JCB Article

Vav2 is required for cell spreading

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Vav2 is a widely expressed Rho family guanine nucleotide exchange factor highly homologous to Vav1 and Vav3. Activated versions of Vav2 are transforming, but the normal function of Vav2 and how it is regulated are not known. We investigated the pathways that regulate Vav2 exchange activity in vivo and characterized its function. Overexpression of Vav2 activates Rac as assessed by both direct measurement of Rac-GTP and cell morphology. Vav2 also catalyzes exchange for RhoA, but does not cause morphologic changes indicative of RhoA activation. Vav2 nucleotide exchange is Src-dependent in vivo, since the coexpression of Vav2 and dominant negative Src, or treatment with the Src inhibitor PP2, blocks both Vav2-dependent Rac activation and lamellipodia formation. A mutation in the pleckstrin homology (PH) domain eliminates exchange activity and this construct does not induce lamellipodia, indicating the PH domain is necessary to catalyze nucleotide exchange. To further investigate the function of Vav2, we mutated the dbl homology (DH) domain and asked whether this mutant would function as a dominant negative to block Rac-dependent events. Studies using this mutant indicate that Vav2 is not necessary for platelet-derived growth factor– or epidermal growth factor–dependent activation of Rac. The Vav2 DH mutant did act as a dominant negative to inhibit spreading of NIH3T3 cells on fibronectin, specifically by blocking lamellipodia formation. These findings indicate that in fibroblasts Vav2 is necessary for integrin, but not growth factor–dependent activation of Rac leading to lamellipodia.

Introduction

Rho family GTP-binding proteins (GTPases) mediate numerous cellular events, including the regulation of actin structures, adherence and motility, gene transcription, and cell cycle progression (Hall, 1998). GTPases undergo a conformational change upon GTP binding that allows them to interact with effector molecules and thereby transmit signals. The exchange of GTP for GDP is the critical step in initiating signaling and is catalyzed by guanine nucleotide exchange factors (GEFs)* (Cerione and Zheng, 1996). Thus, understanding how RhoGEFs are regulated is critical to providing insight into how Rho family GTPases function.

There are >30 RhoGEFs, all of which contain a DH domain, which is necessary to catalyze nucleotide exchange, and an adjacent PH domain (Whitehead et al., 1997). The PH domains in some proteins, including RhoGEFs, bind phosphoinositides and mediate membrane localization (Bottomley et al., 1998). The PH domain of some RhoGEFs is necessary for nucleotide exchange (Freshney et al., 1997). The large number of RhoGEFs and their overlapping specificities for GTP binding proteins raise the questions of how Rho family–dependent functions are coordinated in vivo and whether RhoGEFs are redundant or have specific functions. Although RhoGEFs have been extensively studied, these questions remain largely unanswered.

The RhoGEF Vav1 has been well characterized and two other mammalian family members have been identified recently, Vav2 and Vav3 (Katzav et al., 1989; Henske et al., 1995; Schuebel et al., 1996; Trenkle et al., 1998; Movilla and Bustelo, 1999). The expression of Vav proteins is highly conserved: both *Caenorhabditis elegans* and *Drosophila melanogaster* have Vav homologues (Dekel et al., 2000). All mammalian Vav proteins share the same domain structure. Within the NH_2 terminus of Vav family members is an acidic domain and a region related to calponin that is present in some actin binding proteins. These domains are followed by the DH and PH domains and a COOH terminus with a cysteine-rich region and a single SH2 domain flanked by SH3 domains.

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*Abbreviations used in this paper: DH, dbl homology; EGFP, enhanced green fluorescent protein; GEF, guanine nucleotide exchange factor; GST, glutathione *S*-transferase; HA, hemagglutinin; KD, kinase dead; PAK, p21-activated kinase; PBD, PAK binding domain; PH, pleckstrin homology; PI-3, phosphoinositide 3; RBD, Rho binding domain.

Key words: Vav; Rac; spreading; integrins; growth factors

A truncated form of Vav1 was originally identified as an oncogene and Vav1 is expressed primarily in the hematopoietic system, as well as in the pancreas and lung (Bustelo et al., 1993). Vav1 activates Rac1 and perhaps RhoA and Cdc42 (Crespo et al., 1997; Han et al., 1997). Vav2 is widely expressed in tissues and cell lines. The literature is inconsistent with regards to the GTPases activated by Vav2. Schuebel et al. (1998) reported that Vav2 catalyzed exchange for RhoA, RhoB, and RhoG in vitro, whereas Abe et al. (2000) reported that Vav2 catalyzed exchange for Rac1, RhoA, and Cdc42 in vitro. The development of assays based on the known binding interaction between Rac/Cdc42 and p21-activated kinase (PAK) binding domain (PBD) (Sander et al., 1998), and based on the known binding interaction between RhoA and the Rho binding domain (RBD) of rhotekin (Ren et al., 1999), has permitted the determination of Vav2 exchange activity in vivo. Liu and Burridge (2000) showed that Vav2 is an exchange factor for Rac1, Cdc42, and RhoA in CHO cells in vivo using these assays. Vav3 is expressed predominantly in brain and hematopoietic cells and to a lesser extent in other tissues, and activates RhoG, RhoA, and Rac1 in vitro (Movilla and Bustelo, 1999; Trenkle et al., 2000) and RhoA and Rac1 in vivo (Zeng et al. 2000).

Tyrosine phosphorylation is necessary for the exchange activity of the Vav proteins in vitro and has been used as a surrogate for activation in vivo (Crespo et al., 1997). Vav1 is tyrosine phosphorylated in response to many signals, including B or T cell receptor activation and integrin cross-linking in myeloid cells and platelets (Bustelo, 2000). The kinases that phosphorylate Vav1 include Syk in B cells, Zap70 and Fyn in T cells, and Janus kinases in response to cytokine receptor stimulation (Bustelo, 2000; Huang et al., 2000). Vav proteins are also tyrosine phosphorylated in response to growth factor stimulation, including EGF, PDGF, and insulin (Bustelo et al., 1992; Moores et al., 2000; Pandey et al., 2000). Src family kinases phosphorylate and activate Vav proteins in vitro, but their role in Vav function in vivo is not established. Phosphorylation of Y174 stimulates exchange activity in vitro (Han et al., 1997) by disrupting the inhibitory interaction of Y174 with the DH domain (Aghazadeh et al., 2000). Mutation of Y174 to F activates Vav1, which likely reflects the inability of F174 to bind to and inhibit the DH domain (Lopez-Lago et al., 2000). The Y174F mutant is activated to the same extent as wild-type Vav1 by tyrosine phosphorylation in in vitro exchange assays, indicating that an additional site(s) is phosphorylated to stimulate exchange activity.

Vav proteins activate pathways dependent on Rho family GTP-binding proteins, including protein kinases in the mitogen-activated protein kinase and PAK families, actin rearrangement, and stimulation of transcription by serum response factor and nuclear factor kappa B (Bustelo, 2000). Interestingly, the activation of nuclear factor of activated T cells does not require the exchange activity of Vav1 (Kuhne et al., 2000). In contrast, Vav2 potentiation of nuclear factor of activated T cells requires exchange activity in B cells (Doody et al., 2000). Studies of cells from mice lacking Vav1 indicate that Vav1 is critical for B and T cell signaling (Fischer et al., 1995, 1998; Tarakhovsky et al., 1995; Zhang et al., 1995; Turner et al., 1997). B cells from $Vav^{-/-}$ mice have a defective proliferative response to B cell receptor activation and fail to mount an immune response to nonrepetitive antigens. Cytotoxic T cells from $Vav^{-/-}$ mice show a reduction in CD3- plus CD28-mediated proliferation, a decrease in IL-2 production, and a reduction in cytotoxic T cell responses. Little is known about the biological roles of Vav2 or Vav3.

