β3 Tyrosine Phosphorylation and αvβ3-mediated Adhesion Are Required for Vav1 Association and Rho Activation in Leukocytes*

Received for publication, December 22, 2004, and in revised form, January 18, 2005
Published, JBC Papers in Press, February 7, 2005, DOI 10.1074/jbc.M414457200

Chunlei Gao‡, Erik Schaefer§, Montaha Lakkis¶, and Scott D. Blystone‡¶

From the ‡Department of Cell and Development Biology, State University of New York Upstate Medical University, Syracuse, New York 13210, and §Biosource International, Hopkinton, Massachusetts 01748

Integrin αvβ3-mediated adhesion of hematopoietic cells to vitronectin results in activation of the Rho GTPases. Mutation of β3 tyrosine residue 747, previously shown to disrupt cell adhesion, results in sustained activation of Cdc42 and diminished Rac and Rho activity. We investigated the role of the hematopoietically restricted guanine nucleotide exchange factor Vav1 in αvβ3-mediated adhesion. We find that Vav1, a guanine nucleotide exchange factor for Rac and Rho, associates with αvβ3 upon cell adhesion to vitronectin and that this association requires β3 tyrosine phosphorylation. Expression of exogenous Vav1 demonstrates that Y160F, but not wild type or the Vav1Y174F mutant, inhibits Rac and Rho activation during αvβ3-mediated cell adhesion to vitronectin. Cells expressing Vav1Y160F exhibit a sustained Cdc42 activation similar to nonphosphorylatable β3 mutants. In addition, cytoskeletal reorganization and cell adhesion are severely suppressed in Vav1Y160F-transfected cells, and Vav1Y160F fails to associate with β3 integrins. Furthermore, Vav1 itself is selectively phosphorylated upon tyrosine 160 after αvβ3-mediated adhesion, and the association between Vav1 and β3 occurs in specific response to adhesion to substrate. These studies describe a phosphorylation-dependent association between β3 integrin and Vav1 which is essential for cell progression to a Rho-dominant phenotype during cell adhesion.

The ultimate fate of integrin receptor complexes in adherent cells is their association with the actin cytoskeleton. Although enzymatically inactive, integrins sequester and/or recruit several classes of signaling and adaptor molecules to interaction domains within their cytoplasmic tails, forming functional signaling complexes. One purpose of these complexes is initiation of actin cytoskeletal linkage. In cells that circulate in the vasculature or in tissues during immune surveillance, the assembly of integrin signaling complexes is regulated to prevent unwarranted adhesion that could lead to inappropriate inflammation or thrombosis. We have previously defined one such regulated signal pathway in the αvβ3-mediated adhesion of hematopoietic cells (1). Adhesion to vitronectin mediated by the integrin αvβ3 in hematopoietic cells requires phosphorylation of tyrosine 747 of the β3 cytoplasmic tail (2). This signaling event leads to activation of the small GTPase Rho in a Syk- and phosphatidylinositol 3-kinase-dependent manner (1). Rho activation is required for hematopoietic αvβ3-mediated adhesion and the corresponding formation of actin stress fibers. Phosphorylation of tyrosine 747 of β3 is also required for localization of the actin nucleation complex Arp2/3 to β3 adhesion sites (3). Blockade of Rho activity or its downstream effector ROCK prevents both cell adhesion and the formation of actin stress fibers (1, 4).

It has been reported and is well characterized that activation of Rho family GTPases is involved in the formation of cytoskeletal structures. For example, Rho activation induces stress fiber formation, Rac activation induces lamellipodia formation, and Cdc42 activation induces filopodia formation (5). Rho family GTPases execute their functions by regulating downstream effector proteins such as ROCK and PAK and are themselves subject to upstream regulation. Guanine nucleotide exchange factors (GEFs) participate in the regulation of Rho activity by accelerating the exchange reaction rate between the nucleotide-free and nucleotide-occupied states of these GTPases (6). Among the GEFs that have been identified thus far, the Vav protein family is a novel group that can promote the Rho/Rac, but not the Cdc42, family transition from the inactive state to the active state (7). Vav proteins contain seven domains, including a calponin homology domain, an acidic domain, Dbl homology region in which the catalytic activity resides, an adjacent pleckstrin domain, one zinc finger domain, two Src homology 3 domains, and an Src homology 2 domain (8). The Vav family of GEFs has three members: Vav1, Vav2, and Vav3 (9, 10). Vav1 expression is only detected in cells of hematopoietic origin, whereas Vav3 has broader tissue expression, and Vav2 is expressed ubiquitously (10–12). It has been reported that extracellular stimuli can modify Vav by tyrosine phosphorylation and that Vav proteins have been shown to associate with multiple growth factor receptors upon activation (13). Integrin engagement also leads to Vav1 activation through tyrosine phosphorylation (14). Phosphorylation of tyrosine residues within the amino terminus of Vav has been implicated in its function (8). The Vav1 acidic domain contains three tyrosine residues (tyrosine 142, 160, and 174). Biochemical and structural evidence suggests that tyrosine 174 of the Vav1 acidic region contributes to an autoinhibitory event via intramolecular interaction with the catalytic Dbl homology domain (15, 16). This autoinhibitory interaction is released upon phosphorylation of tyrosine 174 (16).

