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Relationships between Rap1b, Affinity Modulation of Integrin $\alpha_{IIb}\beta_3$, and the Actin Cytoskeleton*

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The affinity of integrin $\alpha_{IIb}\beta_3$ for fibrinogen is controlled by inside-out signals that are triggered by agonists like thrombin. Agonist treatment of platelets also activates Rap1b, a small GTPase known to promote integrin-dependent adhesion of other cells. Therefore, we investigated the role of Rap1b in $\alpha_{IIb}\beta_3$ function by viral transduction of GFP-Rap1 chimeras into murine megakaryocytes, which exhibit inside-out signaling similar to platelets. Expression of constitutively active GFP-Rap1b (V12) had no effect on unstimulated megakaryocytes, but it greatly augmented fibrinogen binding to $\alpha_{IIb}\beta_3$ induced by a PAR4 thrombin receptor agonist ($p < 0.01$). The Rap1b effect was cell-autonomous and was prevented by pre-treating cells with cytochalasin D or latrunculin A to inhibit actin polymerization. Rap1b-dependent fibrinogen binding to megakaryocytes was blocked by POW-2, a novel monovalent antibody Fab fragment specific for high affinity murine $\alpha_{IIb}\beta_3$. In contrast to GFP-Rap1b (V12), expression of GFP-Rap1GAP, which deactivates endogenous Rap1, inhibited agonist-induced fibrinogen binding ($p < 0.01$), as did dominant-negative GFP-Rap1b (N17) ($p < 0.05$). None of these treatments affected surface expression of $\alpha_{IIb}\beta_3$. These studies establish that Rap1b can augment agonist-induced ligand binding to $\alpha_{IIb}\beta_3$ through effects on integrin affinity, possibly by modulating $\alpha_{IIb}\beta_3$ interactions with the actin cytoskeleton.

The $\alpha_{IIb}\beta_3$ integrin is a receptor for adhesive ligands such as fibrinogen and von Willebrand factor, and ligand binding to $\alpha_{IIb}\beta_3$ is required for platelet aggregation and spreading in hemostasis. Ligand binding is regulated by positive and negative "inside-out" signals that converge on $\alpha_{IIb}\beta_3$ to control the integrin activation state through modulation of receptor affinity or avidity (1). Affinity modulation, the dominant mode of regulation in platelets, implies a change in the conformation of the $\alpha_{IIb}\beta_3$ heterodimer to increase access of ligand binding sites, while avidity modulation implies lateral movements of heterodimers in the plane of the plasma membrane, culminating in integrin clustering (2–4). Positive inside-out signals can be initiated by agonist occupancy of several different classes of excitatory receptors that couple to heterotrimeric G proteins and tyrosine kinases (5–10). $\alpha_{IIb}\beta_3$ activation can be negatively regulated by prostacyclin or nitric oxide, whose effects are mediated through cyclic AMP and cyclic GMP, respectively (5).

The signaling molecules directly responsible for regulation of $\alpha_{IIb}\beta_3$ downstream of excitatory receptors are incompletely characterized. Nonetheless, isoforms of protein kinase C and PI[1] 3-kinase as well as cytoplasmic free Ca^{2+} have been identified as key signaling intermediates (1, 5). In addition, $\alpha_{IIb}\beta_3$ function in platelets appears to be regulated in some way by the actin cytoskeleton because inhibition of actin polymerization by low micromolar concentrations of cytochalasin D or latrunculin A increases agonist-dependent fibrinogen binding, whereas higher concentrations of these agents partially inhibit fibrinogen binding (11). Therefore, protein kinase C, PI 3-kinase, and Ca^{2+} may introduce post-translational modifications in signaling/cytoskeletal proteins that associate with and regulate $\alpha_{IIb}\beta_3$ (1, 12). However, major gaps remain in the identification and characterization of integrin regulatory proteins in platelets.

The Ras family GTPase, Rap1b, may be one such integrin regulatory protein. Like other Ras family members, it cycles from an inactive, GDP-bound form to an active, GTP-bound form, with cycling regulated by one or more guanine nucleotide exchange factors (GEFs), guanine nucleotide dissociation inhibitors (GDIs), and GTPase-activating proteins (GAPs) (13). Rap1b is highly expressed in platelets, is rapidly activated in response to agonists such as thrombin, ADP, or epinephrine and partitions along with $\alpha_{IIb}\beta_3$ to the Triton-insoluble core actin cytoskeleton of aggregated platelets (14–16). Rap1b activation in response to thrombin depends initially on Ca^{2+} fluxes into the platelet cytoplasm and subsequently on protein kinase C (17). Although the function of Rap1b in platelets is unknown, it has been implicated in promoting β_1 and β_2 integrin-dependent adhesion of fibroblastic and hematopoietic cell lines and murine thymocytes (18–22). The evidence for this is based largely on overexpression of constitutively active and dominant-negative forms of Rap1, but additional support for Rap1 involvement comes from studies of mouse embryonic fibroblasts deficient in the Rap1 GEF, C3G, which exhibit defective integrin-dependent adhesion that is correctable by expression

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[1] The abbreviations used are: PI, phosphatidylinositol; GFP, enhanced green fluorescent protein; RIPA, radioimmune precipitation assay buffer; GST, glutathione *S*-transferase; IL, interleukin; GEF, guanine nucleotide exchange factors; GAP, GTPase-activating proteins.

of a constitutively active Rap1 mutant (23). Because, cell adhesion by integrins is dependent on a combination of factors, including inside-out signaling, ligand binding, and post-ligand binding events, the precise mechanism(s) whereby Rap1 promotes adhesion may vary with the integrin and the cell type.

Based on these considerations, the present study was carried out to determine whether Rap1b is an effector of inside-out signaling to $\alpha_{IIb}\beta_3$. Because platelets are not amenable to genetic manipulation, we used Sindbis virus vectors to express specific GFP-tagged chimeric proteins in primary, mature murine megakaryocytes. Megakaryocytes are nucleated cells that function primarily to produce platelets, and like platelets they exhibit an inside-out signaling pathway from excitatory receptors to $\alpha_{IIb}\beta_3$ (24–26). The results establish that Rap1b, in concert with platelet agonists, can promote fibrinogen binding to $\alpha_{IIb}\beta_3$. Furthermore, they indicate that Rap1b functions to modulate $\alpha_{IIb}\beta_3$ affinity, possibly through effects on the actin cytoskeleton.

EXPERIMENTAL PROCEDURES

Construction of Sindbis Virus Vectors—Enhanced green fluorescent protein (GFP) was amplified by PCR using pEGFP-C1 as a template (CLONTECH, Palo Alto, CA) and cloned into the *Mlu*I/*Sph*I sites of the Sindbis expression vector, pSinRep5 (Invitrogen, Carlsbad, CA). Full-length human Rap1b (V12) or Rap1b (N17) cDNAs in pGBT9 and pCGN, respectively, were PCR-amplified to generate *Xba*I/*Sph*I fragments and directionally cloned into pSinRep5. Then a *Xba*I/*Xba*I GFP cassette was subcloned in-frame into this plasmid to create GFP-Rap1b (V12) or GFP-Rap1b (N17) fusions. GFP-Rap1GAP in pSinRep5 was generated by cloning PCR-amplified human Rap1GAP (a generous gift from Alan Hall, London) as a *Mlu*I/*Mlu*I fragment, followed by a 5′ in-frame insertion of the GFP cassette. Insert orientations were verified by colony PCR, and all coding sequences were verified in the Sindbis vectors by automated DNA sequencing.

