Rac-1 dependent stimulation of the JNK/SAPK signaling pathway by Vav

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The protein product of the human' *vav* oncogene, Vav, exhibits a number of structnral motifs suggestive of a role in signa! transduction pathways, including a leucine-- rich region, a plekstrin homology (PH) domain, a cysteine--rich domain, two SH3 regions, an SH2 domain, and a central Dbl homology (DH) domain. However, the transforming pathway(s) activated by Vav has not yet been elucidated. Interestingly, **OH** domains are fre-- **quently fonnd in gnanine llUcleotide--exchange factors for small G'.I'.P-binding proteioi; of the Ras and Rho families,** and it has been **recently shown that, whereas Ras controls the activation of mitogen activated kinases (MAPKs),** two **memhers of** the **Rho family of small GTPases, Rae 1 and Cdc42, regulate activity** of **stress activated protein kinases (SAPKs), also termed c:inn *N*- terminal kinases (JNKs). Tbe strnctnral similarity between Vav and ofüer guanine nucleotide exchange factors for small GTP-binding proteins, togetber witb the recen! identification of biochemical routes specific for members of the Ras and Rho family of GTPases, prompted us to explore whether MAPK or JNK are downstream components of the Vav signaling pathways. Using the COS-7 cell transient expression system, we have found that neither Vav nor the product of the** *vav* **proto-oncogene, proto-Vav, can enhance the enzymatic activity of a coexpressed, epitope tagged MAPK. On the other hand, we have observed that, whereas proto-Vav can slightly elevate JNK/SAPK activity, oncogenic Vav potently activates JNK/SAPK to an extent comparable to that elicited by two guanine--nucleotide exchange factors for Rho family members, Dbl and Ost. We also show that point mutations** in **conserved residues within the cysteine rich and OH domains of Vav both preven! its ability to actívate JNK/SAPK and render Vav oncogenically inactive.** In **addition, we found that coexpression of the Rac-1 Nl 7 dominan! inhibitory mutan! dramatically diminisbes JNK/SAPK stimulation by Vav, as well as reduces the focus-fonning ability of Vav in NIH3T3 murine fibroblasts. Taken together, these findings provide the first evidence that Rac-1 and JNK are integral components of the Vav signaling pathway.**

Keywords: Vav; signa] transduction; JNK; MAP kinases; GTP-binding proteins

Introduction

Toe human *vav* gene was first identified by virtue of its oncogenic activation during the course of gene transfer assays when using tumor-derived DNA (Katzav *et al.*, 1989). Toe normal *vav* allele, the *vav* proto-oncogene, encodes a protein of 95 kDa, designated proto-Vav, which is preferentially expressed in cells of the hematopoietic lineage (Katzav *et al.*, 1991; Bustelo *et al.*, 1993). Recently, it has been shown that proto-Vav is rapidly phosphorylated on tyrosine residues opon activation of a variety of growth factor (Bustelo *et al.*, 1992; Bustelo and Barbacid, 1992; Margoiis *et al.*, 1992; Alai *et al.*, 1992; Dosil *et al.*, 1993; Evans *et al.*, 1993; Platanias aud Sweet, 1994) and hematopoietic cell-specific receptors (Margolis *et al.*, 1992; Bustelo and Barbacid, 1992). However, the normal function of Vav is still unknown, although analysis of its primary sequence reveals a number of structural motifs suggestive of a role in signa! transduction pathways. These motifs include a leucine-rich N-terminal region (Coppola *et al.*, 1991), a plekstrin homology (PH) domain (Mussachio *et al.*, 1993), a cysteine-rich domain (Coppola *et al.*, 1991), two SH3 regions flanking a single SH2 domain within its carboxyl- terminus (Bustelo *et al.*, 1992; Margolis *et al.*, 1992), and a central DH domain (Boguski and McCormik, 1993) which bears strong homology to Dbl (Eva *et al.*, 1988), a guanine nucleotide-exchange factor (GEF) for Rho related small GTP-binding proteins.