In this paper, utilizing K562 stable transfection cell lines, we...
carried out a detailed study of Rh family GTPase activity during integrin αβ₂-mediated cell adhesion to vitronectin (Vn). To determine further the requirement for β₂ integrin tyrosine phosphorylation in the activation of Rh family GTPases, we assessed the role of a Rac/Rho GEF, Vav1, in αβ₂-mediated adhesion of hematopoietic cells. We found a specific association between αβ₂ and Vav1 which depends upon the tyrosine phosphorylation of β₂ at tyrosine 747. Expression of wild type Vav1 and the Vav1 tyrosine mutants Vav1Y160F and Vav1Y174F in K562 cells, demonstrates that the tyrosine 160 mutation disrupts cytoskeletal organization formation and impairs activation of Rho and Rac in vivo and inhibits cell adhesion. Association of Vav1 and β₂ integrin is also perturbed by the tyrosine 160 mutation. Further, specific phosphorylation of Vav1 is induced by αβ₂-mediated adhesion, and cell adhesion is required for optimal Vav1-β₂ association. These results suggest that tyrosine 160 of Vav1 plays a unique role in αβ₂-mediated cell adhesion and subsequent cytoskeletal reorganization and possibly involvement in translocation of Vav1 to the β₂ integrin adhesion complex. It is our belief that successful tyrosine 160 phosphorylation of Vav1 and its translocation to β₂ are key components of the β₂ tyrosine phosphorylation-dependent adhesion mechanism of hematopoietic cell types.

**EXPERIMENTAL PROCEDURES**

**Cells and Materials**—K562 cells were stably transfected with cDNA encoding wild type β₂, Y747F β₂, Y759F β₂, and Y747F/Y759F β₂ together with the αV subunit and maintained as described previously (2). αβ₂ expression in K562 cells (Kαβ2, Kαβ7, Y747F, Kαβ7Y759F, Kαβ7Y747F/Y759F) was equivalent between cell lines and monitored by flow cytometry. Vav1Y160F and Vav1Y174F were created using standard PCR mutagenesis from pEGFPN3-Vav1, a gift from Dr. R. Kawahara, University of Nebraska. The Y160F mutation was introduced using 5′-GGAGTGAAGATCTAGTTCGGGTGG3′ and 5′-CCACGCGAGGCAACACGCTGCTTGGG3′ and 5′-GCCGGTGGCGTCCACCAGCTGCAGCTGAGCC3′. A 5′-noncoding EcoRI site was introduced for transfection of Vav1 and Vav1Y160F to pGEX-2T for production of full-length Vav1 fusion proteins in BL21 bacteria. GFP-Vav1, GFP-Vav1Y160F, and GFP-Vav1Y174F to pGEX-2T for production of full-length Vav1 fusion proteins in BL21 bacteria. GFP-Vav1, GFP-Vav1Y160F, and GFP-Vav1Y174F-transfected Kαβ₂ cells were stably transfected with GFP-Vav1, GFP-Vav1Y160F, and GFP-Vav1Y174F-transfected Kαβ₂ cells were adherent to coverslips for 1 h at 37 °C in Iscove’s modified Dulbecco’s medium supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 μg/ml gentamicin, and 2 mM L-glutamine. 200 μg/ml sucrose was added to the cells for 30 min at 37 °C, and the cells were pelleted with 10 mM M NaVO₄ at room temperature. After incubation, cells were lysed and immunoprecipitation of β₂ was performed as described previously. Phosphorylation of β₂ tyrosine 747 was detected by Western blotting using a PSSA recognizing phosphorylated tyrosine 747 of β₂.

**Rho GTPase Activity Assay**—6-well plates were coated with 1 μg/ml Vn, and cells were plated at 2 × 10⁴ cells/well and allowed to adhere for the indicated times at 37 °C. Rho GTPase activity was assessed as described previously (1). Briefly, cells were lysed in ice-cold buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM MgCl₂, 1% Nonidet P-40, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride. Cell lysates were then clarified by centrifugation at 12,000 × g for 15 min at 4 °C. GTP-bound Rho, Rac, and Cdc42 were affinity purified from lysates using a glutathione S-transferase (GST) fusion construct of the Rho binding domain of trout Ken (GST-RB) or glutathione S-transferase bound to the target PAK1 (kindly provided by M. A. Schwartz, Scripps Institute, La Jolla, CA). Lysates were incubated with GST-RBD/GST-PBD (25 μg) beads for 1 h at 4 °C. GTP-bound Rho, Rac, and Cdc42 were separated using 12% SDS-polyacrylamide gels under reducing conditions. Total Rho, Rac, and Cdc42 were determined by loading 1/10 total volume of cell lysate on a 12% SDS-polyacrylamide gel under reducing conditions. Proteins were transferred to polyvinylidene difluoride membranes and subjected to Western blotting. Antibodies used for Western blotting were polyclonal anti-Rho, anti-Rac, and anti-Cdc42.

**Fluorescent Microscopy**—12-mm glass coverslips were coated overnight at 4 °C with 1 μg/ml Vn in PBS. 8 × 10⁴ Kαβ₂ cells, GFP-Vav1, GFP-Vav1Y160F, or GFP-Vav1Y174F-transfected Kαβ₂ cells were adherent to coverslips for 1 h at 37 °C in Iscove’s modified Dulbecco’s medium supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. Cells were fixed with 1% formaldehyde for 1 h, cells were permeabilized with 0.005% Nonidet P-40 for 15 s at 4 °C followed by two washes with PBS. For actin filament staining, rhodamine-phalloidin (1:200) was diluted in PBS and added to the cells for 30 min at 37 °C followed by five washes in PBS. Coverslips were reversed onto o-phenylenediamine in glycerol. Fluorescence was visualized on a Nikon Eclipse E800 fluorescent microscope. Images were digitalized using a Hamamatsu ORCA-ER digital camera (Bridgewater, NJ) and processed with Simple PCI and Adobe Photoshop 5.5 software.

**Cell Adhesion Assays**—96-well microtiter plates (Immunol II, Dynatech, Chantilly, VA) were coated with 1 μg/ml Vn in PBS overnight at 4 °C. Wells were washed twice in PBS and postcoated with 1% casein in PBS for 1 h at room temperature. Cells were suspended in 1 ml of PBS containing 10 mM NaVO₄ for 1 h at room temperature. Crystal violet was dissolved in methanol, and adhesion was quantified by absorbance at 570 nm using an Emax microplate reader (Molecular Dynamics, Sunnyvale, CA).