To produce Sindbis viruses encoding mRNA for the GFP chimeras, the pSinRep5 plasmids and a helper plasmid (DH26S) were linearized and used as a template to synthesize *in vitro* capped and polyadenylated mRNA using an SP6 RNA-polymerase kit (Ambion, Austin, TX). Expression and helper mRNAs were then co-transfected in a 1:1 molar ratio by electroporation into BHK cells, which were cultured for 24 h to allow virion production. Supernatants containing virions were collected, centrifuged at 2000 × *g* for 10 min at 4 °C, and stored in liquid nitrogen in 1-ml aliquots. Viral titers were evaluated by transducing BHK cells and assessing GFP expression 18 h later by flow cytometry. Only viral preparations capable of inducing GFP expression in more then 50% of BHK cells at a 1:3000 dilution of viral supernatant were used for subsequent megakaryocyte experiments.

Characterization of GFP-tagged Proteins—GFP, GFP-Rap1b, and GFP-Rap1GAP proteins were characterized by incubating NIH 3T3 cells with a 1:3 dilution of recombinant virus in 1% Dulbecco's modified Eagle's medium/fetal calf serum for 1 h at 37 °C. After 10-fold dilution in the same medium without virus, cells were cultured another 6 h, and expression of recombinant proteins was assessed in Nonidet P-40 detergent-solublized cell lysates by Western blotting (26). Blots were probed with a monoclonal antibody to GFP (1:500 dilution; CLONTECH) and an horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (1:3000 dilution; Bio-Rad Laboratories). Immunoreactive bands were detected by enhanced chemiluminescence using SuperSignal WestPico reagent (Pierce).

GTP loading of Rap1b was detected by a pull-down assay (14). NIH 3T3 cells were lysed at 4 °C in RIPA buffer containing 75 mM NaCl, 1% Nonidet P-40, 1% deoxycholic acid, 0.2% sodium dodecyl sulfate, 2.5 mM $MgCl_2$, 1 mM sodium orthovanadate, 1 mM phenylmethylsulphonyl fluoride, 2 μM leupeptin, 2 μM aprotinin, and 50 mM Tris/HCl, pH 7.4. After clarification, 500 μg of protein in 500 μl were incubated with 30 μl of a 50% slurry of glutathione-Sepharose beads precoupled to either GST or the GST-Rap binding domain of RalGDS. After three washes with RIPA buffer, the presence of GFP or GFP-Rap1b proteins in the pellets, and the amounts of recombinant or endogenous Rap1 in the starting material were examined by Western blotting with an antibody to either GFP or Rap1 (Santa Cruz Biotechnology, Santa Cruz, CA).

Transduction of GFP-tagged Proteins into Megakaryocytes—Bone marrow cells were harvested from 6 to 8-week-old BALB/c mice and cultured in the presence of thrombopoietin, IL-6, and IL-11 as described (26). After 5 days, mature, polyploid megakaryocytes were enriched by gravity sedimentation for 60 min at 37° in a 50-ml conical polypropylene tube and then applied to a discontinuous density gradient of 1–2-3% bovine serum albumin in phosphate-buffered saline, pH 7.4. After gravity sedimentation for 30 min at 37°, cells were resuspended in complete Iscove's Modified Dulbecco's medium to 2×10^5/ml, and 0.5-ml aliquots were added to 100-mm dishes previously blocked with 1% bovine serum albumin in phosphate-buffered saline. Cells were infected with 2 ml of a 1:1 dilution of recombinant Sindbis virus for 1 h at 37° and then diluted with 5 ml of complete medium and incubated for 6 h in a CO_2 incubator.

Analysis of Fibrinogen Binding to Megakaryocytes—After viral infection, megakaryocytes were collected in 50-ml polypropylene tubes and sedimented by gravity for 60 min at 37°. Cells were gently resuspended in modified Tyrode's buffer (137 mM NaCl, 2.9 mM KCl, 12 mM $NaHCO_3$, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.1% bovine serum albumin, 0.1% glucose, 5 mM HEPES, pH 7.4), and incubated for 20 min at room temperature in a final volume of 50 μl in the presence of 200 μg/ml biotin-fibrinogen, 0.5 or 1 mM PAR4 thrombin receptor-activating peptide (AYPGFK) (27), and 10 μg/ml phycoerythrin-streptavidin (Molecular Probes, Eugene, OR). To assess nonspecific binding, parallel samples were incubated with either 10 mM EDTA or 20 μg/ml 1B5, a function-blocking antibody specific for murine $\alpha_{IIb}\beta_3$ (a gift from Barry Coller, New York) (28). In some cases, cells were pre-incubated for 10 min with either 10 μM cytochalasin D, latrunculin A or an equivalent volume of Me_2SO vehicle before addition of fibrinogen and agonist. Fibrinogen binding to large megakaryocytes was quantified by flow cytometry (26). Surface expression of $\alpha_{IIb}\beta_3$ was determined by flow cytometry after incubating cells with 10 μg/ml biotinylated anti-murine α_{IIb} antibody or an isotype-matched control IgG (BD PharMingen, San Diego, CA).

Development of a Recombinant Antibody Fab Fragment Specific for High Affinity Murine $\alpha_{IIb}\beta_3$—PAC-1 Fab is a recombinant IgG_1, RGD-containing antibody Fab fragment specific for high affinity human $\alpha_{IIb}\beta_3$ (29). To determine whether Rap1b modulates $\alpha_{IIb}\beta_3$ affinity in mouse megakaryocytes, PAC-1 was re-engineered to recognize high affinity murine $\alpha_{IIb}\beta_3$. Specifically, PAC-1 Fab heavy chain cDNA was subjected to splice-overlap extension PCR such that an 11-amino acid stretch of H-CDR-3 (PSYYRGDGAGP) was replaced with a 13-amino acid stretch from kistrin (CRIPRGDMPDDRC), an integrin-binding snake venom peptide (30). When expressed as a secreted heavy chain along with the PAC-1 light chain in *Drosophila* S2 cells (31), the resulting Fab fragment, named POW-2, was found to be selective for high affinity murine $\alpha_{IIb}\beta_3$ (see "Results"). Serum-free S2 culture supernatant containing POW-2 Fab was concentrated 10-fold and dialyzed extensively against phosphate-buffered saline. Preliminary studies showed that the Fab in this preparation was monomeric by size exclusion chromatography on a Sephadex G-200 column (29). Furthermore, forced oligomerization of $\alpha_{IIb}\beta_3$ in a Chinese hamster ovary cell model system did not promote POW-2 Fab binding, indicating that POW-2 was not sensitive to changes in $\alpha_{IIb}\beta_3$ avidity (3). POW-2 Fab interaction with mouse platelets and megakaryocytes was analyzed by flow cytometry (29). For platelets, POW-2 binding was assessed with a secondary goat anti-mouse Ig (H+L) antibody $(Fab')_2$ conjugated with Alexa-488 (31). For megakaryocytes, POW-2 was used to compete for biotin-fibrinogen binding.