DH domains are a common motif in most GEFs for small GTP-binding proteins. As such, these domains are present in both Ras GEFs, Ras-GRF and SOS (Boguski and McCormick, 1993). However, these DH domains appear not to be directly involved in nucleotide exchange activity towards Ras (Shou *et al.*, 1992). DH domains are also present in the Rho/ Cdc42 GEF Ost (Horii *et al.*, 1994), in the yeast Cdc42-specific GEF, Cdc24 (Ron *et al.*, 1991), in the ecr2 oncogene product which associates with RhoA, RhoC and Rae! (Miki *et al.*, 1993), as well as in Abr (Heisterkamp *et al.*, 1993) and Bcr (Ron *et al.*, 1991), both GTPase activating proteins (GAPs) for the Rho GTP-binding protein family. Taken together, these data suggest that DH domains are characteristic of regulatory molecules for small GTPases, thus raising the possibility that Vav might affect the function of GTP-binding proteins. In this regard, it has been suggested that Vav could act as a Ras-specific GEF (Gulbins *et al.*, 1993), although these data are in conflict with recently available reports (Bustelo *et al.*, 1994; Khosravi-Far *et al.*, 1994).

Work from a number of laborator¡es has demonstrated that certain small GTP-binding proteins control

the enzymatic activity of a family of closely related serine-threonine kinases, known as mitogen activated protein kinases (MAPKs) (Cano and Mahadevan, 1995). Toe function of MAPKs is to convert extra- cellular stimuli to intracellular signals which, in mm, control the expression of genes that are essential for many cellular processes, including cell growth and differentiation (Marshall, !995). MAPKs have been classified in three subfamilies: extracellular signa! regnlated kinases (ERKs), including ERKl and ERK2) also known as p44mapk and p42rn[a]P\ respectively (referred to herein as MAPKs); stress activated protein kinases (SAPKs), also termed c-jun N-tenninal kinases (JNKs); and p38 kinase (Cano and Mahadevan, 1995). Whereas Ras controls the activation of MAPK (Marshall, 1995), we and others have recently observed that two members of the Rho family of small GTP- binding proteins, Racl and Cdc42, regulate the activity of JNKs (Coso *et al.,* 1995; Minden *et al.,* 1995). Thus, the similarity between Vav and the large family of DH- containing GEFs for small GTPases, and the identifica- tion of biochemical routes specific for Ras and Rho related GTP-binding proteins, prompted us to explore whether MAPK or JNK are downstream components of the Vav signaling pathways. Here, we report that expression of Vav in COS-7 cells fails to elevate the enzymatic activity of MAPK. However, we show that oncogenic forms of Vav can potently actívate JNK, acting on a Rac-1 dependen\ signaling pathway.