To better understand the function of Vav2, we determined the GTPases activated by Vav2 in vivo and investigated the role of Src and the Vav2 DH and PH domains in regulating Vav2 exchange activity. We used a mutant form of Vav2 that lacks exchange activity to inhibit the function of endogenous Vav2 and found that this mutant blocked spreading of NIH3T3 cells on fibronectin. The cells form filopodia, but do not form lamellipodia. This mutant did not block Rac-dependent effects of PDGF or EGF, suggesting that Vav2 is necessary for integrin-dependent activation of Rac during cell spreading, but not for growth factor–dependent Rac activation.

Results

Exchange activity of Vav2

An important step in understanding the function of Vav2 is to determine the GTPases it activates. Studies of the exchange activity of Vav2 in vitro have yielded conflicting results, as discussed above. Recently, assays have been described that allow measurement of Rac, Cdc42, and RhoA activation in vivo (Sander et al., 1998; Ren et al., 1999). We used these assays to determine the GTP binding proteins activated by Vav2 expression in vivo. NIH3T3 fibroblasts were transfected with Vav2 and myc-Rac, hemagglutinin (HA)-Cdc42, or HA-RhoA. Cells were harvested after 24 h, lysed, and incubated with glutathione *S*-transferase (GST)-PBD or GST-RBD bound to glutathione agarose beads. The amount of activated Rac, Cdc42, or RhoA was determined by probing the Western blots for the GTPases associated with the GST-PBD or GST-RBD constructs. Vav2 activated Rac1 (Fig. 1 A) and to a lesser extent RhoA (Fig. 1 D), but we detected minimal activation of Cdc42, although Dbl did activate Cdc42 as expected (Fig. 1 C). Similar results were obtained when the experiments were done using HEK293T or Cos7 cells. We also compared activation of endogenous Rac by Vav2 and Vav1 and detected a similar extent of activation (Fig. 1 B).

Vav2 induces lamellipodia and activation of Jnk

Since we detected activation of Rac and Rho by Vav2 we investigated the effects of overexpressing Vav2 on actin structures in NIH3T3 cells to determine whether the phenotype suggested a predominance of either Rac or Rho activation. Overexpression of Vav2 resulted in extensive lamellipodia in 80% of transfected cells (Fig. 2 A). No untransfected cells had the extensive lamellipodia seen in the Vav2 transfected cells and less than 20% of untransfected cells had any lamellipodia. These results are similar to Moores et al. (2000), but in contrast to Schuebel et al. (1998), who reported that overexpression of wild-type Vav2 had no effect on actin. We detected few cells that had prominent stress fibers or were contracted, suggesting that under these conditions there was little activation of RhoA relative to Rac. We also assayed Jnk activity in COS7 cells transfected with Vav2 and found that

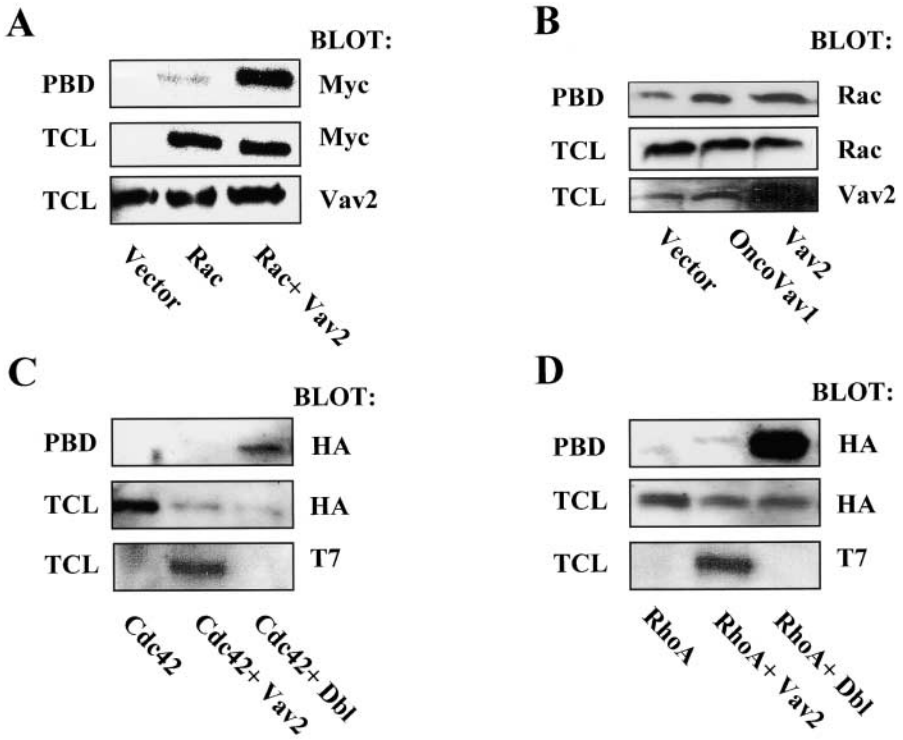

Figure 1. **Activation of GTPases by Vav2 expression in vivo.** NIH3T3 fibroblasts (A, C, and D) or HEK293T cells (B) were transfected with Vav2, Vav1, or Dbl plus myc-Rac (A), HA-Cdc42 (C), or HA-RhoA (D) at a ratio of 3:1 (RhoGEF/GTPase). The effect of Vav1 and Vav2 on endogenous Rac was determined in HEK293T cells (B). Cells were harvested after 24 h and lysates incubated with GST-PBD (A–C) or GST-TRBD (D) bound to glutathione beads for 40 min at 4°C and then washed and the proteins separated by SDS-PAGE. Activation of Rac, RhoA, or Cdc42 was determined by Western blotting with Rac, myc, or HA antibodies. Total cell lysates (TCL) were also Western blotted for expression of Vav2 with Vav2 antibody and GTPases as described in Material and methods. These results are representative of three experiments.

overexpression of Vav2-activated Jnk (Fig. 2 B). Since activation of Jnk in COS7 cells is dependent on Rac or Cdc42 but not Rho (Coso et al., 1995), and we detect minimal activation of Cdc42 by Vav2, stimulation of Jnk by Vav2 in COS7 cells indicates that Vav2 activates Rac in vivo.

We used a Vav2 construct containing the T7 epitope at the COOH terminus in overexpression studies. Since overexpression of wild-type Vav2 alone activated Rac1, we wanted to be certain that the T7 epitope tag did not stimulate Vav2. NIH3T3 cells were cotransfected with either T7-tagged or untagged Vav2 and an enhanced green fluorescent protein (pEGFP) to identify the transfected cells, and the number of transfected cells with lamellipodia were counted. Both untagged and T7Vav2 constructs caused ~80% of the transfected cells to form lamellipodia (data not shown), indicating that the epitope tag does not activate Vav2.