**Actin Sedimentation Assays**—6-well plates were coated with 1 μg/ml Vn, and cells were plated at 1 × 10⁴ cells/well and allowed to adhere for 1 h at 37 °C. Cells were lysed with 0.5 ml of lysis buffer (150 μM KCl, 20 mM imidazole, 5 mM MgCl₂, pH 7.2; fresh EGTA was added to a final concentration of 0.1 mM and fresh dithiothreitol to a final concentration of 2 mM, 1% Nonidet P-40, 2 mM phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2 μg/ml phallodin). Cell lysates were performed on ultracentrifugation at 100,000 × g for 1 h. Cells were subjected to ultracentrifugation supernatant were trichloroacetic acid precipitated and loaded along with pellets from ultracentrifuged sample on 10% SDS-polyacrylamide gel for electrophoresis. Actin was determined by loading 10% total volume cell lysates. Actin was detected by Western blotting with anti-actin monoclonal antibody.

**In Vitro Tyrosine Phosphorylation and Guanine Nucleotide Exchange Exchange Assay**—GST-Vav1 and GST-Vav1Y160F were incubated with 10 ng/ml phorbol 12-myristate 13-acetate-treated Kαβ₂ lysate for 20 min at room temperature in the presence or absence of 6 units of Src (Sigma). After glutathione-Sepharose pull-down, proteins were transferred to polyvinylidine difluoride membranes and subjected to Western blotting. Antibodies used for Western blotting were monoclonal antibody 7G2 (anti-β₂), monoclonal anti-phosphotyrosine antibody 4G10, polyclonal β₂, PSSA recognizing phosphorylated tyrosine 747, polyclonal Vav1 PSSA recognizing phosphorylated Vav1 tyrosine 160, and polyclonal anti-Vav1 antibody. β₂ Phosphorylation—2 × 10⁴ K562 cells expressing wild type or Y747F/Y759F β₂ were suspended in 1 ml of PBS and incubated with or without 2 μM Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP) peptide, in the presence of 40 μM NaVO₄ for 1 h at room temperature. After incubation, cells were lysed, and immunoprecipitation of β₂ was performed as described previously. Phosphorylation of β₂ tyrosine 747 was detected by Western blotting using a PSSA against phosphorylated tyrosine 747 of β₂.
A detailed examination of the three major Rho family GTPases in cell lysates revealed Vav1 phosphorylation. For guanine nucleotide exchange assay, GST, GST-Vav1, GST-Vav1Y160F, and GST-Vav1Y174F were incubated with GST-PBD or GST-RBD-agarose beads to pull down active Rac and Rho. GTP-Rac and GTP-Rho were detected by Western blotting, and 1/10 of the total lysates were probed for Cdc42 to demonstrate equal loading. B and C, Rac and Cdc42 were incubated with GST-PBD and GST-RBD-agarose beads to pull down active Rac and Cdc42. Parallel samples of lysates used to pull down Rac and Cdc42 were allowed to adhere for 10, 30, or 60 min at 37 °C. Cell lysates were incubated with GST-PBD immobilized on agarose beads. GST-Cdc42 was detected by Western blotting, and 1/10 of the total lysates were probed for Cdc42 to demonstrate equal loading. B and C, Rac and Cdc42 were incubated with GST-PBD and GST-RBD-agarose beads to pull down active Rac and Cdc42. Parallel samples of lysates used to pull down Rac and Cdc42 were allowed to adhere for 10, 30, or 60 min. Cell lysates were incubated with GST-PBD and GST-RBD-agarose beads to pull down active Rac and Rho. GTP-Rac and GTP-Rho were detected by Western blotting, and 1/10 of the total lysates were probed for Cdc42 to demonstrate equal loading. Shown are representative examples of multiple experiments.

polyclonal antibody recognizing phosphorylated Vav1 at tyrosine 160 to reveal Vav1 phosphorylation. For guanine nucleotide exchange assay, GST, GST-Vav1, GST-Vav1Y160F, and GST-Vav1Y174F were incubated with Kαβ3 integrin antibody and monoclonal anti-Cdc42. Antibodies used for Western blots were polyclonal anti-Rho, anti-Rac, and anti-Cdc42. Parallel samples of lysates used to pull down Rac and Cdc42 were allowed to adhere for 10, 30, or 60 min. Cell lysates were incubated with GST-PBD and GST-RBD-agarose beads to pull down active Rac and Rho. GTP-Rac and GTP-Rho were detected by Western blotting, and 1/10 of the total lysates were probed for Cdc42 to demonstrate equal loading. B and C, Rac and Cdc42 were incubated with GST-PBD and GST-RBD-agarose beads to pull down active Rac and Cdc42. Parallel samples of lysates used to pull down Rac and Cdc42 were allowed to adhere for 10, 30, or 60 min. Cell lysates were incubated with GST-PBD and GST-RBD-agarose beads to pull down active Rac and Rho. GTP-Rac and GTP-Rho were detected by Western blotting, and 1/10 of the total lysates were probed for Cdc42 to demonstrate equal loading. Shown are representative examples of multiple experiments.