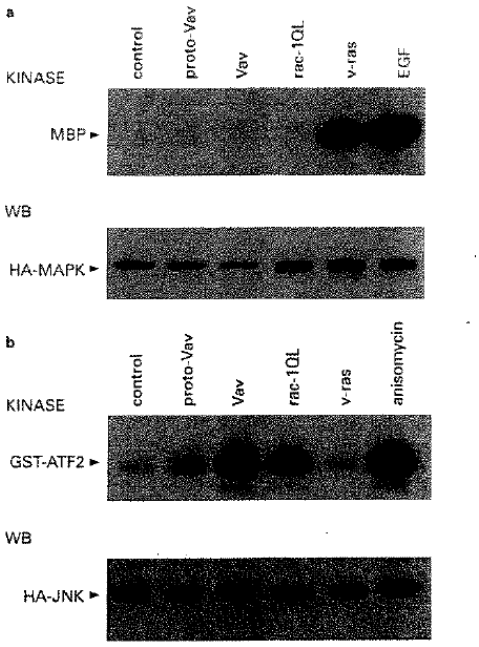

Figure 1 Effect of Vav on MAPK and JNK activity. COS-7 cells were transfected with pcDNA3-HA-JNKl (1 µg/plate) or pcDNA3-HA-MAPK (l µg/plate) for **(a)** MAPK and (b) JNK assays, respectively, together Wlth pcDNA3 vector (control) or expression vectors carrying cDNAs for·proto-Vav, Vav, *v-ras,* or a constitutively active fonn of Rac-1 (Rac-1 QL) (2 µg per plate in each case), as indicated. Treatment of cells with 10 µg/ml of anisomycin for 20 min or 100 ng/m.l EGF was u.sed as a control. Kinase reactions were performed in anti-HA immunoJ)recipitates from the corresRonding cellular lysates as described in Materials and methods. :! P labeled products are indicated. Equ.al levels of expression of HA MAPK or HA-JNK were ascertained by Western Blotting as described in Materials and methods. Similar results were obtained in four independent experiments

Results

Vav activates JNK but not MÁPK

The transforming pathway(s) activated by Vav has not been yet elucidated, but recen! reports have suggested that Vav might act as a Ras-specific GEF (Gulbins *et al.,* 1993). On the other hand, a wide array of studies have positioned Ras upstream of MAPK and shown that Ras is capable of potently elevating the activity of this serine-threonine kinase (Marshall, 1995). Thns, we hypothesized that if Vav were a Ras GEF, it would be expected to induce signals leading to the activation of MAPK. To test that, we took advantage of a simple *in vivo* model, consisting of the transient expression of Vav and an epitope-tagged MAPK in COS-7 cells, as previously reported (Crespo *et al.,* 1994; Coso *et al.,* 1995). Cells were cotransfected with the expression vectors for p95 proto-Vav or its oncogenically activated form, p85 Vav, together with a hemoagglti- nin-tagged p42 MAPK (HA-MAPK). Expression of the HA-MAPK was assessed by Western blot analysis of total cell lysates with the anti-epitope murine monoclonal antibody, and found to be similar for each transfected cell (Figure la). Under these experimental conditions, we found that, whereas coexpression of activated Ras, v-Ras, or EGF treatment potently elevates the MBP phosphorylating activity of the HA-MAPK, neither proto-Vav nor Vav had any demonstrable effect on MAPK. These data strongly suggest that in COS-7 cells Vav does not actívate that MAPK signaling pathway.

Recent work from our laboratory has demonstrated that small GTP binding proteins of the Rho family, Rae! and Cdc42, can control the activity of a proline-directed kinase related to but distinct from MAPK, known as JNK or SAPK (Coso *et al.,* 1995). The finding that Vav fails to actívate MAPK, and the presence of a domain bearing homology to GEFs for Rho-like GTPases, DH domain (Ron *et al.,* 1991), prompted us to explore whether Vav could actívate JNK. As an experimental approach, we coexpressed the Vav proteins with HA-JNK in COS-7 cells. Our results expressed that, while proto-Vav could induce JNK activity poorly, Vav causes a dramatic increase in JNK activity (sixfold), toan extent comparable to that exerted by a constitutively active Rac-1 mntant (Rac-lQL) (Figure lb). Increased JNK activity cannot be attributed to differences in expression ofHA-JNK, as equal levels ofHA-JNK were detected in lysates from each transfected cell culture.

It has been recently shown that other DH-containing proteins such as Ost and Dbl can potently actívate JNK (Coso *et al.,* 1995). Thus, we next set out to investigate how does JNK activation by Vav compare with that induced by Ost and DbL As shown in Figure 2, we found that_ when equal amounts of expression