Src is necessary for Vav2 activity

Coexpression of activated Lck with wild-type Vav2 promotes colony formation and induces lamellipodia (Schuebel et al., 1998), and the exchange activity of Vav proteins is activated by Src-like kinases in vitro, but it is not known whether Src kinases are necessary for Vav2 activation in vivo. To determine whether Src family members are necessary for Vav2 activity, HEK293T cells were transfected with Vav2 and kinase-dead (KD) Src, and Rac activation was compared with cells transfected with Vav2 alone. As shown in Fig. 3 A, KD Src blocked Vav2-dependent Rac activation. We did not detect increased Rac activation when Vav2 was cotransfected with activated Src, although Vav2 tyrosine phosphorylation was increased (data not shown), indicating that endogenous Src activity is sufficient to fully activate Vav2. We also determined the effect of KD Src and the Src inhibitor, PP2, on the formation of Vav2-dependent lamellipodia. NIH3T3 were transfected with Vav2 and KD Src or with Vav2 alone and treated with PP2. PP2 treatment or cotransfection of Vav2 and KD Src reduced the number of Vav2 transfected cells with lamellipodia by 80 and 70%, respectively, and in those cells in which lamellipodia were present, they were much less prominent (Fig. 3 B). These data strongly suggest that Src or an Src family member is necessary for Vav2 activation of Rac in vivo.

The PH domain is necessary for Vav2 activity

The activity of Vav1 is stimulated by phosphoinositide products of phosphoinositide 3 (PI-3) kinase binding to the PH domain, however, mutations in the PH domain of Vav1 do not completely inhibit exchange activity and a mutation in the PH domain of Vav3 does not impair its function (Han et al., 1998; Movilla and Bustelo, 1999). The role of the PH domain in Vav2 function is not known. To determine whether the PH domain is necessary for Vav2 activity we mutated R425 to G, based on mutations in Vav1 that disrupt the binding of phosphoinositides (Han et al., 1998). HEK293T cells were transfected with R425G Vav2 and Rac1 activation was measured. This construct did not activate Rac (Fig. 4 A) and or cause lamellipodia (compare Fig. 4 B with 2 A), indicating that unlike Vav1 and Vav3, the PH domain is required for Vav2 function.

The necessity of the PH domain could reflect a requirement for phosphoinositide binding. To determine if the products of PI-3 kinase are necessary for Vav2 function, we tested whether wortmannin blocked Vav2-dependent Rac activation. HEK293T cells were transfected with Vav2 and Rac activation compared in cells treated with wortmannin (200 nM) for 1 h to cells left untreated. As shown in Fig. 4 C, wortmannin did not affect Vav2-dependent Rac activation. These results were confirmed by examining lamellipo-

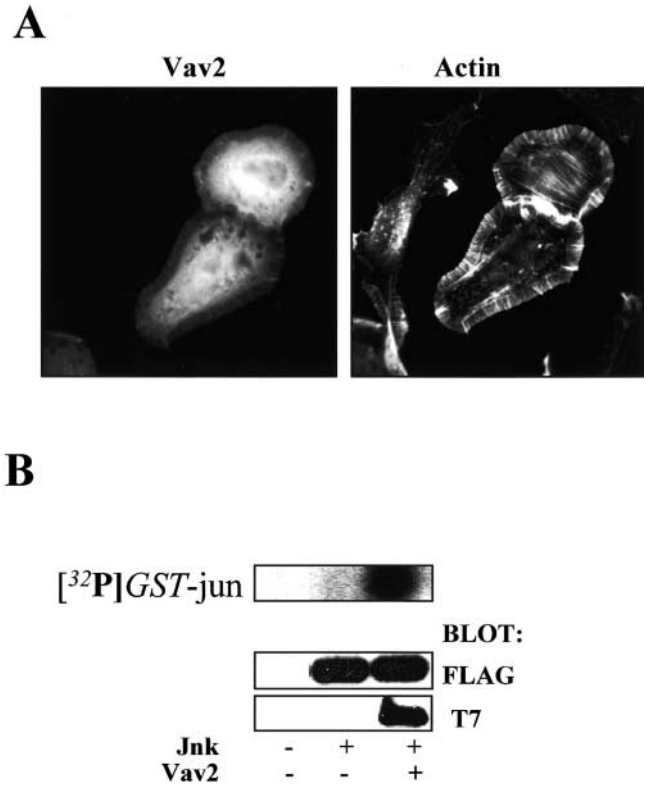

Figure 2. **Vav2 stimulates lamellipodia and Jnk activity.** (A) NIH3T3 cells were transfected with Vav2, fixed after 24 h, and stained for Vav2 expression with the T7 antibody and for actin with rhodamine-phalloidin. (B) COS7 cells were transfected with Vav2 and FLAG epitope–tagged Jnk followed by immunoprecipitation of Jnk with FLAG antibody. Jnk activity was determined as described in Materials and methods by phosphorylation of GST-jun (top). Cell lysates were Western blotted to determine Jnk (middle) and Vav2 (bottom) expression. These results are representative of four experiments.

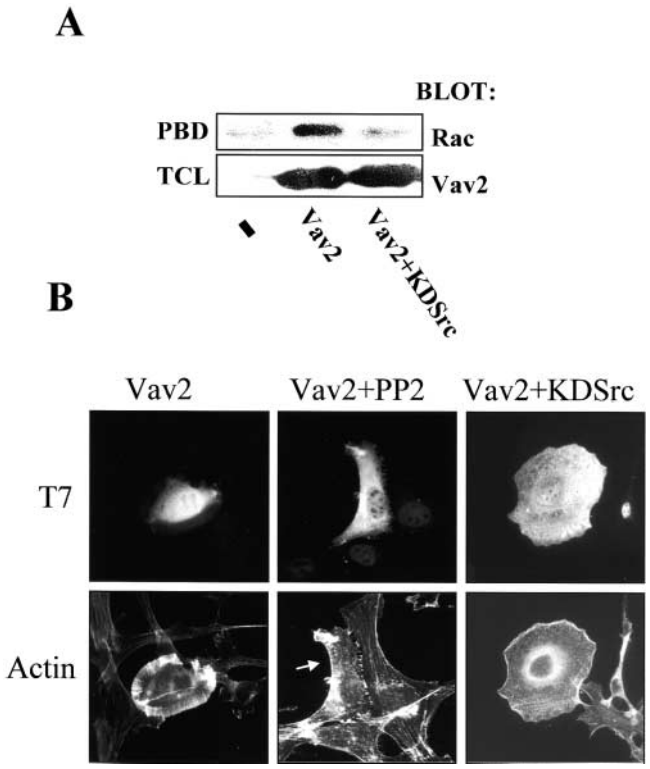

Figure 3. **Activation of Vav2 is Src-dependent.** (A) HEK293T cells were transfected with Vav2 or Vav2 and KD and harvested after 24 h. Activation of endogenous Rac (PBD) was assayed as described in Materials and methods. Total cells lysates (TCL) were also Western blotted with T7 antibody for Vav2 expression. (B) NIH3T3 cells were transfected with Vav2 and KD Src or Vav2 transfected cells were treated with PP2 (2 μM) for 30 min or left untreated. The cells were fixed and then stained for Vav2 expression with T7 antibody and for actin with rhodamine-phalloidin. The arrow indicates the transfected cell. These results are representative of three experiments.

dia in NIH3T3 cells overexpressing Vav2 after treatment with wortmannin. Concentrations of wortmannin as high as 1 μM did not inhibit Vav2-mediated lamellipodia, although the contracted appearance of untransfected cells indicated that the wortmannin was active (Fig. 4 D). PI-3 kinase is not required for Vav2 activation of Rac or lamellipodia formation in contrast to Vav1, where wortmannin partially impairs Vav1-dependent ruffling (Miranti et al., 1998).