RESULTS

Rho Family GTPases Activation and β3 Integrin Phosphorylation—Integrin engagement has been shown previously to regulate Rho GTPase activation, and β3 integrin phosphorylation plays a critical role in Rho activation during αβ3 integrin-mediated cell adhesion to Vn (1). In this study, we carried out a detailed examination of the three major Rho family GTPases activation during αβ3 integrin-mediated cell adhesion to Vn. To investigate the relationship between Rho family GTPases activation and β3 integrin phosphorylation, we adhered Kαβ3, Kαβ3Y747F, Kαβ3Y759F cells, and Kαβ3Y747F/Y759F cells to Vn for 10, 30, and 60 min and examined Cdc42 activity in all four cell types by GST-PBD pull-down, as described under “Experimental Procedures.” Distinct responses of Cdc42 activity were observed among these cells during their adhesion time course (Fig. 1A). In Kαβ3 and Kαβ3Y759F cells, Cdc42 activity reached its peak at 30 min and declined afterward. Contrastingly, in Kαβ3Y747F and Kαβ3Y747F/Y759F cells, with nonphosphorylatable tyrosine 747, Cdc42 displayed continually increasing activity through 60 min of adhesion, as shown in Fig. 1A. We also examined Rho and Rac activity in adhering Kαβ3 and Kαβ3Y747F/Y759F cells at 10, 30, and 60 min. We found that Rac was activated as early as at 10 min in both Kαβ3 and Kαβ3Y747F/Y759F cells. In Kαβ3 cells, Rac activity kept increasing for 60 min. In contrast, Rac activity was diminished in Kαβ3Y747F/Y759F cells at both 30 and 60 min (Fig. 1B). We also found that Rho was not activated until late in adhesion in Kαβ3 cells, corresponding to organization of actin stress fibers in these cells (3). At 60 min, we were able to detect Rho activity in Kαβ3 cells, whereas in Kαβ3Y747F/Y759F cells, Rho activity was inhibited throughout the time course (Fig. 1C).

Vav1 Association with β3 Integrins—Rho GEFs play an important role in the pathway from extracellular stimuli to Rho GTPase activation. Given the dependence of Rho GTPases activation on β3 tyrosine phosphorylation, we investigated whether activation of their upstream regulators, Rho GEFs, was also dependent upon the phosphorylation status of β3. Because it has been reported that Vav1 of the Vav family of GEFs is hematopoietically restricted (11), we evaluated the role of Vav1 in β3-dependent Rho GTPase activation. To investigate the relationship between αβ3 and Vav1 activation, we utilized a Vav1 polyclonal antibody to immunoprecipitate Vav1 from Kαβ3 and Kαβ3Y747F/Y759F cells after their adhesion to Vn for 1 h. Western blotting analysis for associated β3 showed that Vav1 associated with αβ3 only under conditions where β3 was tyrosine-phosphorylated (Fig. 2, A and B). Phosphorylation of β3 was detected with a PSSA against β3 tyrosine 747 (3). Vav1 was tyrosine-phosphorylated both in Kαβ3 and Kαβ3Y747F/Y759F cells, as shown in Fig. 2A by anti-phospho-tyrosine antibody 4G10. These results suggest that although association between Vav1 and β3 depends on the status of β3 tyrosine phosphorylation, Vav1 tyrosine phosphorylation is not disturbed in the absence of β3 tyrosine residues.

Rho Family GTPase Activity and Vav1 Phosphorylation—Previous studies have shown that the activation of Vav1 GEF activity is mediated by its tyrosine phosphorylation (15). Phosphorylation at Vav1 tyrosine 142 has been shown previously to relieve an inhibition to the Vav1 Dbl homology domain and expose this catalytic domain to Rho GTPases (16). Little work has been done to determine the roles that other tyrosine residues play in Vav1 activity regulation. Some reports suggest that tyrosine 142 and tyrosine 160 might have synergistic effects on Vav1 activity inhibition because of
Rho and Rac activation comparable with K
Wild type Vav1 and Vav1Y174F-transfected cells exhibited
ined the activation status of Rho, Rac, and Cdc42 in K
160 may be mechanistically related.

Having determined that the mutation of Vav1 tyrosine
zation—

Vav1Y160F displayed a pattern of Rho GTPase activation
after 60 min of cell adhesion (Fig. 3
in Vav1Y160F-expressing cells remained at a high level
ated. This suggests that for

Cdc42 activity and reduced Rac and Rho activity. K
and deficient actin organization exhibited by the Vav1Y160F
and Cdc42 in K, cells expressing wild type Vav1 (Fig. 4

In agreement with actin visualization studies using rhodamine-phalloidin, sediment of cell lysates from adherent K, or K cells expressing wild type, Y160F, or Y174F Vav1 revealed that the Y160F mutation resulted in a large increase in soluble actin and a reduction in polymerized actin compared with cells expressing wild type Vav1 (Fig. 4C). This suggested that tyrosine 160 phosphorylation of Vav1 is an important step in the mechanism leading to actin cytoskeletal organization during α,β-mediated leukocyte adhesion.

To determine whether or not the abnormal GTPases profile and deficient actin organization exhibited by the Vav1Y160F mutant cells were reflected in cell adhesion, K, or K cells expressing wild type, Y160F, or Y174F Vav1 revealed that a double tyrosine mutation in the cytoplasmic tail which prevented Vav1 association with β3 (Fig. 2) were permitted to adhere to Vn in a quantitative adhesion assay (Fig. 4B). Mutation of tyrosine 160 of Vav1, but not tyrosine 174, dramatically reduced cell adhesion in this assay. As shown previously (18), mutation of the β3 cytoplasmic tyrosines also reduced cell adhesion. These results demonstrate that tyrosine 160 of Vav1 is important to α,β-mediated adhesion and may relate to the defective adhesion, Rho activation, and cytoskel-

defective adhesion, Rho activation, and cytoskeletal organization demonstrated previously in tyrosine mutant β3 integrins.

Disturbance of Vav1 Association with β3 Integrin by Vav1Y160F Mutation—To determine further the function of Vav1 tyrosine 160 phosphorylation and its role in β3 integrin-

FIG. 3. Rho GTPase activity and Vav1 phosphorylation. A and B, K and Kα,β cells expressing wild type GFP-Vav1, GFP-Vav1Y160F, or GFP-Vav1Y174F were plated on 6-well plates coated with 1 μg/ml Vn and allowed to adhere for 1 h at 37 °C. Cell lysates were incubated with GST-RBD and GST-PBD immobilized on beads. GTP-Rho and GTP-Rac were detected by Western blotting, and 1/10 of the total lysates were probed for Rho and Rac to demonstrate equal loading. C, K and Kα,β cells expressing wild type GFP-Vav1, GFP-Vav1Y160F, or GFP-Vav1Y174F were treated as in A for 1 h, and cell lysates were incubated with GST-PBD beads to pull down active Cdc42. GTP-Cdc42 was detected by Western blotting, and 1/10 of the total lysates were probed for Cdc42 to demonstrate equal loading. Shown are representative examples of multiple experiments.

