Vav GEFs are required for beta2 integrin-dependent functions of neutrophils

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Vav GEFs are required for β2 integrin-dependent functions of neutrophils

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Integrin regulation of neutrophils is essential for appropriate adhesion and transmigration into tissues. Vav proteins are Rho family guanine nucleotide exchange factors that become tyrosine phosphorylated in response to adhesion. Using Vav1/Vav3-deficient neutrophils (Vav1/3 ko), we show that Vav proteins are required for multiple β2 integrin-dependent functions, including sustained adhesion, spreading, and complement-mediated phagocytosis. These defects are not attributable to a lack of initial β2 activation as Vav1/3 ko neutrophils undergo chemotactant-induced arrest on intercellular adhesion molecule-1 under flow. Accordingly, in vivo, Vav1/3 ko leukocytes arrest on venular endothelium yet are unable to sustain adherence. Thus, Vav proteins are specifically required for stable adhesion. β2-induced activation of Cdc42, Rac1, and RhoA is defective in Vav1/3 ko neutrophils, and phosphorylation of Pyk2, paxillin, and Akt is also significantly reduced. In contrast, Vav proteins are largely dispensable for G protein-coupled receptor–induced signaling events and chemotaxis. Thus, Vav proteins play an essential role coupling β2 to Rho GTPases and regulating multiple integrin-induced events important in leukocyte adhesion and phagocytosis.

Introduction

Neutrophil recruitment from the blood is a central event in the innate immune responses to invading pathogens and tissue damage. Regulation of cellular adhesive activity by the leukocyte β2 integrins is an essential element of neutrophil recruitment. Circulating neutrophils maintain their integrins in a low affinity, nonadhesive state and, in response to local inflammatory stimuli, are rapidly activated to bind ligand, a process known as “inside-out” activation. Once arrested on venular endothelium, neutrophils must subsequently strengthen their adhesion to resist detachment by disruptive shear flow, while extravasating through the endothelial barrier for migration into target tissues and clearance of invading microbes by phagocytosis. Studies in mice have indicated that β2 integrins are required for both neutrophil recruitment (Lu et al., 1997) and cytotoxic functions such as complement-mediated phagocytosis (Coxon et al., 1996). The importance of integrin-mediated adhesion for proper leukocyte function is emphasized in leukocyte adhesion deficiency patients, who suffer from severe bacterial infections due to decreased β2 integrin expression and/or function (Bunting et al., 2002). Thus, integrins are critical mediators of neutrophil function and innate immunity; however, the molecular mechanisms by which integrins regulate adhesive events in neutrophils are largely undefined.

The Vav family of guanine nucleotide exchange factors (GEFs) activates Rho GTPases (Rho, RhoG, Rac, and Cdc42) by catalyzing the exchange of GDP for GTP. The three mammalian Vav proteins (Vav1, -2, -3) differ in their tissue distribution: Vav1 is expressed predominantly in hematopoietic cells, whereas Vav2 and Vav3 are more broadly expressed. Each Vav protein shares the same set of structural domains (calponin homology, Dbl homology, pleckstrin homology, cysteine-rich, and Src homology 2 and 3 [SH2, SH3] domains) and display overall 50–60% sequence homology (Bustelo, 2000). Studies of lymphocytes from mice lacking vav1, vav2, vav3, or combinations of these genes indicate

Abbreviations used in this paper: AAM, adhesion assay media; fMLP, formyl-Met-Leu-Phe; GEF, guanine nucleotide exchange factor; GPCR, G protein-coupled receptor; ICAM, intercellular adhesion molecule; IRR, immune response receptor; LTB₄, leukotriene B₄; MLC, myosin light chain; SFK, Src family kinase; TCR, T cell receptor.

The online version of this article contains supplemental material.

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that whereas certain functions of Vav proteins are redundant, individual Vav proteins also serve specialized functions in lymphocytes (Bustelo, 2000; Fujikawa et al., 2003). It has not been established which targets of individual Vav proteins mediate the specific functions in lymphocytes nor is it known which specific Rho family GTPases serve as direct substrates for each Vav family member in vivo.

The function of Vav proteins has been best characterized downstream of immune response receptors (IRRs). Targeted disruption of Vav1 results in severe impairment of T lymphocyte function in response to antigen receptor stimulation (Bustelo, 2000) and impaired FcεR-induced degranulation and cytokine production in mast cells (Manezet al., 2001). In particular, T cell receptor (TCR)-induced IL-2 production, proliferation, and calcium mobilization are impaired in vav1−/− T lymphocytes (Bustelo, 2000). Interestingly, actin cytoskeletal rearrangements such as TCR capping and actin patch formation are defective in vav1−/− lymphocytes (Fischer et al., 1998; Holsinger et al., 1998), suggesting Vav1 is specifically required to couple the TCR to the actin cytoskeleton. A double deficiency in Vav1/2 or Vav1/3 causes distinct phenotypic effects in lymphocytes, with Vav2 loss causing more severe effects in B cells and Vav3 loss further enhancing T cell defects (Doody et al., 2001; Tedford et al., 2001; Fujikawa et al., 2003). Deletion of all Vav family members prevents production of all mature B and T cells (Fujikawa et al., 2003). Therefore, Vav proteins serve critical functions downstream of IRR signaling in lymphocytes. However, the role of Vav proteins downstream of receptors other than IRRs has not been extensively examined.

