Role of p190RhoGAP in β2 Integrin Regulation of RhoA in Human Neutrophils

Karim Dib, Fredrik Melander and Tommy Andersson

J Immunol 2001; 166:6311-6322; ;
http://www.jimmunol.org/content/166/10/6311
Role of p190RhoGAP in β2 Integrin Regulation of RhoA in Human Neutrophils

Karim Dib, Fredrik Melander, and Tommy Andersson

We found that engagement of β2 integrins on human neutrophils induced activation of RhoA, as indicated by the increased ratio of GTP/GDP recovered on RhoA and translocation of RhoA to a membrane fraction. The clustering of β2 integrins also induced a time-dependent increase in GDP bound to RhoA, which correlated with β2 integrin-induced activation of p190RhoGAP. The activation of p190RhoGAP was completely blocked by [4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d][pyrimidine] (PP1), a selective inhibitor of Src family tyrosine kinases. However, clustering of β2 integrins did not increase the basal tyrosine phosphorylation of p190RhoGAP, nor did it affect the amount of p120RasGAP bound to p190RhoGAP. Instead, the β2 integrin-induced activation of p190RhoGAP was accompanied by increased tyrosine phosphorylation of a p190RhoGAP-associated protein, p120RasGAP, and accumulation of both p120RasGAP and p190RhoGAP in a membrane fraction. PP1 blocked the β2 integrin-induced phosphorylation of p120RasGAP, as well as the translocation of p190RhoGAP and p120RasGAP, but it did not affect the accumulation of RhoA in the membrane fraction. In agreement with the mentioned findings, PP1 also increased the GTP:GDP ratio recovered on RhoA immunoprecipitated from β2 integrin-stimulated cells. Thus, in neutrophils, β2 integrin-induced activation of p190RhoGAP requires a signal from a Src family tyrosine kinase, but it does not occur via the signaling pathway responsible for activation of RhoA. The Journal of Immunology, 2001, 166: 6311–6322.

Members of the β2 integrin family are the dominating integrins expressed on neutrophils, and they play a major role in cell-cell and cell-matrix adhesion of neutrophils. β2 integrins are noncovalently associated heterodimers composed of a common β-chain, CD18, and one of four unique α-chains; CD11a, CD11b, CD11c, or CD11d, with CD11b/CD18 being the most prominent on neutrophils (1, 2). The signal transduction properties of these integrins are vital for the migration of neutrophils, but to date have only been partly elucidated.

Engagement of β2 integrins on neutrophils has been accomplished in experimental models by plating the cells on surface-bound anti-β2 integrin Abs or on ICAM-1 or other types of coated surfaces. The results show that β2 integrin engagement triggers various signal transduction events, including tyrosine phosphorylation of several different proteins (3–8), activation of the Src family tyrosine kinases p59/56hck and p58 c-fgr (3, 9), and activation of p21ras (7). These signals are associated with a profound alteration in cell morphology that leads to cell spreading and locomotion. It has been reported that these morphological changes are driven by dynamic β2 integrin-induced rearrangement of the actin cytoskeleton (10), but little is known about the nature of the signal(s) generated by β2 integrins regulating cytoskeletal rearrangements. However, in other cell types, small GTP-binding proteins that be-long to the Rho subfamily, members of the Ras superfamily of small GTPases, have been strongly implicated (11). These small GTP-binding proteins cycle between a GDP-bound inactive form to a GTP-bound active form (12). Guanine nucleotide exchange factors (GEFs),3 activated by extracellular stimuli, are responsible for the GDP-GTP exchange, a transition that allows translocation of the monomeric G proteins to the plasma membrane (13, 14). In their GTP-bound state, these proteins interact with specific effectors to initiate downstream signals and functions. The subsequent hydrolysis of bound GTP to GDP is mediated via the intrinsic GTPase activity of these GTP-binding proteins, which, however, is so low that the proteins are totally dependent on GTPase-activating proteins (GAPs) to achieve adequate hydrolysis of GTP (12).