RESULTS

Expression of GFP-Rap1 Chimeric Proteins—The purpose of this investigation was to determine the role of Rap1b in $\alpha_{IIb}\beta_3$ function. Toward this end, RNA Sindbis virus vectors encoding either GFP, constitutively active GFP-Rap1b (V12), dominant-negative GFP-Rap1b (N17), or GFP-Rap1GAP were introduced into murine megakaryocytes, and fibrinogen binding to $\alpha_{IIb}\beta_3$ was examined. GFP was fused to the N termini of these constructs to facilitate flow cytometric identification of transduced cells. Prior to megakaryocyte studies, recombinant protein expression was verified by transducing murine NIH 3T3 cells and examining Western blots of cell lysates with an antibody to GFP. In each case, a single immunoreactive band with the appropriate electrophoretic mobility was observed (Fig. 1*A*). To determine whether GFP-Rap1b (V12) was active in the sense that it was loaded with GTP, lysates from virally infected cells were incubated with glutathione-Sepharose beads coated with the GST-Rap1 binding domain of RalGDS, a Rap1b effector (20). GFP-Rap1b (V12) bound to these beads, whereas GFP-Rap1b (N17) and GFP did not (Fig. 1*B*). Binding of GFP-Rap1b

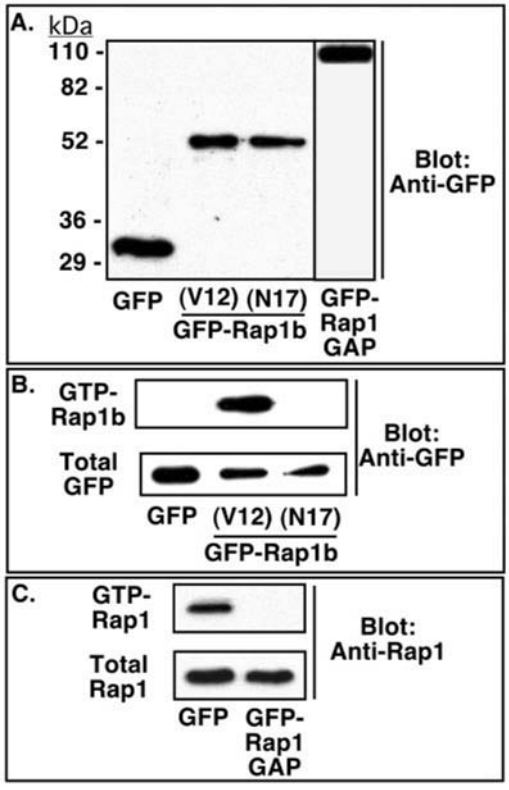

FIG. 1. **Expression of GFP-Rap1 proteins in murine cells.** As described under "Experimental Procedures," NIH 3T3 cells were incubated for 6 h with Sindbis viruses encoding either GFP, GFP-Rap1b (V12), GFP-Rap1b (N17), or GFP-Rap1GAP. In *panel A*, cells were lysed in Nonidet P-40 buffer and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting using anti-GFP antibody as a probe. In *panel B*, cells were incubated with viruses encoding GFP, GFP-Rap1b (V12), or GFP-Rap1b (N17), lysed in RIPA buffer, and then subjected to a pull-down assay using glutathione-Sepharose beads coated with GST-Rap1 binding domain of RalGDS. After washing, GFP-tagged proteins retained on the beads (*e.g. GTP-Rap1b*) were analyzed on a Western blot using an anti-GFP antibody. In parallel, the amount of recombinant GFP proteins in 10 μg of each cell lysate (*e.g. total GFP*) was assessed using an antibody to GFP. In *panel C*, infection of cells with viruses encoding GFP or GFP-Rap1GAP was carried out in the presence of 10% fetal calf serum, the latter added to stimulate endogenous Rap1. After cell lysis, GTP-Rap1 retained on the beads and total Rap1 in 10 μg of cell lysate were assessed by Western blotting using an antibody to Rap1.

(V12) was specific because it failed to bind to beads coated with GST (not shown). GFP-Rap1GAP was functional in that it eliminated GTP-loading of endogenous Rap1 (Fig. 1*C*). On the basis of these results, these viral vectors were used to introduce the GFP-tagged chimeras into megakaryocytes.

Rap1b and Inside-out $\alpha_{IIb}\beta_3$ Signaling in Megakaryocytes—Large, mature megakaryocytes derived from bone marrow cultures were incubated for 6 h with Sindbis viruses encoding the GFP-tagged chimeras, and fibrinogen binding was then examined by flow cytometry (26). The light scattering mode of the flow cytometer was used to identify large megakaryocytes, the FL1 fluorescence channel to identify virally transduced, GFP-positive cells, the FL2 channel to quantify the binding of biotin-fibrinogen (using phycoerythrin-streptavidin), and the FL3 channel to exclude propidium iodide-positive, dead cells. After viral infection, the percentage of large megakaryocytes that expressed the recombinant GFP proteins ranged from 10 to 50% from experiment to experiment. Fig. 2 shows the fibrinogen binding data for a single experiment in the form of dot plots, where cells expressing the recombinant protein are above the horizontal lines and cells not expressing the recombinant protein are below the horizontal lines. Note that megakaryocytes that had been incubated with the control Sindbis virus encoding GFP (Fig. 2, *panels A* and *D*) bound little or no fibrinogen unless the cells were stimulated during the fibrinogen binding step with a PAR4 thrombin receptor-activating peptide (AYPGKF) (27). Both GFP-positive and GFP-negative cells appeared to respond in a similar fashion. Unstimulated megakaryocytes expressing either GFP-Rap1b (V12), which is constitutively active, or GFP-Rap1GAP, which inactivates endogenous Rap1 (18), also bound little fibrinogen (Fig. 2, *panels B* and *C*). In contrast, when stimulated with AYPGKF, megakaryocytes expressing GFP-Rap1b (V12) appeared to bind relatively more fibrinogen than non-expressing cells (Fig. 2, *panel E*), while megakaryocytes expressing GFP-Rap1GAP appeared to bind relatively less fibrinogen than non-expressing cells (Fig. 2, *panel F*).