The exchange activity of Vav proteins is regulated by phosphorylation of regulatory tyrosine residues in the amino terminus (Bustelo, 2000). In addition to IRRs, stimulation of receptor tyrosine kinases, cytokine receptors, and G protein-coupled receptors (GPCRs) induces tyrosine phosphorylation of Vav1, the best characterized family member (Bustelo, 2000). Thus, Vav proteins can couple to diverse signaling receptors and, due to their exchange activity and adaptor domains, are ideally suited to transduce signals to Rho GTPases and the actin cytoskeleton. Previous studies have implicated a kinase cascade involving the tyrosine kinases Src and Syk in vav1−/− integrin-induced Vav1 phosphorylation and cytoskeletal rearrangements (Miranti et al., 1998; Obergell et al., 2002). Inducible Vav1 phosphorylation in response to β1 and β2 integrin ligation has also been reported in myeloid cells (Gotoh et al., 1997), T cells (Yron et al., 1999), and platelets (Cichowski et al., 1996). In addition, overexpression studies have implicated Vav proteins in integrin-dependent cell spreading (Marignani and Carpenter, 2001; del Pozo et al., 2003). Thus, Vav proteins can be activated by integrins in multiple hematopoietic cell types and may regulate intracellular signaling pathways important for proper integrin function.

In this paper, we have examined the role of Vav proteins in β2 integrin- and GPCR-dependent biological functions of neutrophils using mice deficient in both Vav1 and Vav3. We show that Vav proteins are required for multiple β2 integrin-induced functions, including sustained adhesion, spreading, and complement-mediated phagocytosis, but are not essential for initial agonist-induced inside-out activation. β2 integrin-induced activation of Cdc42, Rac1, and RhoA is defective in the absence of Vav1 and Vav3. In addition, Pyk2 and paxillin phosphorylation, Akt activation, and phosphorylation of the Rho family GTPase effectors PAK and myosin light chain (MLC) are impaired in the absence of Vav proteins. The requirement for Vav is specific for integrin-dependent events, as GPCR-induced signaling pathways and chemotaxis are largely unaffected in Vav-deficient neutrophils. Thus, our work has defined a novel role for Vav proteins as critical regulators of integrin function in neutrophils.

**Results**

**Activation of Vav proteins in response to adhesion in primary neutrophils**

Vav GEFs can be activated by tyrosine phosphorylation via Syk and Src family kinases (SFKs) and previous work from our laboratory has implicated a signaling cascade involving these kinases in integrin-induced phosphorylation of Vav1 in αIIbβ3-reconstituted CHO cells (Miranti et al., 1998; Moores et al., 2000). To determine whether integrin engagement induces Vav phosphorylation in primary phagocytes, we first examined which Vav proteins are expressed in neutrophils. Using noncross-reactive antibodies against specific family members, all three Vav proteins were detected in lysates of primary mouse neutrophils, with Vav1 and Vav3 in greatest abundance (Fig. 1 A). Quantitation based on the relative affinities of the Vav-specific antisera indicated that the relative abundance of the isoforms is Vav3 > Vav1 and Vav1/3. Because integrin receptors on resting neutrophils are in a low affinity state and require signals from proinflammatory stimuli to induce activation and adhesion to their ligands, cells were treated with TNFα to induce integrin receptor adhesive activity. TNFα stimulation in suspension did not result in phosphorylation of Vav proteins (Fig. 1 B). However, upon adhesion to C3bi, all three Vav family members became inducibly tyrosine phosphorylated

![Figure 1. Expression and β2 integrin-induced activation of Vav proteins in primary mouse neutrophils.](image-url)
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(50x253) contrast to reported findings for their ability to adhere and spread on two

C3bi (Fig. 2 A) and ICAM-1 (not depicted) in response to formyl

neutrophil functions,

To examine the role of Vav proteins in integrin-dependent

spreading of WT neutrophils–maximum spreading of Vav1/3ko

neutrophils occurred after 30 min of stimulation ver-

30 min of stimulation revealed that Vav1/3ko neutrophils are indeed able to

undergo an initial adhesion to the ligand-coated surfaces, suggesting that the integrin receptors are competent to bind ligand. In addition, unstimulated wild-type (WT) neutrophils displayed a low level of adhesion on polyRGD (Fig. 2 B), most likely due to the direct cross-linking of integrins by the high valency of this ligand, which allows adhesion in the absence of additional stimuli (Mocsai et al., 2002). However, adhesion of unstimulated Vav1/3ko neutrophils to polyRGD was significantly reduced (Fig. 2 B), suggesting that Vav is required for signals downstream of direct integrin cross-linking. Furthermore, although treatment of Vav1/3ko cells with phorbol ester (PMA), which can bypass upstream signaling events and induce inside-out integrin activation, could rescue the spreading defect of Vav1/3ko neutrophils, this effect was significantly delayed relative to PMA-induced spreading of WT neutrophils–maximum spreading of Vav1/3ko neutrophils occurred after ~30 min of stimulation versus 5 min in WT neutrophils (unpublished data). Therefore, this rescue may be a secondary effect of PMA stimulation and not integrin activation per se.

To investigate inside-out activation, we examined the distribution of β2 integrins in suspended neutrophils stimulated with fMLP. First, αMβ2 was constitutively clustered in unstimulated WT neutrophils and was not notably altered by fMLP stimulation. In contrast, αMβ2 was uniformly distributed in unstimulated WT neutrophils; however, we were able to detect clustering in response to fMLP in only a very small percentage of cells (unpublished data). Therefore, chemotactant-induced, ligand-independent β2 integrin clustering does not seem to significantly contribute to neutrophil adhesion under the conditions examined in this work.