Mutation of the Vav2 DH domain

All exchange factors for Rho family of GTPases contain a DH domain that is required to catalyze nucleotide exchange. We wished to make a Vav2 DH mutant that lacked exchange activity and thus might function as a dominant negative to allow us to identify Vav2-dependent functions. Mutations in the DH domain of several RhoGEFs disrupt catalysis of GDP/GTP exchange (Hart et al., 1994; Liu et al., 1998; Ma et al., 1998). Based on these mutations, we mutated R335 to G and the L342/L343 residues to R342/S343 in the DH domain of Vav2. The R335G mutant functioned very much like wild-type Vav2 in assays of Rac activation, stimulation of Jnk and induction of lamellipodia (data not shown), which is somewhat surprising since a similar mutant in the Trio DH domain markedly inhibited exchange activity in vitro (Liu et al., 1998). The L342R/L343S mutant did not activate Rac (Fig. 5 A), or Rho (data not shown) and did not cause lamellipodia in transfected NIH3T3 cells (Fig. 5 B) or Jnk activation in transfected COS7 cells (data not shown, see Fig. 7 C).

L342R/L343SVav2 does not inhibit formation of Vav1-dependent actin structures or Rac activation

The purpose of designing a Vav2 DH domain mutant was to use this reagent as a dominant negative in order to identify a function for Vav2. L342R/L343SVav2 should be recruited to sites where Rac would be activated by Vav2 and thereby block recruitment of endogenous Vav2 and thus Rac activation by endogenous Vav2. However, this construct could act nonspecifically, particularly if it sequestered Rac. To be certain that any inhibitory effects of L342R/L343SVav2 were specific for Vav2, we investigated whether it would block Vav1-mediated Rac activation or lamellipodia formation. HEK293T cells were transfected with OncoVav1 or OncoVav1 and L342R/L343SVav2, and activation of endogenous Rac was determined using the PBD assay. L342R/L343SVav2 did not block Vav1-dependent Rac activation (Fig. 6 A). Similarly, L342R/L343SVav2 coexpression did not affect the frequency or extent of lamelli-

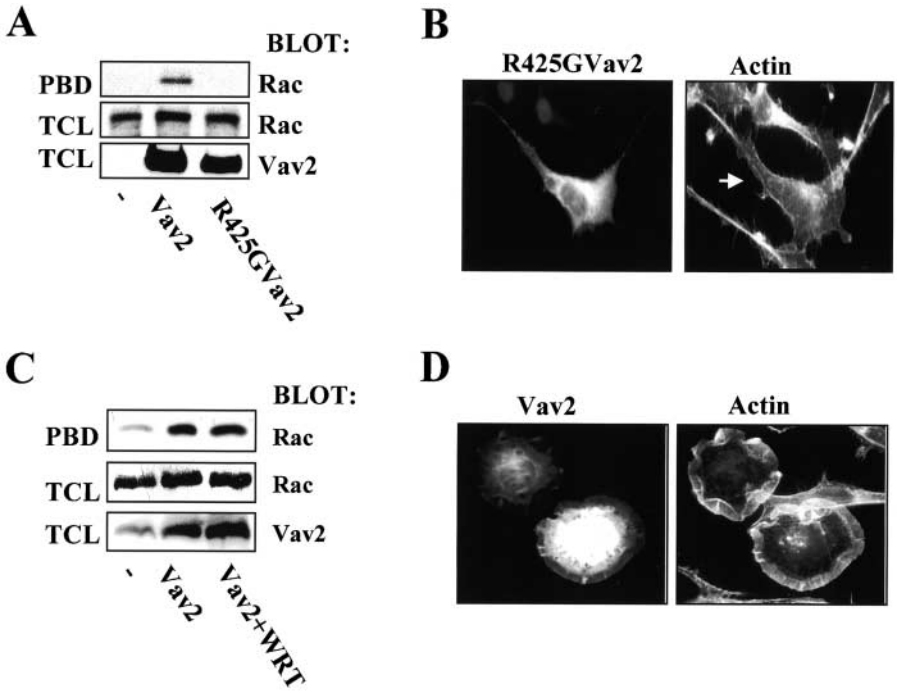

Figure 4. **The PH domain is necessary for Vav2 exchange activity.** (A) HEK293T cells were transfected with R425GVav2 and exchange activity for endogenous Rac (PBD) was determined as described in Materials and methods. Total cell lysates (TCL) were also Western blotted for Rac and Vav2 expression with Rac and T7 antibodies, respectively. (B) NIH3T3 cells were transfected with R425GVav2, fixed 24 h later, and stained for R425GVav2 expression with T7 antibody and for actin with rhodamine-phalloidin. The arrow indicates the transfected cell. (C) HEK293T cells were transfected with Vav2 and treated after 24 h with wortmannin (200 nM) for 1 h. Activation of endogenous Rac (PBD) was determined as described in Materials and methods. Total cell lysates were Western blotted for Rac and Vav2 expression using Rac and Vav2 antibodies, respectively. (D) NIH3T3 cells were transfected with Vav2, and after 24 h the cells were treated with wortmannin (200 nM) for 1 h and then fixed and stained for Vav2 expression with T7 antibody and for actin with rhodamine-phalloidin. These results are representative of three experiments.

podia or stress fibers in OncoVav1 transfected cells (Fig. 6 B). Thus, the L342R/L343SVav2 mutant could function as a dominant negative specific for Vav2.

Vav2 is not necessary for EGF- or PDGF-induced ruffling or Jnk activation

Tyrosine phosphorylation of Vav family members is important for activation in vitro and Vav1, Vav2, and Vav3 are tyrosine phosphorylated in response to EGF and PDGF stimulation (Margolis et al., 1992; Schuebel et al., 1996; Pandey et al., 2000). Therefore, we investigated whether Vav2 might be necessary for PDGF- or EGF-dependent activation of Rac. We first confirmed that PDGF and EGF stimulated tyrosine phosphorylation of Vav2. NIH3T3 or COS7 cells were treated with PDGF or EGF, respectively. Cells were lysed and endogenous Vav2 was immunoprecipitated and Western blotted for phosphotyrosine. PDGF and EGF stimulated tyrosine phosphorylation of Vav2, as has been described previously (Fig. 7 A; Schuebel et al., 1998; Pandey et al., 2000). To determine whether Vav2 was necessary for PDGF- or EGF-dependent lamellipodia, NIH3T3 or COS cells were transfected with L342R/L343SVav2, serum starved, and then treated with PDGF (15 ng/ml) or EGF (33 nM) for 15 min. The cells were then fixed and stained for L342R/L343SVav2 expression using the T7 antibody and for actin with rhodamine phalloidin. Expression of L342R/L343SVav2 did not affect the morphology of serum-starved cells and did not block either PDGF (Fig. 7 B, top) or EGF-dependent lamellipodia (Fig. 7 B, bottom). L342R/L343SVav2 did not inhibit EGF-dependent Jnk activation either (Fig. 7 C). These results indicate that Vav2 does not mediate EGF- or PDGF-dependent lamellipodia or EGF-dependent Jnk activation and demonstrate that L342R/L343SVav2 does not block all Rac-dependent functions. Growth factor stimulation did result in a perinuclear vesicular staining pattern of Vav2, which might be explained by internalization with growth factor receptors (Fig. 7 B).

