonucleotide pairs were boiled for 5 minutes at 95°C, allowed to cool to 40°C, and mixed together and incubated at 25°C overnight. The annealed product (~230 bp) was amplified by PCR and inserted into *myc*-pRK5 vector using 5′ *Bam*HI and 3′ *Eco*RI sites. Oligonucleotide sequences were the following. A: 5′-GATCCAAGCACCCCGACACCA-AGAAGAACACCAGGAAGCGACACTCCTTCACCTC-CCTCACCATGGCCAACAAGTCTT-3′. B: 5′-GGGACCCC-TGGGAAGACTTGTTGGCCAT GGTGAGGGAGGTG-AAGGAGTGTCGCTTCC TGGTGTTCTTCTTGGTG-TCGGGGTGCTTG-3′. C: 5′-CCC AGGGGTCCCAGAACC-GCCACTCCATGGAGATCAGCCCTCCTGTGCTCAT-CAGTTCCAGCAACCCCACAGCCG-3′. D: 5′-CGCTGAT-GCGGGCTGCGG CTGTGGGGTTGCTGGAACTGATGAG-CACAGGAGGGCTGATCTCCATGGAGTGGCGGTTCT-3′. E: 5′-CAGCCCGCATCAGCGAACTGTCCGGGCTCTCC-TGCAGCGCCCCGTCTCAGGTCCATATAAGCAC-CACTGGGATCG-3′. F: 5′-AATTCGATCCCAGTGGTGC-TTATATGGACCTGAGACGGGGCGCTGCAGGA-GAGCCCGGACAGTT-3′. Sequence fidelity was verified by automated DNA sequencing.

Cell Culture, Transfection, and Transformation Assays

NIH 3T3 cells were cultured in DMEM supplemented with 10% fetal calf serum. DNA transfections were done by calcium phosphate precipitation as described previously (80). For each assay, cognate empty vectors were used as controls. For focus formation analyses, transfected NIH 3T3 cells were maintained in growth medium for 12 to 14 days. The dishes were stained with crystal violet (0.5%) and the number of foci was then quantitated. The growth medium was supplemented with the U0126 MEK inhibitor at a final concentration of 30 nmol/L, the SP600125 JNK inhibitor (Biomol Research Laboratories, Inc., Plymouth Meeting , PA) at a final concentration of 10 or 20 μmol/L, or the SB203580 p38 inhibitor (Calbiochem, La Jolla, CA) at a final concentration of 5 or 10 μmol/L. Each inhibitor was added to the cells every other day for the transformation assays or immediately after glycerol shocking for the transient expression reporter gene assays. All assays were done at least three independent times.

Western Blot Analyses

To establish stable cell lines that express pCTV3 and pCTV3 encoding ΔN-186 Vav, transfected NIH 3T3 cells were selected in growth medium supplemented with 400 μg/mL hygromycin and multiple drug–resistant colonies were pooled together after 10 days of selection to establish mass populations of stably transfected cells. For use in Western blot analysis, nearly confluent dishes of selected cells were lysed in lysis buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 0.5% NP40, 50 mmol/L NaF, 1 mmol/L $NaVO_3$, 1 mmol/L DTT, 10 μg/mL

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leupeptin, 10 μg/mL aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride]. Lysates were normalized and separated by SDS-PAGE (50 μg), transferred to Immobilon-P (Millipore, Bedford, MA) membrane and incubated with the appropriate antibodies. The expression of ΔN-186 Vav was confirmed with an anti-hemagglutinin antibody, HA.11 (Covance, Princeton, NJ). The antibodies for JNK (#9252), phospho-JNK (#9255), p38 (#9212), and phospho-p38 (#9211) were all obtained from Cell Signaling Technology (Beverly, MA). Protein concentrations were determined with the BCA kit (Pierce, Rockford, IL) and proteins were detected by chemiluminescence (Amersham, Arlington Heights, IL).

Transient Expression Reporter Gene Assays

Transient expression transcriptional assays were done as described previously (81). Briefly, NIH 3T3 cells were transfected by calcium phosphate precipitation in six-well 30-mm dishes (5×10^5 cells per well). The cells were serum starved (0.5% calf serum) 14 to 15 hours before lysing with luciferase lysis buffer (Amersham). Lysates were analyzed using enhanced chemiluminescence reagents and a Monolight 2010 luminometer (Analytical Luminescence, Ann Arbor, MI). All the assays were done at least thrice.

The reporter plasmid constructs used for these assays have been described previously. To determine Elk-1 or c-Jun activity, we used plasmids encoding the Gal4 DNA binding domain fused to either Elk-1 or c-Jun NH_2-terminal transactivation domain (Gal4-Elk-1 or Gal4-Jun, respectively) together with the 5× Gal4-Luc luciferase gene reporter plasmid where luciferase gene expression was under the control of a *fos* minimal promoter that contains tandem copies of the Gal4 DNA binding motif (82). The HIV NF-κB-Luc reporter has been described previously (83).

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