Neutrophils rolling on selectins in vitro and in vivo rapidly arrest in response to proinflammatory stimuli by binding to ICAMs such as ICAM-1 via inside-out activation of β2 integrins. To address whether the phenotype of Vav1/3ko neutrophils was attributable to reduced initial adhesion due to defective inside-out β2 integrin activation, we compared the ability of WT and Vav1/3ko neutrophils to arrest on coimmobilized P-selectin/ICAM-1 in response to LTB4 under low shear (1 dyne/cm²) in vitro. LTB4-mediated neutrophil arrest on ICAM-1 requires β2 integrin function, as β2−/− neutrophils did not arrest in response to this stimulus (unpublished data). Vav1/3ko neutrophils rapidly arrested on

(Fig. 1 B), indicating that all three Vav proteins are activated in response to αMβ2 integrin ligation in neutrophils.

Vav proteins are required for spreading and sustained adhesion

To examine the role of Vav proteins in integrin-dependent neutrophil functions, vav1−/− (Vav1ko), vav3−/− (Vav3ko) and vav1−/−vav3−/− (Vav1/3ko) neutrophils were analyzed for their ability to adhere and spread on two β2 integrin ligands, C3bi and intercellular adhesion molecule (ICAM)-1 (αMβ2 and αLβ2 ligand), in response to multiple stimuli. In contrast to reported findings for vav1−/− T lymphocytes (Krawczyk et al., 2002; Ardonin et al., 2003), neutrophils deficient in either Vav1 alone or Vav3 alone exhibited no defects in spreading (Fig. 2 A and not depicted). However, Vav1/3ko neutrophils were severely defective in spreading on C3bi (Fig. 2 A) and ICAM-1 (not depicted) in response to formyl-Met-Leu-Phe (fMLP) and leukotriene B4 (LTB4; not depicted), and TNFα (Fig. 2 A). Neutrophil spreading on the synthetic integrin ligand polyRGD was also impaired (not depicted). The defect in spreading was not merely due to a delay in cell spreading because Vav1/3ko neutrophils did not spread even after prolonged incubation in the presence of agonist (unpublished data). This spreading defect was associated with a significant reduction in the strength of adhesion, as fewer Vav1/3ko neutrophils remained adherent on the ligand-coated surfaces after repeated washes (Fig. 2 B). The reduced adhesion was not attributable to decreased integrin expression or impaired integrin receptor mobilization to the cell surface as we did not detect any differences in the level of αM or β2 integrins in resting neutrophils and saw a similar increase after agonist stimulation based on FACS analysis (unpublished data). Furthermore, neutrophils deficient in individual Vav proteins did not exhibit reduced adhesive strength over a range of ICAM-1-coating concentrations (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200404166/DC1). Thus, Vav proteins may be functionally redundant in neutrophils and are required for sustained adhesion and spreading in vitro.

Proinflammatory stimuli transduce “inside-out” signals that modulate the affinity and avidity of integrins toward their ligands by inducing a high affinity conformational state as well as lateral integrin mobilization/clustering. Once engaged, integrins transduce “outside-in” signals that mediate coupling to the actin cytoskeleton and the generation of an adhesive force. Visual observation of neutrophils after stimulation revealed that Vav1/3ko neutrophils are indeed able to undergo an initial adhesion to the ligand-coated surfaces, suggesting that the integrin receptors are competent to bind ligand. In addition, unstimulated wild-type (WT) neutrophils displayed a low level of adhesion on polyRGD (Fig. 2 B), most likely due to the direct cross-linking of integrins by the high valency of this ligand, which allows adhesion in the absence of additional stimuli (Mocsai et al., 2002). However, adhesion of unstimulated Vav1/3ko neutrophils to polyRGD was significantly reduced (Fig. 2 B), suggesting that Vav is required for signals downstream of direct integrin cross-linking. Furthermore, although treatment of Vav1/3ko cells with phorbol ester (PMA), which can bypass upstream signaling events and induce inside-out integrin activation, could rescue the spreading defect of Vav1/3ko neutrophils, this effect was significantly delayed relative to PMA-induced spreading of WT neutrophils–maximum spreading of Vav1/3ko neutrophils occurred after ~30 min of stimulation versus 5 min in WT neutrophils (unpublished data). Therefore, this rescue may be a secondary effect of PMA stimulation and not integrin activation per se.

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ICAM-1 in response to LTB₄, followed by infusion of 100 nM LTB₄ (Fbgn), C3bi, and ICAM-1 was induced with 1 mM Mn²⁺. ICAM-1 in response to LTB₄ indistinguishably from WT and Vav1/3 ko neutrophils adhered to immobilized fibrinogen, C3bi and ICAM-1 in response to manganese treatment (Fig. 3 B), indicating that Vav deficiency does not affect the intrinsic ability of β₂ integrins to bind ligand.