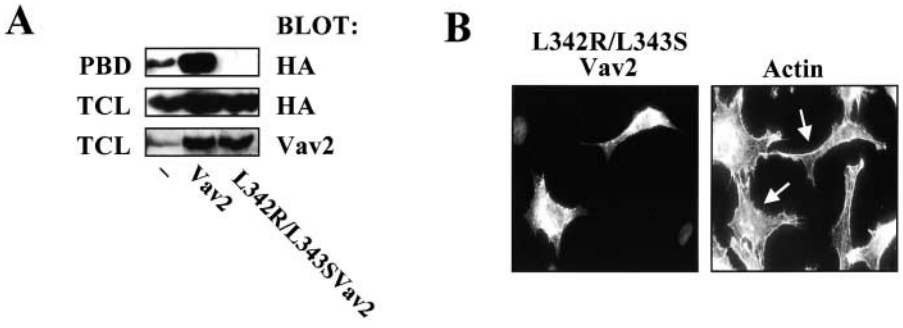

Figure 5. **Vav2 DH mutant lacks exchange activity.** (A) HEK293T cells were transfected with L342R/L343SVav2 and HA wild-type Rac, and 24 h later Rac activation was determined as described in Materials and methods using the PBD assay. Total cell lysates (TCL) were also Western blotted with HA and Vav2 antibodies to determine protein expression. (B) NIH3T3 cells were transfected with L342R/L343SVav2, fixed after 24 h, and stained for Vav2 expression with T7 antibody and for actin with rhodamine-phalloidin. The arrows indicate the transfected cells. These results are typical of four experiments.

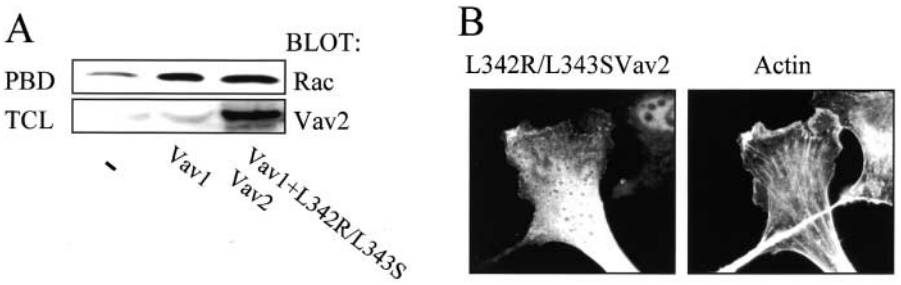

Figure 6. **L342R/L343SVav2 does not inhibit Vav1.** (A) HEK293T cells were transfected with OncoVav1 or OncoVav1 and L342R/L343SVav2 at a DNA ratio of 1:3 (Vav1/Vav2). 24 h later activation of endogenous Rac was determined as described in Materials and methods using the PBD assay. Total cell lysate (TCL) was also Western blotted with Vav2 antibody to determine protein expression. (B) NIH3T3 cells were transfected with OncoVav1 and L342R/L343SVav2 at a DNA ratio of 1:3 (Vav1/Vav2) and fixed 24 h later. The cells were stained for Vav2 expression with T7 antibody and with rhodamine-phalloidin. These results are representative of three experiments.

L342R/L343SVav2 mutant blocks cell spreading on fibronectin

Spreading of cells on fibronectin involves Cdc42-dependent filopodia formation followed by Rac-dependent lamellipodia and then Rho activation (Clark et al., 1998; Price et al., 1998). To determine whether Vav2 is necessary for cell spreading, NIH3T3 cells were transfected with L342R/L343SVav2 and 24 h later they were trypsinized, allowed to recover, and then plated onto fibronectin. At various time points the cells were fixed and stained for Vav2 expression and for actin. The number of cells that were unspread, partially spread, or fully spread was then determined. L342R/L343SVav2 significantly impaired the spreading of NIH3T3 cells on fibronectin, indicating that Vav2 is necessary for this process (Fig. 8 A).

To determine why cells expressing L342R/L343SVav2 failed to spread on fibronectin, we examined these cells using live-time microscopy. NIH3T3 cells were transfected with L342R/L343SVav2 and pEGFP to visualize the transfected cells by EGFP expression. The cells were trypsinized, rested, and plated onto fibronectin and images were captured at various time points. The normal sequence of cell spreading can be seen in untransfected cells in which cells extend filopodia, followed by lamellipodia (Fig. 8 B, white arrow). Cells transfected with L342R/L343SVav2 (Fig. 8 B, black arrows) failed to form lamellipodia or spread. Examination of L342R/L343SVav2-expressing cells at higher power by phase contrast of live cells or actin staining of fixed cells showed that filopodia did form and contained actin (Fig. 8 C). Untransfected cells formed filopodia, but also formed lamellipodia and spread (Fig. 8 C). These results suggest that Vav2 is necessary for lamellipodia formation and thus Rac activation in response to integrin signals.

Since tyrosine phosphorylation has been correlated with Vav family activity, we also determined whether plating of NIH3T3 cells on fibronectin induced tyrosine phosphorylation of Vav2. NIH3T3 cells were trypsinized, rested, and then plated onto fibronectin-coated plates. At the times after plating, Vav2 was immunoprecipitated and tyrosine phosphorylation of Vav2 and total cell lysate was assessed by Western blotting. We detected a decrease in tyrosine phosphorylation of Vav2 at early time points, followed by a return to basal levels (Fig. 8 D). Vav2 protein levels were similar for each time point (data not shown). Tyrosine phosphorylation of proteins in the total cell lysate increased in response to plating as expected.

Discussion

Vav2 is a ubiquitously expressed member of the Vav family of RhoGEFs, whose function is largely unknown. We determined the GTPases activated by Vav2 overexpression, the domains necessary for its activity and have used a dominant negative construct to investigate the function of Vav2. Several groups have shown that either NH_2-terminal truncation of Vav2 or coexpression of wild-type Vav2 with activated Lck was necessary for Vav2-dependent lamellipodia (Schuebel et al., 1998; Abe et al., 2000). In contrast, and similar to the results of Liu and Burridge (2000), we found that overexpression of wild-type Vav2 was sufficient to induce lamellipodia and activate Jnk. Using baculovirus-derived recombinant protein, Schuebel et al. (1998) found that Vav2 activates RhoA, RhoB, and RhoG, but not Rac1 or Cdc42, as determined by exchange assays in vitro. Abe et al. (2000) used truncated Vav2 produced in bacteria and found that it catalyzed exchange for Rac1, RhoA, and Cdc42 in vitro. Liu and Burridge (2000) found that Vav2 expressed in CHO cells activates Rac1, RhoA, and Cdc42 in vivo. We found that Vav2 activates Rac1 and, to a lesser extent, RhoA, but has a minimal effect on Cdc42 in HEK293T or NIH3T3 cells. The varying results from these studies likely reflect both differences between in vitro and in vivo assays and cell-specific effects that may influence the ability of Vav2 to activate particular GTPases in vivo, such as colocalization and GAP activity. Since Vav2 activates RhoG in vitro (Schuebel et al., 1998) and RhoG can activate both Cdc42 and Rac (Gauthier-Rouviere et al., 1998), we cannot rule out the possibility that the effects we detect are mediated by RhoG. However, the lack of Cdc42 activation in our experiments argues against this possibility. In contrast to both Schuebel et al. (1998) and Liu and Burridge (2000) we did not detect an increase in stress fibers in cells overexpressing Vav2. This could reflect either a difference in RhoGAP activity in the cell lines or the extent of Rho activation.

The tyrosine kinases Syk, Zap70, and Janus kinase likely phosphorylate and activate Vav1 in vivo, but the kinase(s) that stimulate Vav2 in vivo are not known. Inhibition of Vav2-dependent Rac exchange and lamellipodia by both KD Src and the Src inhibitor PP2 indicate that Src is necessary for the activity of overexpressed Vav2. Therefore, it is likely that Src, or a family member, is also necessary for the function of endogenous Vav2.