To validate the conclusions drawn from this single experiment, the results of five such experiments are summarized in Fig. 3, which depicts specific fibrinogen binding, defined as binding inhibited by 10 mM EDTA. Neither GFP-Rap1b (V12) nor GFP-Rap1GAP significantly influenced basal fibrinogen binding to unstimulated megakaryocytes (Fig. 3*A*). However, compared with megakaryocytes expressing GFP, those expressing GFP-Rap1b (V12) bound significantly more fibrinogen in response to AYPGKF ($p < 0.01$), whether a subsaturating (0.5 mM) or a saturating (1 mM) concentration of the agonist was employed (Fig. 3*A*). Identical results were obtained if 20 μg/ml 1B5, a function-blocking anti-$\alpha_{IIb}\beta_3$ antibody, was used instead of EDTA to determine specific fibrinogen binding (not shown). In contrast to the results with GFP-Rap1b (V12), cells expressing GFP-Rap1GAP bound 46% less fibrinogen than GFP-expressing cells in response to 1 mM AYPGKF ($p < 0.01$). These effects of GFP-Rap1b (V12) or GFP-Rap1GAP were confined to the subpopulation of megakaryocytes in each sample that had been successfully transduced (compare Fig. 3, *A versus B*). In addition, in three independent experiments, transduction of megakaryocytes with GFP-Rap1b (N17), which acts in a dominant-negative fashion by binding to some Rap1 GEFs (32), inhibited fibrinogen binding induced by 1 mM AYPGKF by 27 ± 8% ($p < 0.05$). Taken together, these results establish that Rap1b promotes agonist-induced fibrinogen binding to $\alpha_{IIb}\beta_3$, and this effect is cell-autonomous.

The observed effects of Rap1b and Rap1GAP expression on fibrinogen binding to megakaryocytes suggest that Rap1b modulates $\alpha_{IIb}\beta_3$ affinity and/or avidity. However, similar results might be obtained if Rap1b were to modify expression levels of $\alpha_{IIb}\beta_3$ or if Sindbis virus infection, *per se*, were to somehow alter the responsiveness of the megakaryocytes to agonists. Therefore these potential confounding variables were investigated. The levels of $\alpha_{IIb}\beta_3$ expressed on the surface of megakaryocytes were measured with an antibody to the α_{IIb} subunit. None of the Sindbis virus constructs affected $\alpha_{IIb}\beta_3$ expression, either before or after stimulation with AYPGKF (Fig. 4*A*). Furthermore, transduction of megakaryocytes with Sindbis virus encoding GFP did not affect agonist-induced fibrinogen binding when the responses of GFP-positive cells were compared with GFP-negative cells (Fig. 4*B*), or when cells exposed to virus were compared with mock-transfected cells (not shown). We conclude that Rap1b modulates fibrinogen binding to $\alpha_{IIb}\beta_3$ by an effect on integrin affinity and/or avidity.

Mechanistic Aspects of Rap1b Function in Inside-out Signaling—To begin to investigate the mechanism by which Rap1b modulates fibrinogen binding to megakaryocytes, we developed a novel reagent capable of reporting on changes in the affinity of murine $\alpha_{IIb}\beta_3$. PAC-1 is a murine IgMκ monoclonal antibody specific for activated human $\alpha_{IIb}\beta_3$ (33). Because it is multim-

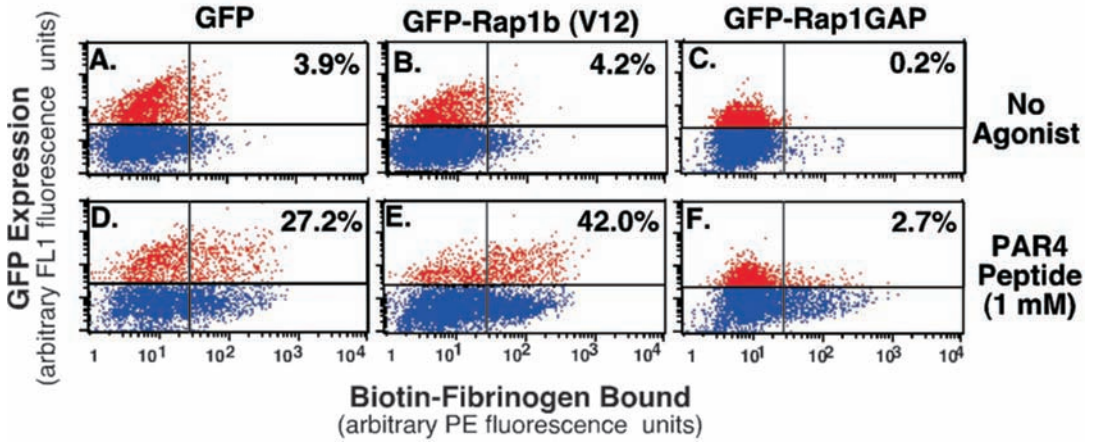

FIG. 2. **Effect of Rap1b on fibrinogen binding to megakaryocytes.** Megakaryocytes were incubated for 6 h with Sindbis viruses encoding either GFP (*panels A* and *D*), GFP-Rap1b (V12) (*panels B* and *E*) or GFP-Rap1GAP (*panels C* and *F*). Cells were then incubated for 20 min with 200 μg/ml biotin-fibrinogen in the presence or absence of 1 mM PAR4 receptor-activating peptide (AYPGKF), and fibrinogen binding was assessed by flow cytometry using phycoerythrin-strepavidin as the fluorophore. Each *panel* is a dot plot representing 10,000 large megakaryocytes. *Blue dots* below the horizontal lines represent cells not expressing the recombinant protein, and *red dots* above the horizontal lines are cells expressing the protein. The number in each dot plot represents the percentage of all GFP-positive events present in the *upper right hand quadrant*.

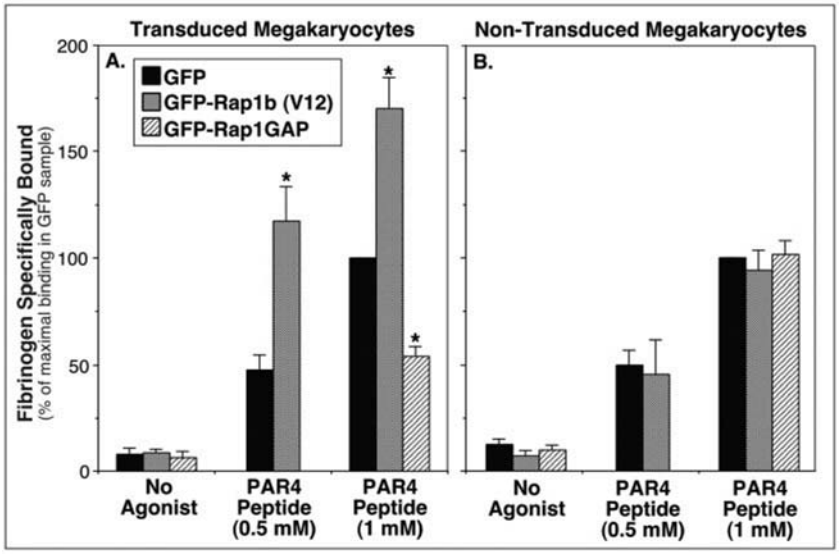

FIG. 3. **Effect of Rap1b on specific fibrinogen binding to megakaryocytes.** Data for five independent experiments of the kind illustrated in the legend to Fig. 2 are summarized. Specific fibrinogen binding was defined as that inhibited by 10 mM EDTA. It was expressed relative to the binding observed with agonist-stimulated megakaryocytes incubated with the Sindbis/GFP virus, which was arbitrarily set at 100%. Data are the means ± S.E. *Asterisks* represent significant differences from the GFP sample, determined by Student's *t* test ($p < 0.01$).