In vivo, circulating leukocytes survey the environment for proinflammatory stimuli by selectin-mediated rolling on the endothelium. At sites of tissue inflammation, rolling leukocytes are quickly converted to firmly adherent leukocytes through β₂ integrin-dependent attachment to ICAM-1, a major cell adhesion molecule on endothelial cells mediating chemoattractant-induced leukocyte arrest in vivo (Foy and Ley, 2000). To directly determine whether neutrophil adhesion was affected in vivo, leukocyte adhesion was induced in WT and Vav1/3 ko neutrophils using serum-opsonized fluorescent E. coli, which binds to α₅β₁. To examine whether Vav is required for this process, we compared the phagocytic activity of WT and Vav-deficient neutrophils using serum-opsonized fluorescent E. coli. Although there was no detectable defect in Vav1 ko or Vav3 ko neutrophils (not depicted), phagocytosis was severely impaired in Vav1/3 ko neutrophils (Fig. 4 A and B). PMA pretreatment was able to only partially rescue this defect in that Vav1/3 ko cells contained significantly fewer fluorescent bacteria per neutrophil (Fig. 4 A). The cells were imaged in the presence of trypan blue to quench any extracellular, unphagocytosed bacteria, as demonstrated by the absence of signal in cells incubated with unopsonized E. coli (Fig. 4 A). To determine whether the impaired phagocytic activity of Vav1/3 ko neutrophils was due to reduced particle binding, neutrophils were incubated with serum-opsonized fluorescent E. coli in the presence of the actin polymerization inhibitor, Latrunculin B, which blocks particle ingestion (Fig. 4 A) but not binding. A similar percentage of WT and Vav1/3 ko neutrophils contained bound E. coli (76% WT vs. 81% Vav1/3 ko) and the amount of bound E. coli per cell was also comparable (Fig. 4 C). Thus, the defect in complement-mediated phagocytosis of Vav1/3 ko neutrophils is not attributable to decreased particle binding.

**Defective complement-mediated phagocytosis in Vav1/3 ko neutrophils**
Neutrophils are professional phagocytes that efficiently bind and clear foreign particles. Serum opsonization leads to efficient fixation of complement C3bi on the surface of bacteria and renders them susceptible to complement-mediated phagocytosis via α₅β₁. To examine whether Vav is required for this process, we compared the phagocytic activity of WT and Vav-deficient neutrophils using serum-opsonized fluorescent E. coli. Although there was no detectable defect in Vav1 ko or Vav3 ko neutrophils (not depicted), phagocytosis was severely impaired in Vav1/3 ko neutrophils (Fig. 4 A and B). PMA pretreatment was able to only partially rescue this defect in that Vav1/3 ko cells contained significantly fewer fluorescent bacteria per neutrophil (Fig. 4 A). The cells were imaged in the presence of trypan blue to quench any extracellular, unphagocytosed bacteria, as demonstrated by the absence of signal in cells incubated with unopsonized E. coli (Fig. 4 A). To determine whether the impaired phagocytic activity of Vav1/3 ko neutrophils was due to reduced particle binding, neutrophils were incubated with serum-opsonized fluorescent E. coli in the presence of the actin polymerization inhibitor, Latrunculin B, which blocks particle ingestion (Fig. 4 A) but not binding. A similar percentage of WT and Vav1/3 ko neutrophils contained bound E. coli (76% WT vs. 81% Vav1/3 ko) and the amount of bound E. coli per cell was also comparable (Fig. 4 C). Thus, the defect in complement-mediated phagocytosis of Vav1/3 ko neutrophils is not attributable to decreased particle binding.

**Multiple β₂ integrin-dependent signaling pathways are affected in Vav1/3 ko neutrophils**
Vav family proteins have multiple structural domains in addition to the catalytic Dbl homology domain that mediates...
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GEF activity. To examine Vav-dependent signaling pathways downstream of integrin ligation, WT and Vav1/3 ko neutrophils were primed in suspension with TNFα and adhered to C3bi. Adhesion-induced tyrosine phosphorylation was significantly reduced in lysates from Vav1/3 ko neutrophils (Fig. 5 A). Because two of the tyrosine phosphorylated bands displayed the same mobility as the focal adhesion-associated proteins Pyk2 and paxillin, we directly examined the phosphorylation status of these proteins using phospho-specific antibodies. In accordance with the phosphotyrosine profile, the phosphorylation of both Pyk2 and paxillin was significantly reduced in Vav1/3 ko neutrophils (Fig. 5 B), but not in Vav1 ko or Vav3 ko neutrophils (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200404166/DC1). Integrin ligation results in the accumulation of 3-phosphorylated phosphoinositides, catalyzed by the enzyme PI 3-kinase (King et al., 1997). Activation of the S/T kinase Akt requires 3-phosphorylated phosphoinositides and can therefore be used as a read out for PI 3-kinase activity. Adhesion-induced activation of PI 3-kinase/Akt was also decreased in Vav1/3 ko neutrophils, but not singly deficient neutrophils (Fig. 5 B and Fig. S2). In contrast, β2 integrin-induced activation of SFKs in Vav1/3 ko neutrophils was comparable to WT (Fig. 5 B), indicating that signaling pathways upstream and independent of Vav remain intact.

It is well documented that Rho family GTPases play an important role in integrin-mediated adhesion, spreading, and migration by regulating the formation and maturation of focal adhesions and of actin-based structures (Ridley, 1999). Vav GEFs can induce activation of multiple members of the Rho family when overexpressed in fibroblasts (Bustelo, 2000) and reduced Rac-GTP levels have been observed in vav1 /vav3 thymocytes after TCR stimulation, implicating endogenous Vav proteins as Rac GEFs in T cells (Reynolds et al., 2002). Therefore, we examined the activation status of Rho GTPases after integrin engagement by affinity isolating the GTP-bound form using GST-PBD (p21-binding domain of PAK) or GST-RBD (Rho binding domain of rhotekin). Adhesion onto C3bi induced the activation of Cdc42, Rac1, and RhoA was analyzed by GST-PBD and GST-RBD pull-down. Activation of the downstream effectors PAKα and MLC was analyzed using pS19/8/8203 and pS19 antibodies, respectively.