All RhoGEFs contain a PH domain immediately COOH-terminal to the DH domain. A mutation in the PH domain

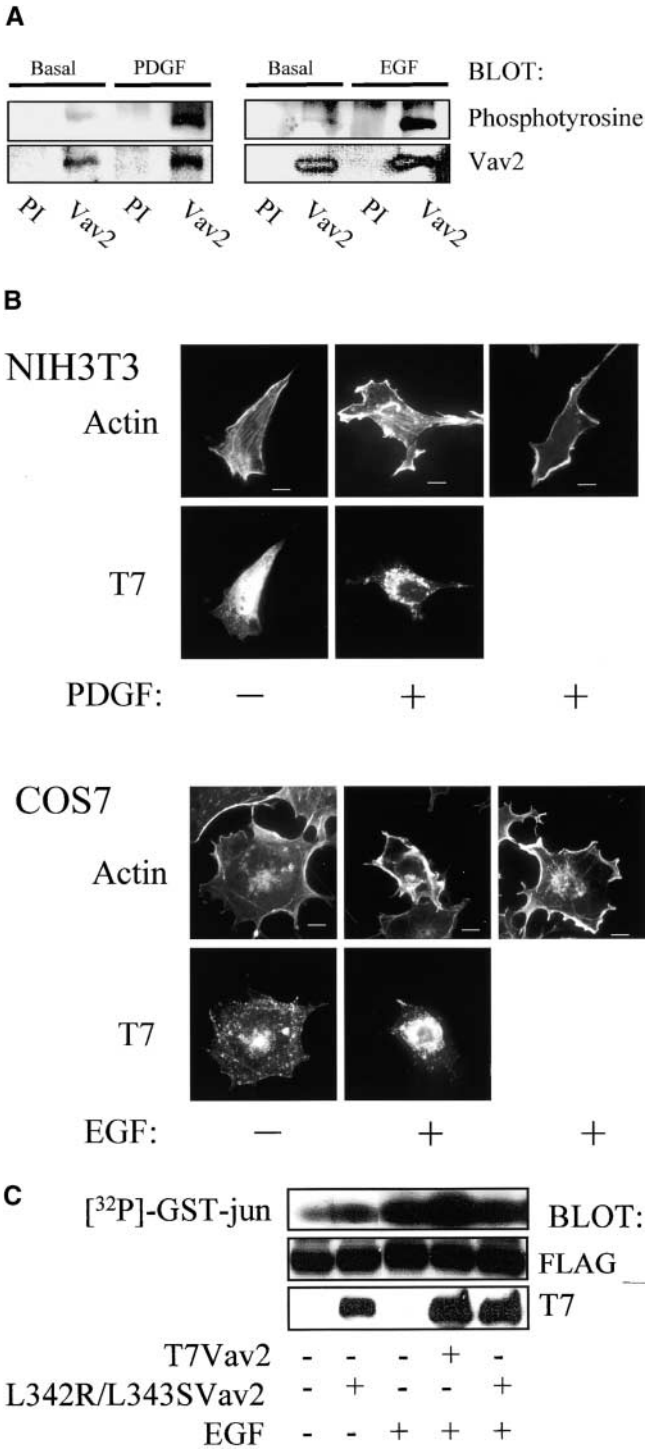

Figure 7. **Vav2 is not necessary for PDGF-dependent ruffling or EGF-dependent Jnk activation.** (A) NIH3T3 cells were stimulated with PDGF or COS7 cells were stimulated with EGF and immunoprecipitations were done with preimmune serum (PI) or Vav2 antibody (Vav2). The proteins were separated by SDS-PAGE and tyrosine phosphorylation of Vav2 was determined by Western blotting. (B) NIH3T3 or COS7 cells were transfected with L342R/L343SVav2, serum-starved, and then NIH3T3 cells were stimulated with PDGF (top) and COS7 cells were stimulated with EGF (bottom). Cells were stained for Vav2 expression with T7 antibody and with rhodamine-phalloidin. The left panels are quiescent cells expressing L342R/L343SVav2, the middle panels are growth factor–stimulated cells expressing L342R/L343SVav2, and the right panels are untransfected cells stimulated with growth factor. (C) COS7 cells were transfected with L342R/L343SVav2 and FLAG-Jnk. The cells were serum starved and stimulated with EGF and Jnk activity ([32P]GST-jun) was determined as described in Material and methods. Cell lysates were also western blotted with FLAG antibody to determined Jnk expression and with T7 antibody to determine Vav2 expression. These results are representative of four experiments. Bar = 10 μm.

of Vav2 predicted to disrupt phosphoinositide binding based both on the crystal structure of PH domains and the effects of a similar mutation in Vav1 (Ferguson et al., 1995; Hyvonen and Saraste, 1997; Han et al., 1998) lacks exchange activity, indicating that the PH domain of Vav2 is necessary for its function. This is in contrast to Vav1 and Vav3, where mutation of the PH domain does not affect exchange activity (Han et al., 1998; Movilla and Bustelo, 1999). Some exchange factors appear to require the PH domain to properly bind the GTP binding protein (Soisson et al., 1998), but the PH domain could be required to interact with phosphoinositides. However, we found that the PI-3 kinase is not necessary for Rac activation by overexpressed Vav2, in apparent contrast to Vav1, where wortmannin partially inhibits lamellipodia in cells cotransfected with Syk and Vav1 (Miranti et al., 1998). It is likely that PI-3 kinase products are not required for the activity of endogenous Vav2, but we cannot exclude the possibility that PI-3 kinase may further stimulate activated Vav2 or play a role in localization. Neither have we excluded the possibility that PtdIns-4,5-P_2 is necessary for Vav2 exchange activity or that a PH domain–dependent protein–protein interaction is required.

We mutated the DH domain of Vav2 to develop a construct that would allow us to investigate the function of endogenous Vav2 using a dominant negative approach. The R335 to G mutant retained exchange activity, but the double mutation of L342/L343 to R/S, respectively, eliminated Vav2 exchange activity for both Rac1 and RhoA. This mutant did not activate Jnk or induce lamellipodia, strongly indicating that the DH domain is necessary for these functions of Vav2. To determine if this mutant could function as a specific dominant negative, we determined whether it blocked Vav1-dependent nucleotide exchange for Rac or actin organization and found that it did not. These results indicate that the DH mutant of Vav2 does not sequester Rac and thereby block all Rac-dependent events.

Since Vav2 is tyrosine phosphorylated in response to EGF and PDGF stimulation, we thought that Vav2 might be necessary for stimulation of Rac and thus growth factor–induced lamellipodia and Jnk activation. However, L342R/L343SVav2 Vav2 did not block lamellipodia induced by either PDGF or EGF, indicating that Vav2 is not necessary for Rac activation by these growth factors. L342R/L343SVav2 did not block Jnk activation by EGF. Tyrosine phosphorylation of Vav2 in response to growth factor treatment may reflect activation of Vav2 for a function other than stimulation of lamellipodia or Jnk activation, or it could also be inhibitory.