FIG. 4. Vav1 tyrosine phosphorylation and cytoskeletal reorganiza-
tion. A, Kα,β cells and Kα,β cells expressing wild type (WT) GFP-Vav1, GFP-Vav1Y160F, or GFP-Vav1Y174F were adhered to 1 μg/ml Vn-coated coverslips for 1 h. Cells were stained with rhodamine-
phalloidin to detect actin organization as described under “Experimental Procedures.” Bar, 5 μm. B, microtiter plates (96-well) were coated with 1 μg/ml Vn. Kα,β, Kα,β,Y47F/Y759F cells, and Kα,β cells expressing wild type GFP-Vav1, GFP-Vav1Y160F, or GFP-Vav1Y174F were adhered to Vn-coated coverslips for 1 h. Cells were lysed, and lysates were subjected to ultracentrifugation at 100,000 x g. Proteins recovered from both supernatants and pellets after ultracentrifugation were detected by Western blotting with monoclonal antibody to actin. Shown are representative examples of multiple experiments.

Vav1 Tyrosine Phosphorylation and Cytoskeletal Reorganiza-
tion—Having determined that the mutation of Vav1 tyrosine 160 alters GTPase activity during α,β-dependent adhesion, we examined whether cells expressing this mutant Vav1 exhibited a phenotype that corresponded with elevated levels of Cdc42 activity and reduced Rac and Rho activity. Kα,β and Kα,β cells expressing GFP-conjugated wild type, Y160F, or Y174F Vav1 were permitted to adhere to Vn for 1 h, and the degree of cell spreading, cellular morphology, and arrangement of the actin cytoskeleton were visualized by rhodamine-phalloidin staining. As shown in Fig. 4A, cells expressing wild type Vav1 or Vav1Y174F exhibited spreading and overall morphol-
mediated adhesion, we permitted \( \kappa_3 \beta_3 \) cells expressing wild type Vav1, Vav1Y160F, or Vav1Y174F to adhere to Vn for 1 h and immunoprecipitated both endogenous and exogenous Vav1 from these cells. As shown in Fig. 5, \( \beta_3 \) association with Vav1 was not disturbed in either wild type Vav1 or Vav1Y174F cells, whereas in cells expressing Vav1Y160F, expression of this Vav1 mutant led to reduced association between \( \beta_3 \) integrin and Vav1. Using an anti-GFP antibody to pull down the exogenous GFP-Vav1 proteins, we found that mutation of Vav1 tyrosine 160 obliterates the association between Vav1 and \( \beta_3 \) integrin. Examination of total Vav1 recovery from these cells showed reduced levels of \( \beta_3 \) integrin association in Vav1Y160F cells. The incomplete inhibition of the Vav1-\( \beta_3 \) association suggests that the Y160F mutant does not have a competitive advantage over endogenous Vav1. Examination of recovered GFP-Vav1 demonstrated altered electrophoretic mobility in both the tyrosine 160 and tyrosine 147 mutants compared with GFP-Vav1. This suggests that both of these residues are phosphorylated during adhesion.

Vav1 Phosphorylation and Association with \( \beta_3 \) Integrin Are Induced upon \( \alpha_\beta_\gamma \)-mediated Adhesion—Tyrosine phosphorylation of \( \beta_3 \) is induced upon integrin binding with \( \alpha_\beta_\gamma \) peptide ligand Arg-Gly-Asp (RGD) (18) or when adherent to \( \alpha_\beta_\gamma \) ligand, such as Vn (2). To determine whether Vav1 phosphorylation and \( \beta_3 \) association were dependent upon \( \alpha_\beta_\gamma \)-mediated adhesion or \( \beta_3 \) phosphorylation, we treated \( \kappa_3 \beta_3 \) cells in suspension with or without 2.0 mM GRGDSP peptide or permitt them to adhere to Vn. Experimental buffers included 40 mM NaVO₄, a concentration of phosphatase inhibitor which does not permit accumulation of spontaneous \( \beta_3 \) tyrosine phosphorylation (Fig. 6A, Control) but allows visualization of stimulated phosphorylation. Under these conditions, we immunoprecipitated \( \beta_3 \) and examined the phosphorylation status of Vav1 and \( \beta_3 \) and their association with one another. Utilizing a Vav1 PSSA recognizing phosphorylated Vav1 at tyrosine 160, we found that significant Vav1 phosphorylation at tyrosine 160 was only seen after cell adhesion to Vn (Fig. 6A). Moreover, association of Vav1 phosphorylated at tyrosine 160 with \( \beta_3 \) was also only evident after adhesion to Vn (Fig. 6A). This is surprising because we demonstrated previously that Vav1 association with \( \beta_3 \) requires \( \beta_3 \) phosphorylation at tyrosine 747, a condition met after treatment with RGD peptide (Fig. 6A). These results suggest that although \( \beta_3 \) phosphorylation is induced by ligand contact in suspended or immobilized form, the phosphorylation of Vav1 at tyrosine 160 requires other aspects of cellular activation provided only by adhesion to substrate. Phosphorylated Vav1 at tyrosine 160 could be detected beginning at 30 min of adhesion and was maintained for at least 1.5 h (data not shown).