Figure 5. Multiple β2 integrin-activated signaling pathways are affected in Vav1/3 ko neutrophils. WT (+/+) or Vav1/3 ko (−/−) neutrophils were primed with TNFα in suspension (S) and plated (P) on C3bi for the indicated time (min). Lysates were analyzed for total phosphotyrosine (A); pY881 Pyk2, pY118 Paxillin, pS473 Akt, and pY416 Src (B). (C) Adhesion-induced activation of the Rho GTPases Cdc42, Rac1, and RhoA was analyzed by GST-PBD and GST-RBD pull-down. Activation of the downstream effectors PAKα and MLC was analyzed using pS19/8/8203 and pS19 antibodies, respectively.
Vav proteins did not exhibit reduced phosphorylation of PAK or MLC (Fig. S2), indicating that individual Vav proteins are functionally redundant in neutrophils. Thus, β2 integrin-dependent activation of Rac1, Cdc42, and RhoA and downstream effectors of these GTPases is dependent on Vav1 and Vav3 in neutrophils.

Vav proteins are not required for chemotaxis

One of the first steps of directional motility is the formation of a leading edge which is stabilized by integrin attachments to the substratum. Furthermore, the creation and turnover of these integrin-based focal adhesions is a tightly coordinated process during cell migration (Webb et al., 2002). Given the defects in spreading and firm adhesion in Vav1/3ko neutrophils, we examined fMLP-dependent chemotaxis of these cells in vitro using transwell filters coated with integrin ligands. Surprisingly, we observed no defects in chemotaxis of Vav1/3ko neutrophils over a range of chemotactic concentrations on either C3bi- or fibronectin-coated transwell filters (Fig. 6 A). To specifically examine whether Vav1/3ko neutrophils exhibited altered cell polarization, speed, or direction of migration, we monitored chemotaxis by video microscopy using Zigmond chambers (Zigmond, 1988) coated with C3bi. Similar to the transwell assays, Vav1/3ko neutrophils migrated efficiently in the direction of the gradient (Fig. 6 B; Videos 1 and 2, available at http://www.jcb.org/cgi/content/full/jcb.200404166/DC1). Furthermore, analysis of the migration tracks of these cells revealed that similar percentages of both WT and Vav1/3ko neutrophils polarized and migrated up the chemotactic gradient of fMLP (Fig. 6 C, 94% vs. 92%). Notably, Vav1/3ko neutrophils on average migrated slightly faster than WT cells and traversed a longer distance (Fig. 6 D). Similar results were obtained with LTB4 (not depicted). Thus, despite significant β2 integrin adhesion defects, Vav1/3ko neutrophils are able to efficiently polarize and migrate in response to a chemotactic gradient.

**Activation of multiple GPCR-dependent signaling pathways in Vav1/3ko neutrophils**

Because Vav1 can couple to diverse classes of signaling receptors, it is possible that Vav proteins may also be required for integrin-independent signaling pathways in neutrophils. Many neutrophil chemoattractants are GPCRs agonists and require Rac activity to mediate multiple biological effects (Roberts et al., 1999; Glogauer et al., 2003). Therefore, we examined whether GPCR-induced signaling pathways were affected in Vav1/3ko neutrophils in response to fMLP and LTB4. fMLP stimulation induced a robust activation of Rac1, Rac2, and Cdc42 in Vav1/3ko neutrophils that was indistinguishable from WT (Fig. 7 A). Similarly, activation of PAK, Akt, and Erk1/2 occurred normally in Vav1/3ko neutrophils stimulated with fMLP (Fig. 7 B) or LTB4 (not depicted). We were unable to detect activation of RhoA in WT neutrophils in response to fMLP (not depicted). These data suggest that Vav proteins are dispensable for many adhesion-independent signaling events in mouse neutrophils.

**Discussion**

In this paper, we demonstrate that Vav GEFs are required for multiple β2 integrin-dependent functions of neutrophils. Although all three Vav proteins are expressed in neutrophils, Vav1 and Vav3 account for ~99% of total Vav protein. Deficiency in a single family member does not lead to any detectable neutrophil dysfunction; however, a combined deficiency in Vav1 and Vav3 results in defective chemoattractant-induced sustained adhesion and integrin-mediated phagocytosis. Adhesion assays under flow and intravital microscopy of the cremaster muscle revealed that chemoattractant-induced arrest is unaffected in Vav1/3ko neutrophils, indicating that the adhesion defect is likely not due to a loss in initial inside-out integrin activation. β2 integrin-dependent activation of multiple Rho GTPases—Rac1, Cdc42, and RhoA—is impaired in the absence of

Figure 6. In vitro chemotaxis of Vav1/3ko neutrophils. (A) Chemotaxis of WT (white bars) and Vav1/3ko (black bars) in response to fMLP on C3bi- or fibronectin (FN)-coated transwell filters. Values are the mean percentage of cells migrated ± SEM. (B) Video microscopy of neutrophils plated on C3bi-coated Zigmond chambers. fMLP increases in concentration from left to right. Arrows indicate the migration of a particular neutrophil. See online supplemental material to view videos. (C) The migration paths of cells from B were tracked and the percentage of cells that underwent a net positive displacement in the direction of the gradient is indicated. (D) The average velocity and distance migrated were quantified with Metamorph. Values are mean ± SEM of 50 cell tracks.