eric, PAC-1 IgM is sensitive to changes in both $\alpha_{IIb}\beta_3$ affinity and avidity. In contrast, and as might be predicted, a recombinant, monomeric, and monovalent Fab fragment of PAC-1 is sensitive only to changes in $\alpha_{IIb}\beta_3$ affinity (3, 29). Because PAC-1 Fab is specific for human $\alpha_{IIb}\beta_3$, we re-engineered it to recognize high affinity murine $\alpha_{IIb}\beta_3$. By removing an 11-amino acid segment from the H-CDR3 of PAC-1 Fab and replacing it with a 13-amino acid RGD-containing segment from the disintegrin, kistrin, a new recombinant Fab fragment called POW-2 was created (Fig. 5*A*). Recombinant POW-2 bound to $\alpha_{IIb}\beta_3$ on agonist-activated mouse (or human) platelets. Binding was specific for high affinity $\alpha_{IIb}\beta_3$ because minimal binding was observed to unstimulated platelets, to stimulated platelets incubated with EDTA, kistrin or antibody 1B5 to block fibrinogen binding to $\alpha_{IIb}\beta_3$ or to stimulated platelets incubated with dibutyryl cyclic AMP to inhibit platelet activation (Fig. 5*B*). Although kistrin recognizes $\alpha_V\beta_3$ and $\alpha_5\beta_1$ in addition to $\alpha_{IIb}\beta_3$, POW-2 only recognized $\alpha_{IIb}\beta_3$ in murine cells (not shown).

Therefore, POW-2 Fab was used to compete with biotin-fibrinogen for binding to AYPGKF-stimulated mouse megakaryocytes, with the rationale that it would compete successfully only for binding to high affinity $\alpha_{IIb}\beta_3$. POW-2 Fab was used at 130 μg/ml, a concentration that in preliminary studies inhibited the specific binding of 50 μg/ml biotin-fibrinogen to AYPGKF-stimulated mouse platelets by >95%. As shown in Fig. 5*C*, POW-2 Fab inhibited agonist-dependent fibrinogen binding to GFP-expressing megakaryocytes by 75% and to GFP-Rap1b (V12)-expressing megakaryocytes by 82%. In fact, POW-2 was almost as good a competitor of biotin-fibrinogen as was an excess of unlabeled fibrinogen (Fig. 5*C*). This substantial blockade of fibrinogen binding by POW-2 Fab suggests that AYPGKF and Rap1b (V12) modulate the affinity of $\alpha_{IIb}\beta_3$.

Because $\alpha_{IIb}\beta_3$ interacts with and may be regulated by components of the platelet actin cytoskeleton (11, 34), we examined whether inhibition of actin polymerization affected Rap1b (V12)-dependent fibrinogen binding to stimulated megakaryocytes.

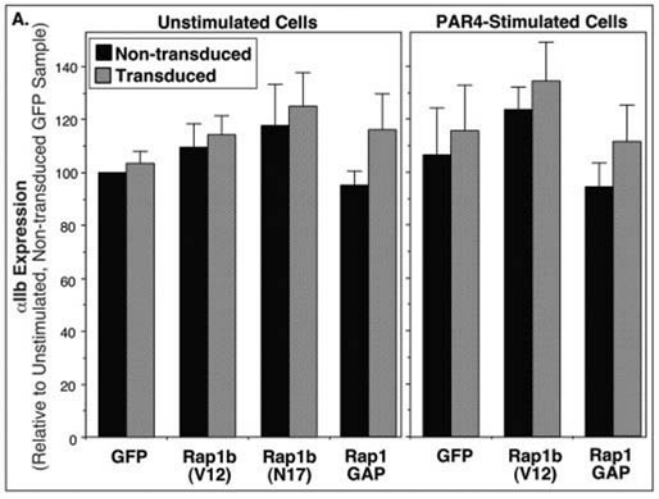

FIG. 4. **Effect of Sindbis virus infection on $\alpha_{IIb}\beta_3$ expression and function in megakaryocytes.** In *panel A*, megakaryocytes were incubated for 6 h with the indicated Sindbis viruses, and surface expression of $\alpha_{IIb}\beta_3$ was determined by flow cytometry, using an antibody specific for the α_{IIb} subunit. Where indicated, cells were stimulated with 1 mM PAR4 receptor-activating peptide (AYPGKF) during the binding assay. Data are presented as specific binding of the anti-α_{IIb} antibody and expressed relative to the binding observed with unstimulated, non-transduced (*GFP-negative*) megakaryocytes that had been incubated with the Sindbis/GFP virus. In *panel B*, megakaryocytes were incubated with Sindbis virus encoding GFP. Biotin-fibrinogen binding was then assessed in the presence or absence of AYPGKF and 10 mM EDTA, as indicated. Binding is expressed as mean fluorescence intensity in arbitrary units. Data represent the means ± S.E. of 3–7 experiments.

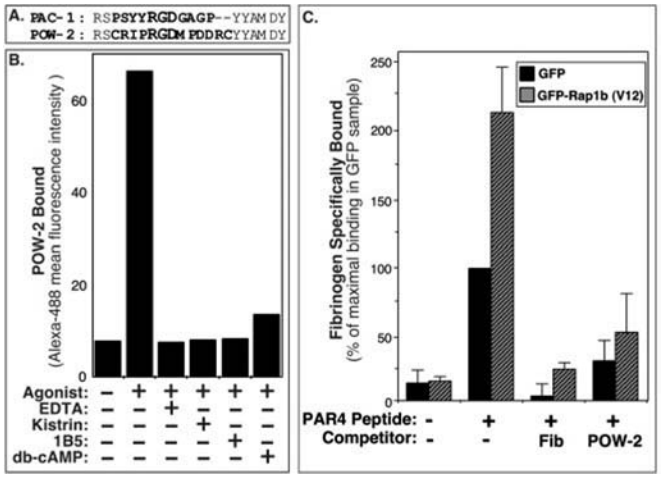

FIG. 5. **Characterization of POW-2 Fab and its effect on fibrinogen binding to megakaryocytes.** *Panel A* shows the amino acid sequences within the H-CDR3 regions of PAC-1 and POW-2 Fabs. The *bold letters* represent the swapped sequences that converted PAC-1 Fab into POW-2 Fab. *Panel B* validates the binding specificity of POW-2 Fab for $\alpha_{IIb}\beta_3$ using murine platelets and flow cytometry, as described under "Experimental Procedures." Incubation of platelets with 130 μg/ml POW-2 Fab was carried out for 30 min in the presence of an agonist and inhibitors, as indicated. The agonist was 1 mM AYPGKF, EDTA was used at 10 mM, kistrin at 5 μM, 1B5 at 20 μg/ml, and dibutyryl cyclic AMP (*db-cAMP*) at 1 mM. *Panel C* shows the effect of 130 μg/ml POW-2 Fab on the specific binding of 50 μg/ml biotin-fibrinogen to megakaryocytes. Where indicated, 0.5 mg/ml of unlabeled fibrinogen was used instead of POW-2 Fab as a competitor. Data represent the means ± S.E. of three experiments.