We have shown previously that tyrosine 747 of \( \beta_3 \) is the major phosphorylation site in leucocyte \( \alpha_\beta_\gamma \) and that mutation of this residue prevents adhesion. To determine whether or not Vav1 association with phosphorylated \( \beta_3 \) was specific to either cytoplasmic tyrosine, \( \kappa_\alpha_\beta_3 Y747F \) and \( \kappa_\alpha_\beta_3 Y759F \) cells were permitted to adhere to Vn, and \( \beta_3 \) was recovered from these cells after 1 h. Vav1 phosphorylation at tyrosine 160 was associated with \( \beta_3 \) only when \( \beta_3 \) tyrosine 747 was intact (Fig. 6B). To determine whether or not an intact actin cytoskeleton was required for Vav1 phosphorylation, \( \kappa_\alpha_\beta_3 \) cells were either kept in suspension or permitted to adhere to Vn in the presence or absence of cytochalasin D. Preventing actin fiber assembly with cytochalasin D had no effect on the phosphorylation of Vav1 tyrosine 160 or its association with \( \beta_3 \), showing that phosphorylation of Vav1 and its recruitment to \( \beta_3 \) integrin are upstream of actin filament formation (Fig. 6C).

To determine the specificity of PSSA against Vav1 tyrosine 160 we employed in previous experiments, we first purified full-length wild type and Y160F-substituted Vav1 as GST fusion proteins. Despite the large size of these constructs, suitable quantities of a single large expression product were purified by glutathione-Sepharose precipitation (Fig. 6D). To demonstrate specificity of the Vav1Y160p PSSA, a quantity of Sepharose-bound GST-Vav1 and GST-Vav1Y160F were incubated with phospholipid 12-myristate 13-acetate-activated lymphocytes from \( \kappa_\alpha_\beta_3 \) cells in the presence or absence of active Src kinase. Blotting pull-downs with the Vav1Y160p PSSA antibody revealed Src-induced phosphorylation of wild type Vav1, but not a mutant Vav1 bearing a phenylalanine substitution for tyrosine 160 (Fig. 6D).

**FIG. 5. Disruption of Vav1 and \( \beta_3 \) association in Vav1Y160F mutants.** Total Vav1, GFP-Vav1, GFP-Vav1Y160F, and GFP-Vav1Y174F were immunoprecipitated (IP) from \( \kappa_\alpha_\beta_3 \) cells expressing wild type (WT) GFP-Vav1, GFP-Vav1Y160F, or GFP-Vav1Y174F after adhering to 1 \( \mu \)g/ml Vn-coated 6-well plates for 1 h and were subjected to SDS-PAGE and Western blot using monoclonal \( \beta_3 \) antibody as described under “Experimental Procedures.” The equivalence of Vav1 phosphorylated or unphosphorylated was demonstrated under “Experimental Procedures.” The equivalence of Vav1 recovery from immunoprecipitations was determined by blotting samples with a polyclonal antibody to Vav1. Migration of endogenous Vav1 and GFP-Vav1 is indicated. Shown is a representative of multiple experiments.

**DISCUSSION**

We have defined ligand-induced phosphorylation of \( \beta_3 \) cytoplasmic tyrosine 747 as a hematopoietic-specific requirement for \( \alpha_\beta_\gamma \)-mediated adhesion. The phosphorylated \( \beta_3 \) cytoplasmic tail serves as a platform for the assembly of a signaling complex that controls integrin linkage to the actin cytoskeleton. Recently we demonstrated that \( \beta_3 \) phosphorylation permits Rho activation and association of phosphatidylinositol 3-kinase with the integrin complex. Phosphatidylinositol 3-kinase activation and its association with \( \beta_3 \) are required for Vav1 phosphorylation, \( \kappa_\alpha_\beta_3 \) cells were either kept in suspension or permitted to adhere to Vn in the presence or absence of cytochalasin D. Preventing actin fiber assembly with cytochalasin D had no effect on the phosphorylation of Vav1 tyrosine 160 or its association with \( \beta_3 \), showing that phosphorylation of Vav1 and its recruitment to \( \beta_3 \) integrin are upstream of actin filament formation (Fig. 6C).

**Regulation of Vav1 Guanine Exchange Activity by Tyrosine Residues**—To examine the function of Vav1 tyrosine 160 and 174 in vitro, reactions were performed similar to those described in Fig. 6D with the addition of a quantity of GST-PBD or GST-RBD. Blotting of glutathione-Sepharose pull-downs with anti-Rac, Rho, or Cdc42 was used to visualize active GTP-bound Rac, Rho, and Cdc42. No Rac or Rho activity was detected in lysates incubated solely with uncomplexed GST, whereas the addition of wild type Vav1 generated small quantities of active Rac or Rho. Interestingly, mutation of both tyrosines 160 and 174 to phenylalanine in GST-Vav1 generated a large increase in the amount of active GST-bound Rac and Rho in the reaction (Fig. 7, top two and middle two panels). Activation of Cdc42 was observed neither in the presence of wild type GST-Vav1 nor in the presence of GST-Vav1 mutants (Fig. 7, bottom two panels), confirming previous data that Vav1 has no activity toward Cdc42. Examination of each cell lysate by direct blotting for Rac, Rho, and Cdc42 reveals equivalent amounts of total Rac, Rho, and Cdc42 in each sample.
Iscove's modified Dulbecco's medium or allowed to adhere (immunoprecipitations was determined by blotting samples with monoclonal antibody to in Iscove's modified Dulbecco's medium with 40 analysis for phosphorylated tyrosine 160 of Vav1 (Fig. 6). Shown are representative examples of multiple experiments. GST-Vav1 and GST-Vav1Y160F were expressed recombinantly, purified from bacterial lysates by glutathione-Sepharose precipitation, and subjected to SDS-PAGE followed by Coomassie staining to demonstrate homogeneity of purified constructs. GST-Vav1 and GST-Vav1Y160F were incubated with lysate from 10 ng/ml phorbol 12-myristate 13-acetate-stimulated K cells during adhesion to Vn. We found that Vav1 associates with tyrosine residues abolish this association. Vav1 is also found specifically phosphorylated on tyrosine 160. These results suggest that Vav1 is involved in the activation of Rho and Rac after cell adhesion through integrin $\alpha_v\beta_3$. Interestingly, phosphorylation of $\beta_3$ was necessary but not sufficient for Vav1 association because cells in suspension in which $\beta_3$ phosphorylation was induced by soluble peptide did not elicit Vav1 association; rather, $\beta_3$ phosphorylation induced upon adhesion to ligand was required for Vav1 phosphorylation and association with $\beta_3$. Cytochalasin D treatment did not interfere with Vav1 phosphorylation and suggests that this signaling event is upstream of cytoskeletal reorganization.