Vav1/3ko neutrophils did not exhibit reduced phosphorylation of PAK or MLC (Fig. S2), indicating that individual Vav proteins are functionally redundant in neutrophils. Thus, β2 integrin-dependent activation of Rac1, Cdc42, and RhoA and downstream effectors of these GTPases is dependent on Vav1 and Vav3 in neutrophils.
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Impaired integrin-mediated adhesion has also been reported in Vav1-deficient T lymphocytes (Krawczyk et al., 2002; Ardouin et al., 2003). This conclusion was based on defects in static adhesion and in α5β1 clustering on the cell surface in response to TCR stimulation. Because Vav1 plays a critical role in transducing early TCR signals required for most TCR-induced biological activities, including actin polymerization and activation of many downstream effectors of integrins (Bustelo, 2000), the specific effects of Vav deficiency on adhesion and integrin activation per se are difficult to distinguish from those required to activate early receptor-proximal events. In neutrophils, Vav is not required for fMLP- or LTB4-induced chemotaxis, initial adhesion, or activation of Rac, Cdc42, Erk, or Akt. Therefore, loss of Vav does not have broad effects on early receptor-induced events in chemoattractant-stimulated neutrophils as in TCR-stimulated T cells, and our data supports the possibility that Vav specifically regulates integrin-mediated adhesion events.

Vav requirement for integrin-dependent Rho GTPase activation
Rho GTPases are key mediators of actin cytoskeletal rearrangements associated with adhesion and spreading as they nucleate events involved in formation of focal adhesions, lamellipodia, and filopodia (Ridley, 1999). However, the mechanisms responsible for integrin-mediated Rho GTPase activation are poorly defined. Here, we show that β2 integrin-dependent activation of Rac1, Cdc42, and RhoA is defective in Vav1/Vav3 deficient neutrophils, implicating Vav proteins as critical Rho family GEFs downstream of integrins. The loss of integrin-induced activation of RhoA, Rac1, and Cdc42 in Vav1/Vav3 deficient neutrophils could be a consequence of direct effects of Vav-mediated GTP exchange on multiple Rho family members or from indirect Vav regulation of multiple Rho GTPases, for example, due to a cascade of Rho GTPase activation or to generalized effects of Vav deficiency on adhesion and spreading. Vav family proteins have been reported to serve as GEFs for RhoA, RhoG, Rac, and Cdc42; however, the specificity of individual Vav proteins has been difficult to establish for many reasons: lack of consistent results from in vitro experiments in different laboratories, use of distinct domains of Vav for in vitro exchange assays, and complications of interpreting experiments in cultured cells in which distinct Vav proteins have been overexpressed (Bustelo, 2000). Thus, it is not feasible to distinguish, based on previous studies, whether the phenotypic effects observed here are due to direct or indirect effects of Vav on these GTPases. Studies from Olson and coworkers demonstrated that a mutant, constitutively activated variant of Vav1 can independently activate Rho, Rac, and Cdc42 in Swiss 3T3 cells (Olson et al., 1996); however, one cannot extrapolate these findings to the endogenous Rho GTPases activated by integrins in neutrophils. There are also precedents for a hierarchical cascade linking Rho GTPases (Ridley and Hall, 1992; Nobes and Hall, 1995; Price et al., 1998), yet coupling between these proteins has not been examined in neutrophils after attachment to integrin ligands. Thus, further studies will be required to establish the basis for loss of activation of RhoA, Rac1, and Cdc42 in Vav1/Vav3 deficient neutrophils.

Vav regulation of integrin-mediated adhesion
One critical aspect of leukocyte adhesion in response to inflammatory stimuli is the induction of integrin binding activity through inside-out signaling events that convert integrins from a low to a high affinity/avidity ligand-binding state. Our evidence that Vav1/Vav3 deficient neutrophils are able to undergo chemoattractant-induced arrest under flow in vitro and in circulating blood in vivo suggests that Vav is not required for initial events associated with inside-out integrin activation. However, given the complexity of this response (Ley, 2003), we cannot rule out that Vav contributes to full activation of integrins, thus affecting the strength and maintenance of adhesion.
Regardless of whether the loss of RhoA, Rac1, and Cdc42 activation is a direct or indirect result of Vav deficiency, impaired Rho GTPase activation may be the major contributing factor in the phenotypes exhibited in Vav1/3−/− neutrophils. As previously discussed, Vav1/3−/− neutrophils are able to make initial contacts with immobilized integrin ligands, yet these neutrophils are unable to spread. Although the precise downstream intracellular events that coordinate cell spreading are poorly understood, a role for Rac and Cdc42 has been well documented in hematopoietic and nonhematopoietic cells (Allen et al., 1997; Clark et al., 1998; D’Souza-Schorey et al., 1998). In addition, Rac1- and Rac2-deficient neutrophils exhibit defects in integrin-mediated cell spreading (Roberts et al., 1999; Glogauer et al., 2003). In contrast, Rho has been implicated in events required for integrin-mediated firm adhesion and aggregation in lymphocytes and platelets (Morić et al., 1992; Laudanna et al., 1996; Woodside et al., 1998), but not for cell spreading (Ren et al., 1999). Interestingly, Rho, but not Rac or Cdc42, has been shown to be required for complement-mediated phagocytosis in macrophages (Caron and Hall, 1998), suggesting that loss of Rho activation contributes to the defect in phagocytosis in Vav1/3−/− neutrophils. Thus, failure to activate RhoA, Rac1, and Cdc42 may differentially contribute to the loss of distinct integrin-mediated events in neutrophils.