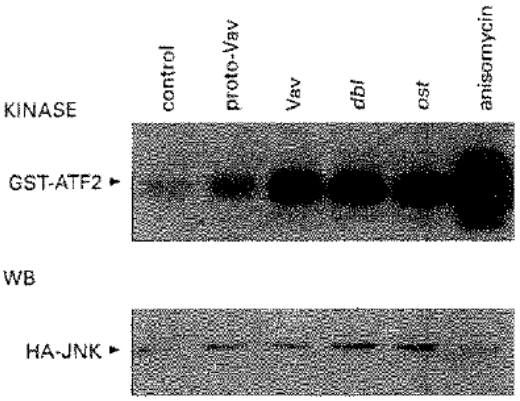

Figure 2 Act.ivation of JNK by expression of Vav and guaninine nucleotide exchange factors for the Rho-family of small GTP-binding proteins. COS-7 cells were transfected with the indicated expression plasmids for Vav, *dbl* and *ost* (2 µg per plate in each case), together with plasmids carrying HA-JNK_ Cells cotraus- fected with the pcDNA3 vector (control) untreated or stimulated with anisomycin (10 µg/ml, 20 min) served as controls. Similar results were obtained in four independent experiments. Expression of each exchange factor was confirmed by Western blotting Wlth the corresponding antibodies (data not shown). Equal levels of expression of HA-JNK were assayed by Western Blotting as described in Materials and methods

plasmids for each of these three proteins were transfected into COS-7 cells together with the HA- JNK DNA construct, the activation of **.11''K** caused by Vav was nearly as potent as that elicited by Ost or DbL

JNK elevation by Vav is inhibited by inactivating mutations within the DH and the cysteine-rich domains

Mutation of the first cysteine residue of the cysteine- rich domain of Vav, Vav C464S, inhibits Vav biological activity (Coppola *et al.*, 1991). Similarly, it has been recently found that substituition in Vav of leucine 213 to glutamine, Vav L213Q, completely abolishes the focus-forming ability of Vav (Bustelo *et al.* manuscript in preparation). Interestingly, sequence alignment analysis revealed that this leucine residue is conserved in al! DH-containing proteins so far described (unpublished observation). Thus, we as- sessed whether these inactivating mutations in the DH or cysteine-rich domain affect signaling to the JNK pathway. As shown in Figure 3a, al! Vav constructs were similarly expressed when transfected into COS-7 cells. However, JNK activation by Vav L213Q and Vav C464S was markedly decreased as compared with **JNK** induction elicited by Vav, although they still exhibited a higher activity than that of proto-Vav (Figure 3b). Thus, these data demonstrate that intact cysteine-rich and DH domains of Vav are necessary to fully actívate **JNK** and also suggest that **JNK** activation correlates with the biological activity of Vav proteins.

Activation of JNK by Vav is blocked by a Rae-] dominilnt inhibitory mutant

Severa! studies have shown that Dbl and other DH-domain bearing proteins such as Ost, act as exchange factors of the Rac/Rho family of small GTP-binding proteins (Hart *et al.*, 1991; Horii *et al.*, 1994).