Cell spreading requires activation of Cdc42 followed by Rac and then Rho (Price et al., 1998). L342R/L343SVav2 blocked the spreading of fibroblasts on fibronectin. Cells expressing L342R/L343SVav2 did not form lamellipodia, indicating that L342R/L343SVav2 acts as a dominant nega-

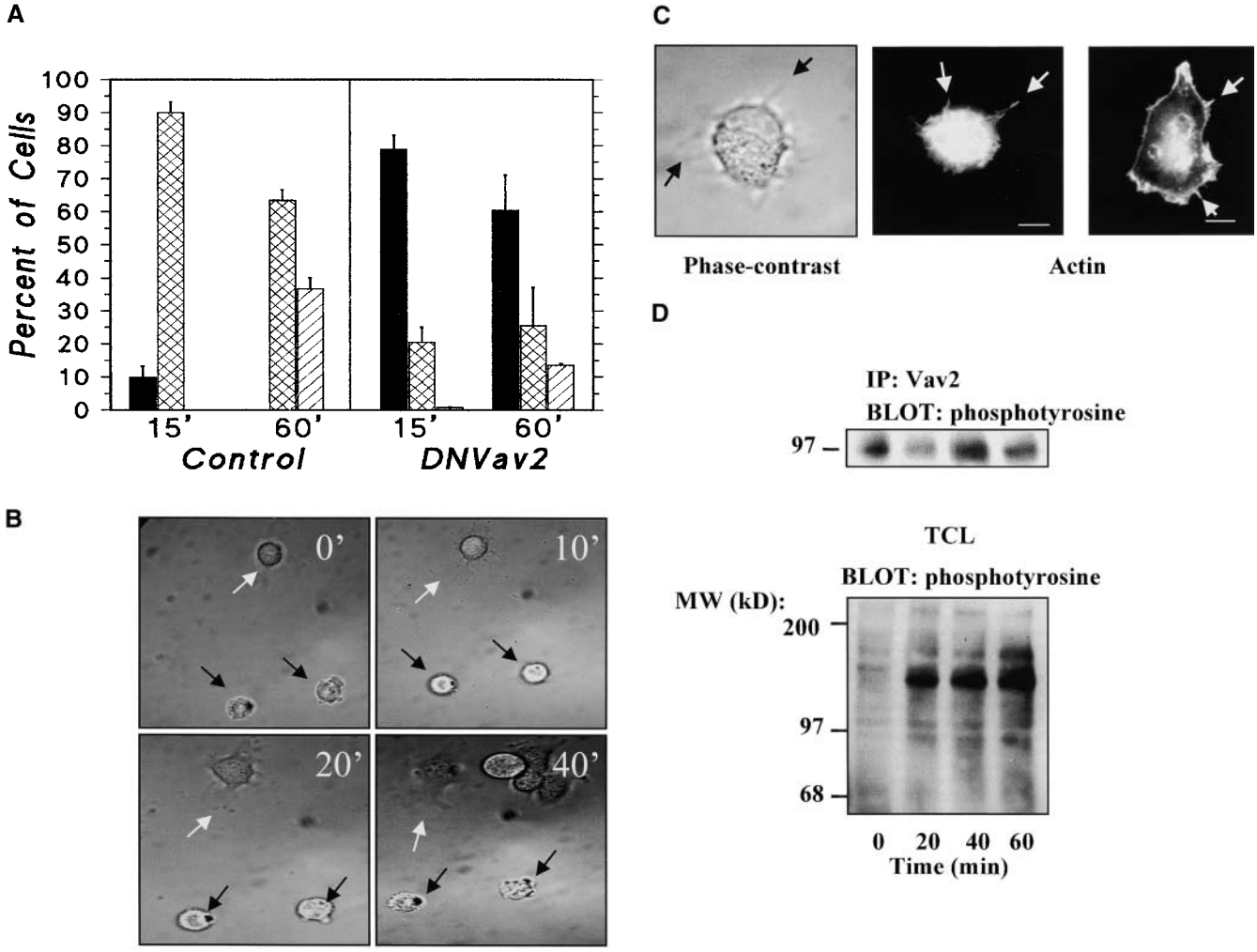

Figure 8. **Effect of L342R/L343SVav2 on cell spreading.** (A) NIH3T3 cells were transfected with vector alone or L342R/L343SVav2. 24 h after transfection they were trypsinized, allowed to recover, and then plated on fibronectin-coated coverslips. 15 or 60 min after plating the cells were fixed and stained for Vav2 expression with T7 antibody and with rhodamine-phalloidin. Cells were counted (100 per condition) and scored for spreading: unspread (black bar), partially spread (crossed bar), or fully spread (back-slash bar). Values are expressed as percent of total cells counted and represent the mean ± SEM of four experiments. (B) NIH3T3 cells were transfected with pEGFP and vector only, or L342R/L343SVav2 at a DNA ratio of 1:3 (pEGFP/Vav2 or vector). Cells were trypsinized 24 h after transfection, allowed to recover, and then plated on fibronectin. Cell spreading was monitored by phase contrast microscopy. Photographs were taken at 0, 10, 20, and 40 min (untransfected cell, white arrow; transfected cells, black arrows). These results are typical of 25 cells examined from 3 separate experiments. (C) NIH3T3 cells were transfected with pEGFP and L342R/L343SVav2, trypsinized 24 h later, allowed to recover, and then plated onto fibronectin-coated coverslips. Cells were examined for the presence of filopodia by phase contrast (left, black arrows indicate filopodia) or fixed and stained with rhodamine-phalloidin (middle). Untransfected cells were also plated and stained with rhodamine-phalloidin (right). (D) NIH3T3 cells were trypsinized, allowed to rest, then plated on fibronectin as described in Materials and methods. Cells were harvested at 0, 20, 40, and 60 min after plating, endogenous Vav2 was immunoprecipitated and tyrosine phosphorylation was determined by Western blotting. Total cell lysates (TCL) were also Western blotted for phosphotyrosine. These results are representative of three experiments. Bars, 10 μm.

tive to block Rac activation by integrins and thus that Vav2 is required for integrin activation of Rac during cell spreading. Since these cells form filopodia and we detected minimal activation of Cdc42 by Vav2, it is likely that another RhoGEF activates Cdc42 in response to integrin activation.

Since we find that Vav2 is necessary for cell spreading and Src activity is necessary for Vav2 activation of Rac and lamellipodia formation, our data suggest a model of Rac activation by integrins that depends on Src phosphorylation of Vav2. Src is known to be important in cell spreading, although some evidence indicates the kinase activity of Src may not be required (Kaplan et al., 1995). In studies in which Src kinase activity did not appear to be necessary for spreading, phosphorylation of focal adhesion proteins was increased, whereas in cells derived from the $Src^{-/-}/Yes^{-/-}/Fyn^{-/-}$ triple knock-out tyrosine phosphorylation of focal adhesion proteins is minimal (Klinghoffer et al., 1999). These results suggest that some level of Src family kinase activity is necessary for adherence and spreading, which could have been provided by Yes or Fyn in $Src^{-/-}$ cells. We did not detect an increase in tyrosine phosphorylation of Vav2 in response to plating of NIH3T3 cells on fibronectin, confirming the results of Liu and Burridge (2000) and Moores et al. (2000). This may reflect concomitant dephosphorylation of inhibitory sites and phosphorylation of sites that stimulate exchange activity, with little net change in total ty-

rosine phosphorylation. It is also possible that integrin-dependent activation of Vav2 is primarily due to cellular localization involving the PH or SH2 or SH3 domains. Based on the induction of lamellipodia in cells overexpressing Vav2, assays of GTPase activation in vivo and the ability of a dominant negative Vav2 to block lamellipodia formation in cells plated on fibronectin, we conclude that Vav2 activates Rac in vivo and is necessary for cell spreading.