**Phenotypic similarities with Syk- and SFK-deficient neutrophils**

In both platelets (Obergfell et al., 2002) and neutrophils (Mocsai et al., 2002), Syk is required for adhesion-induced Vav phosphorylation, implicating Syk as an upstream regulator of Vav. Not surprisingly, Syk- and Vav-deficient neutrophils display multiple phenotypic similarities, including impaired spreading on multiple integrin ligands and reduced Pyk2 activation (Mocsai et al., 2002). Moreover, despite impaired integrin function, SFK-, Syk-, and Vav-deficient neutrophils do not exhibit defects in chemotaxis. The requirement for Syk in neutrophils is limited to integrin-dependent functions, as GPCR-mediated responses are unaffected in syk−/− (Mocsai et al., 2003). This evidence, coupled with the data in this paper, suggests that Vav proteins are critical downstream mediators of integrin-Syk signaling in neutrophils.

**Vav1/3−/− neutrophil migration in vitro and in vivo**

Neutrophils polarize in response to shallow chemotactic gradients by extending a leading edge lamellipodium that is stabilized via integrin-mediated adhesion to the substratum. The evidence that β2 integrins are required for leukocyte migration in multiple mouse inflammatory model systems (Mizgerd et al., 1997; Walzog et al., 1999) demonstrates the importance of these receptors for optimal migration in vivo. Surprisingly, despite significant adhesion defects, Vav1/3−/− neutrophils efficiently polarize and migrate on C3bi-coated glass in response to a chemotactic gradient of either fMLP or LTB4. In comparison to WT, Vav1/3−/− neutrophils on average migrate slightly faster, most likely due to their decreased adhesiveness. These data, coupled with the phenotypes of syk−/− and sfk−/− neutrophils, indicate that optimal integrin function is not required for the amoeboid movement of neutrophils in chemotactic models in vitro. It is possible that the minimal adhesion that occurs in Vav1/3−/− neutrophils is sufficient to mediate this form of chemotaxis in vitro. However, in vivo leukocytes must survey the environment by rolling on venular endothelium and arrest at sites of tissue inflammation firmly enough to withstand the constant shear force from blood flow. Intravital microscopy of the cremaster muscle indicates that Vav1/3−/− leukocytes are not as stably attached as WT to the venular endothelium in response to fMLP. Despite this difference, we have observed similar levels of neutrophil extravasation to the peritoneum in WT and Vav1/3−/− mice treated with thioglycollate in preliminary experiments (unpublished data). Similar results have been reported for syk−/− and sfk−/− mice (Mocsai et al., 2002); however Rac1- and Rac2-deficient mice display defects in chemotaxis in vitro and inflammatory recruitment in vivo (Roberts et al., 1999; Li et al., 2002; Glogauer et al., 2003). Although it is difficult to resolve this discrepancy, these studies suggest that minimal integrin activity may suffice for both in vivo and in vitro neutrophil migration. Alternatively, thioglycollate, an extremely potent inflammatory stimulus, may bypass the need for Syk and Vav signaling in neutrophils, analogous to phorbol ester stimulation in vitro. Therefore, alternative in vivo models are required to resolve this discrepancy.

**Vav proteins in GPCR signaling**

Binding of GPCR agonists to their cognate receptors initiates signaling cascades leading to the activation of Rho GTPases, presumably via activation of a Rho exchange factor. Specifically, Rac2 activity is required for multiple GPCR-induced events in neutrophils, including chemotaxis to fMLP and LTB4 (Roberts et al., 1999). The fact that Vav1/3−/− neutrophils undergo normal chemotaxis in response to these agonists suggests that Vav1 and Vav3 are not critical mediators of GPCR signaling. In addition, GPCR-induced calcium flux, integrin up-regulation, and initial inside-out integrin activation are unaffected in Vav1/3−/− neutrophils. fMLP- and LTB4-induced activation of Rho GTPases, Akt, and Erk are also unaffected, and preliminary experiments with Vav triple knockout neutrophils suggest Vav proteins are completely dispensable for fMLP- and LTB4-induced chemotaxis (unpublished data). Thus, Vav proteins are not critical mediators of many GPCR-induced signaling events in mouse neutrophils, raising the possibility that another GEF, potentially the Gβγ-dependent P-Rex (Welch et al., 2002), regulates Rac activation downstream of GPCRs.

Our results have defined a novel requirement for Vav GEFs as critical mediators of β2 integrin-dependent Rho GTPase activation in neutrophils, in addition to their well-documented role downstream of IRRs. The regulation of neutrophil adhesion and phagocytosis is an important aspect of innate immunity. Thus, Vav proteins may be attractive therapeutic targets for inflammatory diseases.

**Materials and methods**

**Animals**

Germline vav1−/− mice (Tumer et al., 1997) were provided by V. Tybulewicz (National Institute for Medical Research, London, UK). Vav2−/− (provided by F. Alt, Children’s Hospital, Boston, MA) and Vav1/3−/− mice

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are described elsewhere (Fujikawa et al., 2003). Vav1/3−/− mice were bred with 129Sv wild-type mice (Taconic) to generate Vav1/3−/− and were crossed to generate all lines. 8–12 wk old age- and gender-matched mice were used for experiments. All mice were maintained under specific pathogen-free conditions.