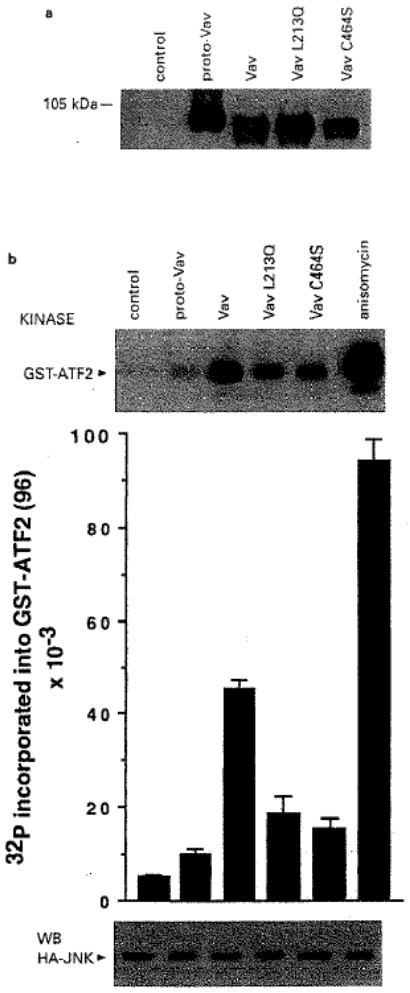

Figure 3 Activation of JNK. by Vav mutants. COS-7 cells were transfected with pcDNA3-HA-JNK (1 µg per plate) togetber with pcDNA3 vector (contról), or with the same expression vector carrying cDNAs far the wild type (próto-Vav) or activated forms of Vav, or Vav L213Q and Vav C464S mutants (2 *µg* per plate in each case). (a) lysates containing 40 *µg* of protein from transfected COS-7 cells were subjected to Western blot analysis with antibodies against Vav (Santa· Cruz). Bands were visualized by the enhanced chemilum.inescence technique using anti rabbit HRP-conjugated goat antiserum. (b) cells cotransfected with the pcDNA3 vector (control) untreated or stimulated with anisomy- cín (10 *µg/ml*, 20 min) served as controls for JNK assays. In the upper panel, a representative autoradiogram. Toe phosphorylated substrate is indicated. In the middle panel, data represent the mean± SEM of five independent experiments, expressed as radioactive counts of ^{32}P incorporated into GST-AFI'2(96) when u.sed as a substrate. Parallel lysates from transfected cells containing 40 *µg* of total cellular protein were subjected to Western blot analysis with anti-Ha antibody 12CA5 (bottom panel)

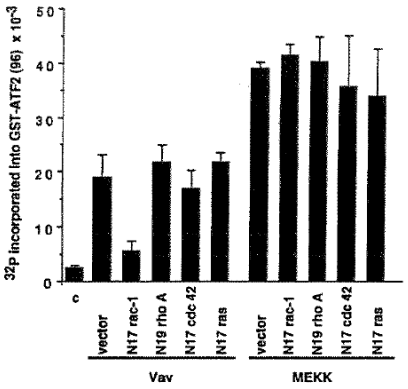

Figure 4 Effect of Ras and Rho family-inhibitory molecules on JNK activation by Vav. COS-7 cells were transfected with expression plasmids for Vav or MEKK (2 µg per plate in each case) and plasmids expressing the dominant inhibitory mutants Rac-1 Nl?, Cdc42 NI?, Rho Nl9 and Ras NI?, together witb pcDNA3-HA-JNK., as indicated. Data represent the mean± SEM of five independent experiments, expressed as radioactive counts of ^{32}P incorporated into GST-ATF2(96)

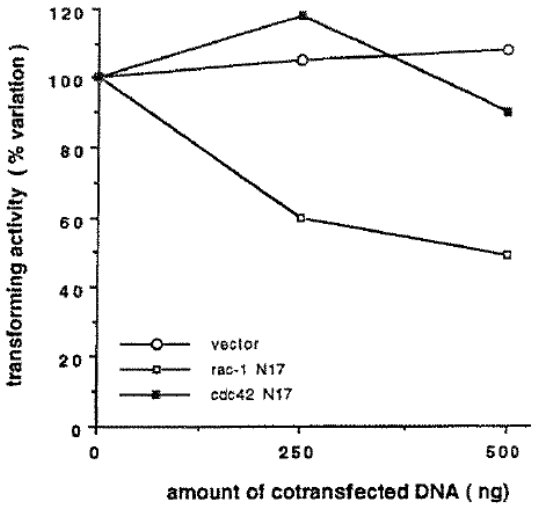

Figure 5 Effects of Rac-1 and Cdc42 dominant inhibitory mutants on vav-induced focus-formation. The indicated amounts of plasmid DNA expressing Rac-1N17, Cdc42 NI7 or the expression vector pMEX were transfected with *50* ng of expression plasmid for Vav into 1"1H3T3 fibroblast as described in Materials and metbods. Foci of transformation were scored 2-3 weeks after transfection. Results represent the average of three independent experiments