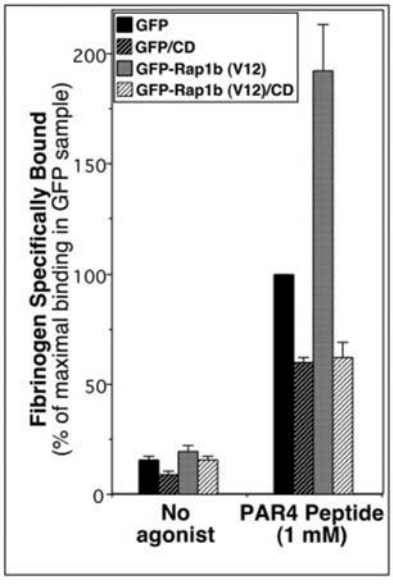

FIG. 6. **Effect of cytochalasin D on specific fibrinogen binding to megakaryocytes.** Megakaryocytes were transduced with Sindbis/GFP or Sindbis/GFP-Rap1b (V12) viruses. Cells were then incubated for 10 min with 10 μM cytochalasin D (*CD*) or Me_2SO vehicle as a control, and fibrinogen binding was determined by flow cytometry. The data represent specific fibrinogen binding to transduced megakaryocytes and is expressed relative to binding observed with agonist-stimulated cells transduced with the Sindbis/GFP virus. Data are the means ± S.E. of three experiments.

Pre-incubation of GFP-expressing megakaryocytes with 10 μM cytochalasin D blocked fibrinogen binding induced by AYPGKF by 40%, consistent with previous studies of this concentration of cytochalasins in platelets (11, 35, 36). More importantly, cytochalasin D completely blocked the increment in fibrinogen binding caused by GFP-Rap1b (V12) (Fig. 6). Similar results were obtained if actin polymerization was blocked with 10 μM latrunculin A instead of cytochalasin D (not shown). Thus, Rap1b may regulate $\alpha_{IIb}\beta_3$ affinity and fibrinogen binding through effects on the actin cytoskeleton.

DISCUSSION

In this study, recombinant human Rap1b constructs were expressed in primary murine megakaryocytes to assess the potential role of this GTPase in inside-out $\alpha_{IIb}\beta_3$ signaling. Megakaryocytes were chosen because they respond to platelet agonists by engaging fibrinogen via $\alpha_{IIb}\beta_3$, but unlike their anucleate platelet progeny, they are amenable to genetic ma-

nipulation (25, 26, 37). The major new findings are the following. 1) Expression of constitutively active Rap1b (V12) augments fibrinogen binding to $\alpha_{IIb}\beta_3$ when megakaryocytes are stimulated through the PAR4 thrombin receptor. In contrast, expression of Rap1GAP, which inactivates endogenous Rap1, or expression of Rap1b (N17), a dominant-negative construct, has the opposite effect. 2) Modulation of fibrinogen binding by Rap1b appears to be due primarily to effects on $\alpha_{IIb}\beta_3$ affinity. 3) Regulation of $\alpha_{IIb}\beta_3$ activation state by Rap1b may depend on the actin cytoskeleton, because no Rap1b effect was observed if actin polymerization was blocked by cytochalasin D or latrunculin A. These results establish a role for Rap1b in affinity modulation of $\alpha_{IIb}\beta_3$, and they raise important new questions about the identities and mechanisms of action of the relevant Rap1b regulators and effectors in megakaryocytes and platelets.

GFP-Rap1b and GFP-Rap1GAP constructs were introduced into mature, bone marrow-derived mouse megakaryocytes using Sindbis viruses. In this system, the recombinant proteins were expressed rapidly, and cell integrity was preserved long enough for functional studies of $\alpha_{IIb}\beta_3$ to be carried out. Indeed, viral transduction, *per se*, had no detrimental effect on $\alpha_{IIb}\beta_3$ surface expression or on agonist-induced fibrinogen binding (Fig. 4). In addition, N-terminal fusion of GFP to these constructs did not adversely affect their function, as exemplified by the ability of Rap1b (V12) to bind GTP and the ability of GFP-Rap1GAP to reduce GTP loading of endogenous Rap1 (Fig. 1). Because expression of GFP alone did not interfere with agonist-induced fibrinogen binding to megakaryocytes, the opposing effects on fibrinogen binding of GFP-Rap1b (V12) and GFP-Rap1GAP provide strong evidence that $\alpha_{IIb}\beta_3$ activation can be modulated by Rap1b (Figs. 2 and 3). Furthermore, although the recombinant proteins used here were human in origin, the high degree of sequence conservation between human and murine Rap1b (*e.g.* 85% overall amino acid identity; 95% in the switch regions)[2] indicates that our results are unlikely to be complicated by species differences.

One caveat in overexpression work with Rap1b (V12) and Rap1b (N17) is that the results obtained may not necessarily reflect the function of endogenous Rap1b. For example, dominant-negative Rap1b (N17) may exert functions in addition to the expected one of titrating Rap1 GEFs, and it may not titrate all Rap1 GEFs (32). Indeed, this may explain why GFP-Rap1GAP was a better inhibitor of agonist-induced fibrinogen binding to megakaryocytes than GFP-Rap1b (N17) (*e.g.* 46% *versus* 27%). Nonetheless, the present study demonstrates unambiguously that Rap1b can promote inside-out signaling to $\alpha_{IIb}\beta_3$ in primary megakaryocytes, thus providing a strong rationale to further evaluate the functional relationships between Rap1b and $\alpha_{IIb}\beta_3$ in platelets. The rapid activation of Rap1b in platelets stimulated with thrombin, ADP, or epinephrine is consistent with a role in inside-out signaling (14–17). Rap1b is a substrate for protein kinase A in platelets (38). While the significance of phosphorylation to Rap1b function is unclear, the phosphorylation of one or more proteins by protein kinase A inhibits agonist-induced ligand-binding to $\alpha_{IIb}\beta_3$ in platelets (39). Perhaps Rap1b is one of the relevant protein kinase A substrates in this context.

This work provides new insights into the role of Rap1b in integrin function. Previous studies in fibroblasts, various hematopoietic cell lines and murine thymocytes have demonstrated that overexpression of Rap1 or its GEFs and GAPs affects cell adhesion, aggregation, and phagocytosis dependent on β_1 or β_2 integrins (18–22, 40). Because all of these responses involve integrin ligation as well as post-ligand binding events, these observed effects of Rap1 do not necessarily pinpoint which phase is targeted by the GTPase. However, in one study employing a B lymphocyte cell line, Rap1 (V12) increased the expression of an activation epitope on $\alpha_L\beta_2$ and the binding of a soluble ICAM-1/IgG fusion protein to the cells, consistent with affinity modulation of $\alpha_L\beta_2$ by Rap1 (19). On the other hand, work in transgenic thymocytes concluded that Rap1 was sufficient to modulate the clustering and avidity, rather than the affinity, of β_1 and β_2 integrins (22).