Isolation of bone marrow neutrophils

Neutrophils were isolated by Percoll density centrifugation as described previously (Mocsai et al., 2003). For a detailed protocol see online supplemental material. Equal cell equivalents were analyzed for Vav expression with rabbit anti-Vav1 (C-14; Santa Cruz Biotechnology, Inc.), anti-Vav2 (2203), and anti-Vav3 (2206; Obergell et al., 2002). Antiserum 2203 to Vav2 was produced by rabbit immunization (Covance) with a bacterially expressed GST-Vav2 fusion protein containing human Vav2 amino acids 573–878. It does not cross react with endogenous or recombinant Vav1 or Vav3 by Western blotting.

Neutrophil adhesion assays

All reagents were purchased from Sigma-Aldrich, unless otherwise indicated. For C3bi coating, 10 µg/ml mouse IgM (BD Biosciences) was absorbed onto 96-well plates (Immulon) overnight at 4°C, washed with PBS (Cellgro) and coated with 33–50% mouse serum. 10 µg/ml mIgC1-Fc (R&D Systems) was bound to protein A–coated wells (Pierce Chemical Co.) according to the manufacturer’s instructions. 20 µg/ml polyRGD was coated overnight at 4°C. Neutrophils in adhesion assay media (AAM) (HBSS1/20 mM Hepes, pH 7.4, 0.1% low endotoxin HSA) were stimulated with 10 µM fMLP, 100 nM LTB4, 10 ng/ml mTNF (R&D Systems), or 100 nM PMA for 30 min at 37°C, washed 2× with AAM, and adherent neutrophils quantified by LDH content using the Cytotox Kit (Promega). For flow assays, the flow apparatus used has been described previously (Luscinakas et al., 1994) with the modifications described in online supplemental material. Cells were infused on-co-immobilized P-selectin/ICAM-1 followed by 100 nM LTB4 and recorded by video microscopy. Arrested cells were defined as those that remained stationary for 6 s and the percentage was calculated by dividing the number of arrested cells by the number of interacting cells (rolling and adherent). Intravital experiments were performed in accordance with protocols approved by the University of Virginia Health Science Center institutional committee for animal use and as described previously (Ley et al., 1995). For a detailed protocol see online supplemental material.

Complement-mediated phagocytosis assays

FITC-E. coli (Molecular Probes) were opsonized with mouse serum for 30 min at 37°C. Neutrophils were treated with 100 ng/ml PMA or 500 nM Latrunculin B (Calbiochem) for 15 min and incubated with E. coli for 30 min. Extracellular fluorescence was quenched with 0.2% trypan blue and representative images were captured using a microscope (model TE300; Nikon) and 10× objective. For quantification, cells were fixed with 3.7% formalin and manually scored by fluorescence microscopy. Cells containing at least one bacterium were scored positive and >200 cells were counted. For binding activity, cells were treated with Latrunculin B and incubated with E. coli for 30 min at 37°C, washed with PBS, and fixed. Cells containing bound E. coli were scored by fluorescence microscopy. Representative images were captured with an ORCA1 CCD camera (Hamamatsu) and 60× objective and overlaid with Metamorph (Universal Imaging Corp.).

Chemotaxis assays

Transwell filters were coated with C3bi or with fibronectin. Assays were performed as described previously (Mocsai et al., 2002), except migrated cells were scored by hemocytometer. For detailed information, see online supplemental material. For video microscopy, cells were plated on C3bi-coated coverslips and mounted onto Zymon slides (Neuropare). The gradient was established with 10 µM fMLP for 10 min. Images were captured at 15 s intervals for >10 min with a 20× DIC objective on a microscope (model TE300; Nikon) modified with a heated stage. Migration paths, velocity, and distance were calculated with Metamorph. 50 tracks/ genotype were analyzed.

Biochemistry experiments

DFP-washed cells were primed with TNFα and plated on C3bi-coated dishes at 37°C. Unattached cells were removed by aspiration. Cells were lysed and lysates normalized by LDH content using the Cytox kit and analyzed by immunoblotting against: phosphophotyrosine (4G10; provided by T. Roberts, Dana Farber Cancer Institute, Boston, MA); pY881 PAK2 and pY118 pxaxillin (Biosource International); pY416 Src, pS473 Akt, and pS19 MLC2 (Cell Signaling); PAKα (Santa Cruz); Pyk2 and pxaxillin (Transduction Laboratories); and pS198/S203 PAKα (a gift from M. Greenberg, Children’s Hospital, Boston, MA). For Vav IPs, equal amounts of lysate were incubated with Vav1 (C-14), Vav2 (2203), and Vav3 (2206) antibodies for 3 h. For GFPase pull-down assays, neutrophils were plated on C3bi and PBD assays performed as described previously (Benard and Bokoch, 2002). Rho assays were performed as described previously (Ren et al., 1999) except cells were lysed in PBD lysis buffer. Pull-downs were blotted with Rac1 and Cdc42 (Transduction Laboratories) or RhoA (Santa Cruz Biotechnol. Inc.) antibodies. Rac2 antibody was a gift from G. Bokoch (Scripps Research Institute, La Jolla, CA). For fMLP stimulation, suspended neutrophils were treated with 10 nM fMLP followed by immediate lysis with 2× lysis buffer.

Online supplemental material

The accompanying videos to the still clips in Fig. 6 show wild-type (Video 1) and Vav1/3−/− (Video 2) neutrophils migrating towards a gradient of fMLP in C3bi-coated Zymon chambers. Analysis of adhesion of single Vav knockout neutrophils to varying concentrations of ICAM-1 is shown in Fig. S1. In addition, activation of multiple integrin signaling pathways in Vav single knockout neutrophils in response to adhesion to C3bi is shown in Fig. S2. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200404166/DC1.

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