Moreover, results from our laboratory support a role for Rac-1 and Cdc42 regulating **JNK** activity (Coso *et al.*, 1995). Thus, it is conceivable that J1"K activation by Vav is mediated by one of these GTPases. To test this hypothesis, we cotransfected Vav together with dominan! inhibitory mutants of Rho A (Rho A Nl9), Rac-1 (Rae-! Nl 7), Cdc42 (Cdc42 Nl 7) and Ras (Ras Nl 7). These mutants are thought to act by competi- tively inhibiting the interaction of small GTPases with their respective GEFs (Feig and Cooper, 1988). When transfected into COS-7 cells, these mutants were found to be expressed at detectable levels (data not shown; Coso *et al.*, 1995). As shown in Figure 4, none of these mutants affected **JNK** activation by MEKK, a kinase acting downstream of the small GTP binding proteins (Lange-Carter *et al.*, 1993), when utilized as a control. In contrast, JNK activation by Vav was markedly decreased upon cotransfection by Rac-1 **NI?,** whereas it was not significantly affected by Cdc42 NI7, Rho Nl9 or Ras NI7. Taken together, these findings suggest that Rae-! function is required for JNK activation by the *vav* oncogene product. Whether Vav directly affects Rae!, or acts upstream of a yet to be characterized Rae! gnanine-nucleotide exchange factor is still unknown and it is cnrrently being investigated.

Transformation by Vav is diminished by a Rac-1 dominant inhibitory mutant

vav behaves as a very potent oncogene when transfected into NIH3T3 cells (Katzav *et al.*, 1991; Coppola *et al.*, 1991). To study whether Rae! participates in the signaling pathway mediating the transforming function of *vav*, we scored vav-induced foci-formation in NIH3T3 fibroblasts upon cotransfection with expression plasmids for the dominant inhibitory mutants of Rae-! and the closely related GTPase, Cdc42. NIH3T3 cells were transfected with 50 ng of *vav* plasmid together with the expression vector (pMex) alone or with increasing concentrations (250 and 500 ng) of plasmids for Rac-1 NI 7 or Cdc42 NI 7. As shown in the Figure 5, cotransfection with Rae-! NI7 results in a significan! reduction of *vav*- transformed foci. On the other hand, cotransfection with Cdc42 Nl 7 cDNA did not sigrtificantly alter the number of foci induced by *vav*, thereby snggesting that Rae-!, but not Cdc42, is necessary for vav-induced transformation.

Discussion

Accumulating evidence snggests that small GTP binding proteins control divergent kinase cascades leading to the activation of each member of the MAPK superfamily. As such, MAPK is regulated by Ras (Marshall, I995), and Rac-1 and Cdc42 control the activation of JNK (Coso *et al.*, 1995; Minden *et al.*, 1995). In addition, Rho regulates the pathway leading to the activation of a putative kinase that affects SRF function (Hill *et al.*, 1995). In this context, GEFs for the Rac/Rho/Cdc42 family such as Dbl and Ost can actívate JNK, whereas GEFs for Ras such as SOS (Aronheim *et al.*, 1994) and Ras-GRF (Famsworth *et al.*, 1995) can actívate MAPKs. Although the function of the protein encoded by the human *vav* oncogene, p85v", is still unknown, it exhibits structural features common to GEFs for GTPases of the Rho and Ras families. Thus, in this study we set out to investigate the Vav signaling pathway by exploring the ability of

Vav to actívate MAPK or JNK, using the transient expression in COS-7 cel!s as a model system,

Our results indicate that neither proto-Vav nor Vav is capable of activating MAPK to any significant extent Severa! studies llave shown otherwise (Khosravi-Far *et al,,* 1994; Gulbins *et al.,* 1994), however, in these cases MAPK activation was determined in stable Vav-transformed cell lines. Thus, it cannot be ruled out that the enhanced MAPK activity reported represents an event secondary to the transformed state of the cells, rather than being a direct effect caused by Vav. Moreover, in those earlier reports (Kliosravi-Far *et al.,* 1994; Gulbins *et al.,* 1994). MAPK activation was scored by change in its electrophoretic mobility in SDS-PAGE geis, a less specific assay than the direct determination of the MBP phosphorylating activity of MAPK. We conclude that Vav is incapable of directly activating MAPK, thus suggesting that Vav <loes not act as a Ras GEF, in agreement with recent studies (Bustelo *el al.,* 1994).