In megakaryocytes, Rap1b increased fibrinogen binding to $\alpha_{IIb}\beta_3$, but only when the cells were stimulated with an agonist (Figs. 2 and 3). This suggests that signals from Rap1b are not sufficient to activate $\alpha_{IIb}\beta_3$, but rather they may be required to achieve maximal $\alpha_{IIb}\beta_3$ activation in response to agonist-triggered signals. Furthermore, the results with POW-2, a monomeric and monovalent ligand-mimetic Fab specific for high affinity murine $\alpha_{IIb}\beta_3$, establish that Rap1b functions, in large part, by modulating $\alpha_{IIb}\beta_3$ affinity in megakaryocytes (Fig. 5). Overall, these results indicate that Rap1 is capable of regulating the activation state of β_1, β_2, and β_3 integrins. However, the precise mechanism of regulation, *e.g.* affinity *versus* avidity modulation, appears to depend on the integrin and the cell type. Future studies should consider the possibility that Rap1 may also influence post-ligand binding events, such as changes in cell shape or polarity (13).

How is Rap1b activated in megakaryocytes and platelets, and which Rap1b effector(s) modulate $\alpha_{IIb}\beta_3$ affinity? Addressing these questions is made complicated by the plethora of Rap1 GEFs and GAPs in various cells and tissues, some of which are not specific for Rap1 (13, 41–43). Furthermore, the effector functions of many of the known proteins that bind selectively to GTP-Rap1 have yet to be completely characterized (13, 44). Most importantly, relatively little information is available about the repertoire of Rap1 regulators and effectors in platelets and megakaryocytes. Because Rap1b (V12) augmented the fibrinogen binding response to a PAR4 thrombin receptor agonist, the relevant Rap1 GEFs or GAPs may themselves be regulated by signaling molecules activated downstream of one or more heterotrimeric G proteins. In this context, the products of phospholipase C-mediated phospholipid hydrolysis, Ca^{2+} and diacylglycerol, have been implicated in agonist-dependent Rap1b activation in platelets (17). Indeed, platelets and megakaryocytes contain a Rap1 GEF (CalDAG-GEFI) likely to be activated by Ca^{2+} and diacylglycerol (41, 45).[3] Potential Rap1 effectors identified in platelets include RalGDS, p110 PI 3-kinase, and Raf-1 (13, 46–48).

Integrins are coupled to actin filaments through actin-binding proteins, such as α-actinin, filamin, and talin (12). Consequently, a conceptual link is often made between changes in the actin cytoskeleton and changes in integrin clustering or avidity (4, 22, 49). We found that inhibition of actin polymerization by 10 μM latrunculin A or cytochalasin D blocked Rap1b-dependent fibrinogen binding to megakaryocytes (Fig. 6). Because fibrinogen binding is controlled primarily by changes in $\alpha_{IIb}\beta_3$ affinity (Fig. 5) (3), we propose that Rap1b may regulate $\alpha_{IIb}\beta_3$ affinity through an effect on actin dynamics or organization. A causal link between changes in actin and changes integrin affinity has not been established unambiguously. However, this idea is consistent with the recent observation that relatively low concentrations of cytochalasin D or latrunculin A, which may release integrins from cytoskeletal constraints, increase agonist-dependent fibrinogen binding to platelets (11).

[2] G. C. White, unpublished observations.

[3] R. Murphy, K. Eto, S. Kerrigan, A. Bertoni, S. Shattil, and A. Leavitt, unpublished observations.

In this regard, at least two Rap1b effectors in platelets, RalGDS and p110 PI 3-kinase, have been proposed to influence actin filament organization (46, 47, 50). Studies in cell lines have demonstrated that Ras family members in addition to Rap1, such as H-Ras and R-Ras, can influence ligand binding to integrins, although the net effects vary considerably among cell types (51–53). Given the suitability of primary megakaryocytes for the molecular analysis of $\alpha_{IIb}\beta_3$ signaling, the experimental system employed here should prove useful for identifying the physiological regulators and effectors of Rap1b responsible for modulating $\alpha_{IIb}\beta_3$ affinity and for determining the roles of other Ras family members in $\alpha_{IIb}\beta_3$ function.