The Vav family of GEFs exhibits activity toward Rac and Rho GTPases, but not Cdc42 (7). We confirmed this finding by showing that although Vav1Y160F disrupted Rac/Rho activity in vivo, it did not interfere with Cdc42 activity. Previous observations demonstrate that Vav1 activity is regulated by phosphorylation of tyrosines in its amino terminus (15). Most recent studies suggest that the Vav1 calponin homology domain plays the major inhibitory role in cis (19). Phosphoregulation of Vav1 is complex and remains incompletely defined. Using transformation to assay Vav1 activity, it has been determined that Vav1 activity is initiated by phosphorylation (15). In its inactive state, the cata-

![Fig. 6. Vav1Y160F phosphorylation and association with $\beta_3$ is induced during $\alpha_\beta_3$-mediated adhesion.](Image 107x193 to 257x369)

![Fig. 7. Regulation of Vav1 guanine exchange activity tyrosine residues.](Image 149x528 to 473x737)
Vav1 Phosphorylation

In addition to in vivo study of the role of Vav1 tyrosine residues 160 and 174, we also evaluated the specificity of Vav1 tyrosine mutants toward Rho GTPases in vitro. Our results agree with previous reports (15) that in vitro environments, phosphorylation on either tyrosine 160 or tyrosine 174 exposes the catalytic site of Vav1. Both Vav1Y160F and Vav1Y174F, which mimic the phosphorylation state of Vav1, have increased GEF activity toward its substrates, Rac and Rho, compared with wild type Vav1 and minimal activity on Cdc42. The discrepancy of Vav1Y160F activity toward Rac and Rho between in vivo and in vitro reinforces our evidence that the role of phosphorylation of tyrosine 160 is to mediate Vav1 translocation and promote the interaction with its substrates, Rho and Rac.

Recent studies on integrin-mediated cell adhesion suggested that Rac is translocated to the cell membrane, where its effectors are closely located (24). Integrins also regulate Rac activity through Rho-GDI dissociation (25). The GDP dissociation inhibitor (GDI) protein family negatively regulates Rho GTPase activity by inhibiting GDP release from Rho GTPases. It is possible that translocation of Vav1 to the site of integrin-mediated adhesion reported in this study allows Vav1 to interact with GDI-free Rac/Rho. Alternatively, Vav1 translocation may also be involved in the GDI dissociation process, and the Vav1 interaction with Ly-GDI through the Vav1 calponin homology domain has been demonstrated previously (26).

Including this report, the ligation of β1, β2, and β3 have been shown to result in Vav1 tyrosine phosphorylation (14, 27, 28). To our knowledge, this is the first report demonstrating Vav1 association with integrins or integrin-mediated site-specific Vav1 phosphorylation. The mechanism whereby phosphorylation of β3 and Vav1 permits coassociation is currently unknown. Tyrosine phosphorylation of β3 in the NPLY motif of its cytoplasmic tail provides a platform for the assembly of a signaling complex leading to actin engagement. Several proteins have been described which interact selectively with phosphorylated β3, including Shc, myosin, Pyk2, and Arp2/3 (3, 29). Although Vav1 direct association with various growth factors such as platelet-derived growth factor and epidermal growth factor, which can also serve as tyrosine kinases, has been shown, it is unlikely that phosphorylated Vav1 binds directly to phosphorylated β3. The linker protein responsible for this event will be of great interest because it may be the proximal binding partner of phosphorylated β3 which initiates the hematopoietic cell-specific adhesion mechanisms of αβ3.

REFERENCES

1. Butler, W., Williams, M. P., and Blystone, S. D. (2003) J. Biol. Chem. 278, 5264–5270
2. Blystone, S. D., Williams, M. P., Slater, S. E., and Brown, E. J. (1997) J. Biol. Chem. 272, 28757–28761
3. Chandhoke, S. K., Williams, M., Schaeffer, E., Zorn, L., and Blystone, S. D. (2004) J. Cell Sci. 117, 1431–1441
4. Maciakwa, M., Ishizaki, T., Nakata, K., Saito, Y., Watanabe, N., Fujita, A., Iwamatsu, A., Ohnita, T., Ohashi, K., Mizuno, K., and Narumiya, S. (1999) Science 285, 895–899
5. Nokes, C. D., and Hall, A. (1995) Cell 81, 53–62
6. Schmidt, A., and Hall, A. (2002) Genes Dev. 16, 1587–1609
7. Movilla, N., Dosil, M., Zheng, Y., and Bustelo, X. R. (2001) Oncogene 20, 8057–8065
8. Bustelo, X. R. (2000) Mol. Cell. Biol. 20, 1461–1477
9. Bustelo, X. R. (1996) Crit. Rev. Oncog. 7, 65–88
10. Movilla, N., and Bustelo, X. R. (1999) Mol. Cell. Biol. 19, 7870–7885
11. Katayama, S., Martin-Zanca, D., and Barbacid, M. (1989) EMBO J. 8, 2283–2290
12. Schuiboel, K. E., Movilla, N., Rosa, J. L., and Bustelo, X. R. (1998) EMBO J. 17, 6608–6621
13. Mores, S. L., Selfors, L. M., Fredericks, J., Breit, T., Fujikawa, K., Alt, F. W., Brugge, J. S., and Swat, W. (2000) Mol. Cell. Biol. 20, 6364–6373
14. Yron, I., Deckert, M., Reif, M. E., Munsch, A., Schwartz, M. A., and Altman, A. (1999) Cell Adhes. Commun. 7, 1–11
15. Lopez-Lago, M., Lee, H., Cruz, C., Movilla, N., and Bustelo, X. R. (2000) Mol. Cell. Biol. 20, 1678–1691
16. Aghazadeh, B., Lowry, E. W., Huang, X. Y., and Rosen, M. K. (2000) Cell 102, 625–633