In contras!, our results indicate that Vav potently activates JNK, and to an extent comparable to that induced by Dbl and Ost, both GEFs for Rho related proteins (Coso *et al.,* 1995). These three proteins share a common structural domain known as the Dbl- homology (DH) domain (Ron *et al.,* 1988; Eva *et al.,* 1988; Boguski and McCormick, 1993), Mutations within this domain impair Dbl activity, abolishing Dbl-induced focus fonnation upon expression in N1H3T3 cells, as well as its ability to stimulate GDP dissociation on Rho when studied by *in vitro* assays (Hart *et al.,* 1994). Here we show that point mutations within the DH domain, Vav L213Q, or the cysteíne- rich domain, Vav C464S, of Vav dramatically diminish JNK stimulation, Interestingly, both Vav L213Q and Vav C464S are biologically ínactive mutants, incapable of transforming N1H3T3 fibroblasts (Coppola *et al.,* 1991; Bustelo *et al.,* manuscript in preparation). Taken together, these findings suggest that the cysteine rich and DH domains of Vav participate in a biologically relevan! pathway, and that JNK activation may contribute to the transforming potential of *vav.*

The structural similarities (Adams *et al,,* 1992) and the fact that the disruption of their respective DH domains impairs Vav and Dbl transforming potential suggests that these two proteins could be acting on the same signaling pathway. Accordingly, Vav and Dbl- transformed fibroblasts have been shown to exhibit similar morphology and nearly identical pattern of distribution of stress fibers (Khosravi-Far *et al.,* 1994), resembling that found in cells transformed by the Rho family of small GTP-binding proteins (Ridley and Hall, 1992; Ridley *et al.,* 1992). In addition, JNK activation by Dbl has been shown to be Rae-! dependent (Minden *et al.,* 1995, our unpublished results). Like- wise, our results indicate that JNK activation by Vav is mediated by Rac-1, as we have observed that the Rae-! Nl 7 dominant inhibitory mutant markedly diminishes JNK activation by Vav, Moreover, we have observed that cotransfection with Rac-1 N17 reduces the focus- forming ability of Vav. Taken together, these data strongly suggest that Rae-! is an integral componen! of the transforming pathway activated by the *vav* oncogene, However, further work will be required to elucidate fully the role of Rae! and JNK in *vav*- mediated transformation.

Stimulation of JNK by externa! stimuli as !L-1, EGF and TNF-a has also been found to be dependent on Cdc42 (Coso *et al.,* l995). This secms not to be the case in Vav signaling, as neither activation of JNK mediated by Vav nor Vav-induced transfonnation was affected by the Cdc42 Nl 7 dominant inhibitory mutant. Thus, Vav appears to have a greater effector specificity when signaling to the JNK cascade, favoring the Rac-1-mediated pathway. However, is Vav an exchange factor for Rae-!? So far, we and others (Gulbins *et al.,* 1993) have been unsuccessful in providing a conclusive answer. Although it is possible that Vav may not be able to induce guanine-nucleotide exchange on Rac-1 in the *in vitro* assays utilized, we cannot rule out that other factors are essential for Vav exchange activity, including the possibilty that Vav may act upstream or in conjunction with additional GEFs to induce Rae-! guanine nucleotide exchange. These issues are under current investigation. Finally, our results may also have implications with respect to the normal function of proto-Vav, raising the possibility that proto-Vav acts as a mediator in the signaling pathways leading to JNK activation hemato- poietic cells, including T and B-lymphocytes, As neither the role of proto-Vav nor the biological consequences of JNK activation in these cells is yet understood, our present study linking these two proteins opens new avenues that warrant further investigation.

Materials and methods

Expression plasmids

An Nrul-Notl DNA fragment from pKLS45 (Coppola *et al.,* 1991), containing the *vav* cDNA, lacking sequences for the aminoterminal 65 aminoacids of proto-Vav, was subcloned into the EcoRV -Notl sites of the pcDNA3 (Invitrogen) mammalian expression vector. pcDNA3 *vav* L213Q was obtained by replacing the *vav* Bglll 1956 bp fragment with the corresponding sequences from pLC4, an expression plasmid carrying a *vav* cDNA mutated in codon 213 (Bustelo *et al.,* manuscript in preparation), pcDNA3 *vav* C464S was generated by replacing the *vav* Bglll fragment with the corresponding sequence from pJCl 7 (Coppola *et al.,* 1991). DNA plasmids expressing epitope- tagged MAPK and JNK, Ras Vl2, Rac-1 QL, Rac-1 N17, RhoA N19, Cdc42 N17, Ras N17, Ost, Dbl and MEKK have been described elsewhere (Coso *et al.,*1995).