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REFERENCES

1. Shattil, S. J., Kashiwagi, H., and Pampori, N. (1998) *Blood* **91,** 2645–2657
2. Xiong, J. P., Stehle, T., Diefenbach, B., Zhang, R., Dunker, R., Scott, D. L., Joachimiak, A., Goodman, S. L., and Arnaout, M. A. (2001) *Science* **294,** 339–345
3. Hato, T., Pampori, N., and Shattil, S. J. (1998) *J. Cell Biol.* **141,** 1685–1695
4. Kucik, D. F., O'Toole, T. E., Zheleznyak, A., Busettini, D. K., and Brown, E. J. (2001) *Mol. Biol. Cell* **12,** 1509–1518
5. Brass, L. F. (2000) in *Hematology. Basic Principles and Practice* (Hoffman, R., Benz, E., Shattil, S., Furie, B., Cohen, H., Silberstein, L., and McGlave, P., eds), 3rd Ed., pp. 1753–1782, Churchill-Livingstone, New York
6. Andrews, R. K., Shen, Y., Gardiner, E. E., Dong, J. F., Lopez, J. A., and Berndt, M. C. (1999) *Thromb. Haemost.* **82,** 357–364
7. Clemetson, K. J., and Clemetson, J. M. (2001) *Thromb. Haemost.* **86,** 189–197
8. Watson, S. P., Asazuma, N., Atkinson, B., Berlanga, O., Best, D., Bobe, R., Jarvis, G., Marshall, S., Snell, D., Stafford, M., Tulasne, D., Wilde, J., Wonerow, P., and Frampton, J. (2001) *Thromb. Haemost.* **86,** 276–288
9. Suzuki-Inoue, K., Ozaki, Y., Kainoh, M., Shin, Y., Wu, Y., Yatomi, Y., Ohmori, T., Tanaka, T., Satoh, K., and Morita, T. (2001) *J. Biol. Chem.* **276,** 1643–1652
10. Phillips, D. R., Prasad, K. S., Manganello, J., Bao, M., and Nannizzi-Alaimo, L. (2001) *Curr. Opin. Cell Biol.* **13,** 546–554
11. Bennett, J. S., Zigmond, S., Vilaire, G., Cunningham, M., and Bednar, B. (1999) *J. Biol. Chem.* **274,** 25301–25307
12. Calderwood, D. A., Zent, R., Grant, R., Rees, D. J. G., Hynes, R. O., and Ginsberg, M. H. (1999) *J. Biol. Chem.* **274,** 28071–28074
13. Bos, J. L., de Rooij, J., and Reedquist, K. A. (2001) *Nat. Rev. Mol. Cell. Biol.* **2,** 369–377
14. Franke, B., Akkerman, J. W. N., and Bos, J. L. (1997) *EMBO J.* **16,** 252–259
15. White, G. C., II, Fischer, T. H., and Duffy, C. M. (1998) *Thromb. Haemost.* **79,** 832–836
16. Lova, P., Paganini, S., Sinigaglia, F., Balduini, C., and Torti, M. (2002) *J. Biol. Chem.* **277**, 12009–12015
17. Franke, B., Van Triest, M., De Bruijn, K. M. T., Van Willligen, G., Nieuwenhuis, H. K., Negrier, C., Akkerman, J. W. N., and Bos, J. L. (2000) *Mol. Cell. Biol.* **20,** 779–785
18. Caron, E., Self, A. J., and Hall, A. (2000) *Curr. Biol.* **10,** 974–978
19. Katagiri, K., Hattori, M., Minato, N., Irie, S., Takatsu, K., and Kinashi, T. (2000) *Mol. Cell. Biol.* **20,** 1956–1969
20. Reedquist, K. A., Ross, E., Koop, E. A., Wolthuis, R. M. F., Zwartkruis, F. J. T., Van Kooyk, Y., Salmon, M., Buckley, C. D., and Bos, J. L. (2000) *J. Cell Biol.* **148,** 1151–1158
21. Suga, K., Katagiri, K., Kinashi, T., Harazaki, M., Iizuka, T., Hattori, M., and Minato, N. (2001) *FEBS Lett.* **489,** 249–253
22. Sebzda, E., Bracke, M., Tugal, T., Hogg, N., and Cantrell, D. A. (2002) *Nat. Immunol.* **3,** 251–258
23. Ohba, Y., Ikuta, K., Ogura, A., Matsuda, J., Mochizuki, N., Nagashima, K., Kurokawa, K., Mayer, B. J., Maki, K., Miyazaki, J., and Matsuda, M. (2001) *EMBO J.* **20,** 3333–3341
24. Zauli, G., Bassini, A., Vitale, M., Gibellini, D., Celeghini, C., Caramelli, E., Pierpaoli, S., Guidotti, L., and Capitani, S. (1997) *Blood* **89,** 883–895
25. Faraday, N., Rade, J. J., Johns, D. C., Khetawat, G., Noga, S. J., DiPersio, J. F., Jin, Y., Nichol, J. L., Haug, J. S., and Bray, P. F. (1999) *Blood* **94,** 4084–4092
26. Shiraga, M., Ritchie, A., Aidoudi, S., Baron, V., Wilcox, D., White, G., Ybarrondo, B., Murphy, G., Leavitt, A., and Shattil, S. (1999) *J. Cell Biol.* **147,** 1419–1430
27. Faruqi, T. R., Weiss, E. J., Shapiro, M. J., Huang, W., and Coughlin, S. R. (2000) *J. Biol. Chem.* **275,** 19728–19734
28. Lengweiler, S., Smyth, S. S., Jirouskova, M., Scudder, L. E., Park, H., Moran, T., and Coller, B. S. (1999) *Biochem. Biophys. Res. Commun.* **262,** 167–173
29. Abrams, C., Deng, J., Steiner, B., and Shattil, S. J. (1994) *J. Biol. Chem.* **269,** 18781–18788
30. Adler, M., Lazarus, R. A., Dennis, M. S., and Wagner, G. (1991) *Science* **253,** 445–448
31. Pampori, N., Hato, T., Stupack, D. G., Aidoudi, S., Cheresh, D. A., Nemerow, G. R., and Shattil, S. J. (1999) *J. Biol. Chem.* **274,** 21609–21616
32. van den Berghe, N., Cool, R. H., Horn, G., and Wittinghofer, A. (1997) *Oncogene* **15,** 845–850
33. Shattil, S. J., Hoxie, J. A., Cunningham, M., and Brass, L. F. (1985) *J. Biol. Chem.* **260,** 11107–11114
34. Calderwood, D. A., Shattil, S. J., and Ginsberg, M. H. (2000) *J. Biol. Chem.* **275,** 22607–22610
35. Peerschke, E. I. B., and Wainer, J. A. (1985) *Am. J. Physiol.* **248,** C466-C472
36. Fox, J., Shattil, S. J., Kinlough-Rathbone, R., Richardson, M., Packham, M. A., and Sanan, D. A. (1996) *J. Biol. Chem.* **271,** 7004–7011
37. Wilcox, D. A., Olsen, J. C., Ishizawa, L., Bray, P. F., French, D. L., Steeber, D. A., Bell, W. R., Griffith, M., and White, G. C. (2000) *Blood* **95,** 3645–3651
38. Fischer, T. H., Gatling, M. N., Lacal, J.-C., and White, G. C. (1990) *J. Biol. Chem.* **265**, 19405–19408
39. Walter, U., Eigenthaler, M., Geiger, J., and Reinhard, M. (1993) *Adv. Exp. Med. Biol.* **344,** 237–249
40. Arai, A., Nosaka, Y., Kanda, E., Yamamoto, K., Miyasaka, N., and Miura, O. (2001) *J. Biol. Chem.* **276,** 10453–10462
41. Kawasaki, H., Springett, G. M., Toki, S., Canales, J. J., Harlan, P., Blumenstiel, J. P., Chen, E. J., Bany, I. A., Mochizuki, N., Ashbacher, A., Matsuda, M., Housman, D. E., and Graybiel, A. M. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 13278–13283
42. Reuther, G. W., and Der, C. J. (2000) *Curr. Opin. Cell Biol.* **12,** 157–165
43. Self, A. J., Caron, E., Paterson, H. F., and Hall, A. (2001) *J. Cell Sci.* **114,** 1357–1366
44. Jin, T.-J., Satoh, T., Liao, Y., Song, C., Gao, X., Kariya, K., Hu, C., and Kataoka, T. (2001) *J. Biol. Chem.* **276,** 30301–30307
45. Dupuy, A. J., Morgan, K., von Lintig, F. C., Shen, H., Acar, H., Hasz, D. E., Jenkins, N. A., Copeland, N. G., Boss, G. R., and Largaespada, D. A. (2001) *J. Biol. Chem.* **276,** 11804–11811
46. Wolthuis, R. M. F., Franke, B., Van Triest, M., Bauer, B., Cool, R. H., Camonis, J. H., Akkerman, J. W. N., and Bos, J. L. (1998) *Mol. Cell. Biol.* **18,** 2486–2491
47. Ohta, Y., Suzuki, N., Nakamura, S., Hartwig, J. H., and Stossel, T. P. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 2122–2128
48. Ezumi, Y., Uchiyama, T., and Takayama, H. (1998) *Eur. J. Biochem.* **258,** 976–985
49. Bazzoni, G., and Hemler, M. E. (1998) *Trends Biochem. Sci.* **23,** 30–34
50. Rittenhouse, S. E. (1996) *Blood* **88,** 4401–4414
51. Zhang, Z., Vuori, K., Wang, H.-G., Reed, J. C., and Ruoshlati, E. (1996) *Cell* **85,** 61–69
52. Shibayama, H., Anzai, N., Braun, S. E., Fukuda, S., Mantel, C., and Broxmeyer, H. E. (1999) *Blood* **93,** 1540–1548
53. Sethi, T., Ginsberg, M. H., Downward, J., and Hughes, P. E. (1999) *Mol. Biol. Cell* **10,** 1799–1809