lytic domain of Vav1 is masked by the amino terminus. Phosphorylation of amino-terminal tyrosines is thought to permit a conformational change leading to exposure of the catalytic domain and enhancement of Vav1 activity (16). This model is examined further in several experiments, which suggest that Vav1 phosphorylation at tyrosine 174 is involved in various signaling pathways such as oncogenic activation, c-Jun NH2-terminal kinase activation, and stimulation of the nuclear factor of T lymphocytes (15). In previous reports, tyrosine 174 was thought to be the primary site of phosphate incorporation. Mutation of tyrosine 174 leads to high levels of cell transformation activity, whereas mutation of tyrosine 160 and tyrosine 142 attenuates this effect (15). Most of the studies of Vav tyrosine mutation are carried out in vitro, and effects of the mutations on Rho family GTPase activity after integrin engagement have not been reported. Although Vav1Y174F represents an active form of Vav1 GEF, it is still phosphorylation-dependent (15). Although integrin ligation has been shown previously to enhance Vav1 phosphorylation, the site of phosphate incorporation was not determined. Here we show that adhesion via αβ3 results in the selective phosphorylation of Vav1 at tyrosine 160, and this phosphorylation permits association of Vav1 with β3. To explore further the role Vav1 tyrosine 160 plays in integrin-mediated cell adhesion, we expressed wild type Vav1, Vav1Y160F, and Vav1Y174F mutants in Kaβ3 cells and determined the effects on cell adhesion and cytoskeletal organization. Interestingly, cells expressing the Vav1Y160F mutant exhibited loss of cell adhesion, impaired cytoskeletal reorganization, and deficient Rho/Rac activation. These results suggest an involvement of Vav1 tyrosine 160 in β3 integrin-mediated cell adhesion and cytoskeletal organization.

Vav proteins are best characterized as GEFs for Rho GTPases. In addition, they also act as adaptor proteins through their carboxyl-terminal Src homology 2 and 3 domains (8, 21, 22). Tyrosine phosphorylation possibly not only initiates Vav activation but also mediates phosphorytrosine binding domain-dependent association of other proteins with Vav. In our experiments, we were unable to observe the phosphorylation of Vav1 at tyrosine 174 because of lack of a reliable PSSA against Vav1 tyrosine 174. We found Vav1 phosphorylation to be time-dependent: total Vav1 tyrosine phosphorylation, by 4G10 and phosphorylation of Vav1 tyrosine 160, by Vav1Y160 PSSA, were detected within 30 min of cell adhesion and increased afterward (data not shown). Phosphorylation at tyrosine 174 may be transient, yet critical for Vav1 activation. Jurkat cells stimulated with anti-CD3 had detectable phosphorylation at Vav1 tyrosine 174 2 min after stimulation with loss of signal after 5 min (15). It has been reported that the primary regulatory tyrosine in Vav1, tyrosine 174, is within a sequence context favored as a substrate of SHP-1 (23). Phosphorylation of Vav1 at other tyrosine residues might be implicated in protein-protein interactions that represent another level of Vav regulation. Expression of Vav1Y160F inhibits endogenous Vav1 activity by preventing its translocation to the β3 integrin adhesion complex. The correlation between the requirements for Vav1 tyrosine 160 phosphorylation and association with β3 and the requirements for hematopoietic αβ3-mediated adhesion are very firm. Furthermore, these requirements, together with the known GEF activity of Vav1 and its demonstrated modulation in tyrosine 160 mutants, strongly suggest that tyrosine 160 phosphorylation of Vav1 regulates Rho/Rac activation during αβ3-mediated adhesion. We conclude that Vav1 tyrosine 160 phosphorylation, in addition to its possible involvement in relief of inhibition to the Vav1 catalytic domain, is also required for Vav1 recruitment to β3 integrin adhesion complexes and for downstream signal transduction.
17. Blystone, S. D., Graham, I. L., Lindberg, F. P., and Brown, E. J. (1994) J. Cell Biol. 127, 1129–1137
18. Blystone, S. D. (2002) J. Biol. Chem. 277, 46886–46890
19. Zugaza, J. L., Lopez-Lago, M. A., Caloca, M. J., Desil, M., Movilla, N., and Bustelo, X. R. (2002) J. Biol. Chem. 277, 45377–45392
20. Miranti, C. K., Leng, L., Maschberger, P., Brugge, J. S., and Shattil, S. J. (1996) J. Biol. Chem. 271, 3856–3862
21. Ye, Z. S., and Baltimore, D. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 12629–12633
22. Stebbins, C. C., Watzl, C., Billadeau, D. D., Leibson, P. J., Burshtyn, D. N., and Leng, E. O. (2003) Mol. Cell. Biol. 23, 6291–6299
23. Del Pozo, M. A., Alderson, N. B., Kiosses, W. B., Chin, H. H., Anderson, K. G., and Schwartz, M. A. (2004) Science 303, 839–842
25. Del Pozo, M. A., Kiosses, W. B., Alderson, N. B., Meller, N., Hahn, K. M., and Schwartz, M. A. (2002) Nat. Cell Biol. 4, 232–239
26. Groysman, M., Russek, C. S., and Katzav, S. (2000) FEBS Lett. 467, 78–80
27. Zheng, L., Sjolander, A., Eckerdal, J., and Andersson, T. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 8431–8436
28. Woodside, D. G., Obergfell, A., Leng, L., Wilsbacher, J. L., Miranti, C. K., Brugge, J. S., Shattil, S. J., and Ginsberg, M. H. (2001) Curr. Biol. 11, 1799–1804
29. Phillips, D. R., Prasad, K. S., Manganello, J., Bao, M., and Nannizzi-Alaimo, L. (2003) Curr. Opin. Cell Biol. 13, 546–554