Kinase assays

Jun-kinase assay Subconfluent COS-7 cells were trans- fected with pcDNA3-HA-JNK and additional DNAs by the DEAE-dextran technique as previously described (Coso *et al.,* 1995). Total arnount of plasrnid DNA was adjusted to 5-10 µg/plate witb vector DNA (pcDNA3, Invitrogen) when necessary. Two days later, transfected COS-7 cells were cultured 2 h in serum-free medium. Cells were then washed witb cold PBS, and lysed at 4ºC in a buffer containing 25 mM HEPES, pH 7.5, 0.3 M NaCI, 1.5 mM MgCl,, 0.2 mM EDTA, 0.5 mM DTT, 1% Triton X-100,
20 mM ,8-glycerophosphate, 1 mM sodium vanadate, 1 mM pbenylmetbylsulfonylfluoride, 20 µg/ml aprotinin, and 20 µg/ml leupeptin. Tbe epitope-tagged JNK was immuno-precipitated from the cleared lysates by incubation with tbe HA-specific antibody 12CA5 (BABCO) for 1 h at 4ºC. Immunocomplexes were recovered with the aid of Gamma-

bind sepharose beads (Pharmacia) and washed three times with PBS containing l %o 1\'P-40 and 2 rnM sodium vanadate, once with 100 mM TRIS, pH 7.5, 0.5 M LiCI, and once in kinase reaction buffer (l2.5 mM MOPS, pH 7.5, 12.5 mM ¡l-glycerophosphate, 7.5 mM MgCI,, 0.5 M EGTA, 0.5 mM sodium fiuoride, 0.5 mM sodium vanadate). The JNK activity present in the immunoprecipitates was determined by resuspension in 30 µ! of kinase-reaction buffer containing 1 μCi [,y-^{1}P]ATP per rcaction and 20 ¡LM of unlabeled ATP, using l μg of GST-ATF2 fusion protein as a substrate, as previously described (Coso *et al.,* 1995). After 20 rnin at 30"C, reactions were terminated by addition of 10 *¡il* 5 x Laemmii buffer. Samples were heated at 95{¡C far 5 min and analysed by SDS-gel eiectrophoresis on 12°/o acrylamide gels. Auto- radiography was performed with the aid of an intensifying screen.

MAPK kinase assay MAPK kinase activity in COS-7 transfected with an epitope-tagged MAPK (HA-ERK2, referred here as HA-MAPK) was determined as previously described (Crespo *et al.,* 1994), using myeiin basic protein (MBP) as a substrate.

Western blors

Lysates containing approximately 40 μg of total cellular protein were analysed by Western-blotting after SDS/ polyacrylamide gel electrophoresis, transferred to nitrocel• lulose and immunoblotted with the corresponding rabbit antisera or mouse monoclonal antibody. lmmunocorn- plexes were visualized by enhanced chemiluminescence detection (Amersham) using goat anti•rabbit or anti- mouse coupled to HRP as a secondary antibody (Cappel). Anti HA-epitope mouse monoclonal antibody (l2CA5) was purchased from BABCO, rabbit polyclonal antibodies against Vav, Rac-1, RhoA and Cdc42, and ami- Ras monoclonal Y13-259 were purchased from Santa Cruz Laboratories.

Focus-forming assays in NJH3T3 cells

Plasmid DNA transfection of NIH3T3 fibroblasts was perfonned by the calcium phosphate precipitation techni- que (Wigler *et al.,* 1977). Transformed foci were scored after 2-3 weeks as previously described (Gutkind *et al.,* 1991).

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