Materials and methods

Plasmids

A Vav2 mammalian expression vector was constructed by subcloning the EcoRI-KpnI fragment of Vav2 in Bluescript (pBS) into EcoRI-KpnI–digested pCMV5. An oligonucleotide containing the sequence for the T7 epitope was designed with SpII-KpnI sites and ligated into SpII-KpnI–digested Vav2 in pCMV5 to provide a COOH-terminal epitope tag. A GST fusion protein containing the COOH terminus of Vav2 was constructed by subcloning the BglII-NotI fragment of Vav2 in pBS into pGEX4T2 (Amersham Pharmacia Biotech). Mutations in the DH and PH domains were generated in T7 epitope–tagged Vav2 in pCMV5 using the Transformer site–directed mutagenesis kit (CLONTECH Laboratories, Inc.). The L342R/L343S mutant was made by changing residue L342 (codon CTC) and L343 (codon TTG) to R (codon CGC) and S (codon TCG), respectively. The PH domain mutant (R425G) was made by mutating residue R425 (codon AGG) to G (codon GGG). The mutations were confirmed by sequencing the constructs. The GST fusion protein of p21 binding domain of PAK was obtained from Dr. Gary Bokoch (The Scripps Research Institute, La Jolla, CA) and the GST fusion protein of the RBD of rhotekin was obtained from Dr. Martin Schwartz (The Scripps Research Institute, La Jolla, CA). Vav1 was obtained from Dr. Stephen Burakoff (Dana-Farber Cancer Institute, Boston, MA) and Dbl was obtained from Dr. Richard Cerione (Cornell University, Ithaca, NY). Constitutively active mammalian Y527F Src was a gift from Dr. Paul Kaplan (Affymetrix, Inc., Santa Clara, CA). KD mammalian Src K295R/Y527F (KD Src) was a gift from Dr. Joan Brugge (Harvard Medical School, Boston, MA).

Antibodies

The Vav2 antiserum was raised in rabbits using the GST fusion protein of the Vav2 COOH terminus as the antigen. Sera did not recognize Vav1 or Vav3. HA (12CA5) antibody was purchased from Boehringer. T7 antibody was purchased from Novagen. FLAG (M2) monoclonal antibody was purchased from Sigma-Aldrich. Antiphosphotyrosine monoclonal antibody was a gift from Dr. Thomas Roberts (Dana Farber Cancer Institute, Boston, MA). Rac and Rho monoclonal antibodies were purchased from Transduction Laboratories.

Cell culture and transfections

NIH3T3 and COS7 cells were grown in DME containing 10% (vol/vol) calf serum. HEK293T cells were grown in DME with 10% (vol/vol) heat-inactivated fetal calf serum. Transient transfections were done using Superfect (QIAGEN) or Lipofectamine Plus Reagent (GIBCO BRL) according to the manufacturer's guidelines.

Cell stimulation and immunoprecipitation

PP2 was used at a concentration of 2 μM for 30 min. In experiments in which growth factor effects on tyrosine phosphorylation of Vav2 were determined, cells were serum starved for 16 h followed by treatment with PDGF (40 ng/ml) or EGF (33 nM) for 15 min. PDGF was used at a concentration of 15 ng/ml for immunofluorescence studies. Immunoprecipitations were done by first washing the cells in cold PBS and then lysing them in a buffer containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP40, 10 mM EDTA, 0.2 mM sodium orthovanadate, 10% glycerol, 4 μg/ml leupeptin, 4 μg/ml pepstatin, and 4 μg/ml AEBSF. Lysates were cleared by centrifugation and incubated with antibody and protein A–Sepharose beads for 3 h at 4°C. The beads were washed three times in lysis buffer and associated proteins were resolved by SDS-PAGE followed by transfer to Immobilon-P membrane (Millipore). Membranes were blocked with 2% (wt/vol) BSA in Tris-buffered saline. The blots were probed with primary antibody and then with horseradish peroxidase–conjugated secondary antibody (Boehringer) and visualized by enhanced chemiluminescence (Dupont) according to the manufacturer's instructions.

Microscopy

NIH3T3 cells on glass coverslips were fixed in 3% paraformaldehyde-PBS for 10 min, permeabilized in 0.2% Triton X-100 for 2 min, and blocked in 2% BSA-PBS for 10 min. The cells were incubated with primary antibodies for 1 h, washed, and then incubated with secondary antibodies (Jackson ImmunoResearch Laboratories) and rhodamine-phalloidin (100 ng/ml; Sigma-Aldrich) for 1 h. Coverslips were mounted onto slides with Fluoromount-G (Southern Biotechnology Associates, Inc.). Images of both live and fixed cells were acquired using a microscope (Diaphot 300; Nikon) mounted with a camera (SenSys CCD; Photometrics) and processed using Vaytek imaging software.

Jnk kinase assays

COS7 cells were transfected and then lysed 24 h later in a buffer containing 40 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 25 mM NaF, 1 mM sodium vanadate, 4 μg/ml aprotinin, and 4 μg/ml leupeptin. FLAG-Jnk was immunoprecipitated from cleared lysates with M2 antibody and protein G–sepharose beads for 1 h at 4°C. Beads were washed and kinase activity was assayed in 30 μl of kinase buffer (25 mM Hepes, pH 7.6, 20 mM $MgCl_2$, 20 mM β-glycerophosphate, 0.1 mM sodium vanadate, 2 mM DTT), 20 μM ATP, 5 μCi [γ-^{32}P]ATP, and 5 μg GST-*c*-jun. Reactions were incubated at 30°C for 30 min and terminated by the addition of 10 μl 5× Laemmli buffer (Ma et al., 1998). The proteins were separated by SDS-PAGE and phosphorylation quantified with a Molecular Imager (Bio-Rad Laboratories).

GTPase activity assays

GST fusion proteins of PBD (GST-PBD; Sander et al., 1998) or rhotekin (GST-RBD; Ren et al., 1999) were as described by (O'Connor et al., 2000). For Rac and Cdc42 activity assays, cells were lysed 24 h after transfection in a buffer containing 20 mM Hepes, pH 7.5, 100 mM NaCl, 0.5% NP-40, 10 mM $MgCl_2$, 10 mM β-glycerophosphate, 10% glycerol, 4 μg/ml leupeptin, and 4 μg/ml pepstatin (Sander et al., 1998). Lysates were cleared and incubated with GST-PBD (to assess Rac or Cdc42 activation) or GST-TRBD (to assess Rho activation) bound to glutathione agarose beads for 30 min at 4°C. For the Rho activity assay, lysates were diluted 1:1 in a buffer containing 50 mM Tris, pH 7.2, 500 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM $MgCl_2$, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM PMSF. The beads were washed three times in lysis buffer and analyzed for bound Rac, Cdc42 and Rho by Western blotting.

Cell spreading on fibronectin

Plates or coverslips were coated with fibronectin (10 μg/ml) for 2 h at 37°C. Cells were prepared for plating by trypsinization and then were washed once in DME with 1 mg/ml soybean trypsin inhibitor. Cells were pelleted, washed twice with DME, and then resuspended in DME and allowed to recover for 40 min at 37°C. Cells were then plated onto fibronectin-coated coverslips or plates. Cells were fixed at 15 and 60 min after plating and stained for Vav2 with T7 antibody followed by AMCA anti–mouse secondary antibody and rhodamine-phalloidin. Vav2-expressing cells were classified as unspread (adherent with no projections), partially spread (adherent with limited lamellipodia), and fully spread.

We thank Drs. David Kwiatkowski for the Vav2 cDNA, Benjamin Neel for critical reading of the manuscript, and Judith Glaven, Sheila Thomas, Kim Tolias, and Andy van Vugt for helpful discussions.

This work was supported by National Institutes of Health grant GM 54389 (CLC).

Submitted: 28 March 2001
Revised: 25 May 2001
Accepted: 30 May 2001

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