The best-characterized Rho-specific GAP is p190RhoGAP, which was originally reported to be a tyrosyl-phosphorylated protein that coprecipitated with p120RasGAP (15). Molecular cloning of the cognate cDNA of p190RhoGAP has revealed an NH2-terminal region that contains several sequence motifs that are shared by all known GTP-binding proteins, and a C-terminal region with a GAP domain specific for the GTP-binding proteins Rho and Rac (16). Several lines of evidence support the idea that p190RhoGAP somehow participates in rearrangement of actin cytoskeleton. For example, microinjection of the C-terminal region of p190RhoGAP into Swiss 3T3 cells has been found to block serum-induced stress fiber formation (17). McGlade et al. (18) reported that constitutive association of GAP-N (i.e., p120RasGAP lacking the C-terminal Ras-binding domain) with p190RhoGAP increased the GAP activity of this complex, caused a subsequent cellular loss of actin stress fibers, and reduced cellular adhesion. In adherent melanoma cells, Nakahara et al. (19) found that engagement of β2 integrins

3 Abbreviations used in this paper: GEF, guanine nucleotide exchange factor; FN, fibronectin; GAP, GTPase-activating protein; PP1, 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine; RAM, rabbit anti-mouse; EGF, epidermal growth factor; PVDF, polyvinylidene difluoride.
induced increased phosphorylation of p190RhoGAP, which enabled association with filamentous actin. In addition, it was demonstrated (20) that increased tyrosine phosphorylation of p190RhoGAP correlated with rapid and exaggerated disassembly of actin stress fibers. The cited authors suggested that the intrinsic RhoGAP activity of p190RhoGAP might be augmented by phosphorylation, but they have not tested that possibility.

In this paper, we investigated the signal transduction pathways involved in β2 integrin-mediated regulation of RhoA activity in neutrophils. In particular, we researched whether a relationship exists between activation of p190RhoGAP and RhoA.

Materials and Methods

Antibodies

The Abs and their sources were as follows: mAb IB4 (mouse anti-human CD18, IgG2a isotype) was obtained from S. Wright (Rockefeller University, New York, NY) (21), mAb 4G10 (mouse anti-phosphotyrosine) and mouse anti-p190RhoGAP mAb used for immunoprecipitation were obtained from Upstate Biotechnology (Lake Placid, NY); mAb PY20 (mouse anti-phosphotyrosine), the anti-p120RhoGAP mAb, and the polyclonal anti-p120RasGAP antiserum were purchased from Transduction Laboratories (Lexington, KY); the anti-CD59 mAb was purchased from Pharmingen (Heidelberg, Germany); rabbit anti-RhoA antiserum was obtained from Santa Cruz Biotechnology (Santa Cruz, CA); and the negative control monoclonal IgG2a Ab (directed against Aspergillus niger glucose oxidase), peroxidase-conjugated Igs, and rabbit anti-mouse (RAM) Igs were purchased from Dakopatts (Glostrup, Denmark).

Isolation of human neutrophils

Blood from healthy donors was collected and isolated under endotoxin-free conditions as previously described (22). The neutrophils were isolated by neutrophils from Buffy coats (7, 23). Briefly, petri dishes were rotated in a cold room for 1 h. Thereafter, 40 μl of a 50% slurry of protein A-Sepharose for 45 min. The beads were subsequently collected by centrifugation and washed three times in a wash buffer (50 mM HEPES (pH 7.4), 1% Triton X-100, 0.1%, SDS, 150 mM NaCl, and 1 mM Na3VO4). The beads were then resuspended in 2× concentrated Laemmli sample buffer and boiled under reducing conditions for 5 min. The immunoprecipitated proteins were subjected to electrophoresis on 7.5% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) transfer membranes. The membranes were blocked in PBS supplemented with 0.2% Tween 20 and 3% BSA, incubated for 1 h with a primary Ab (1/5,000 dilution of the anti-phosphotyrosine Ab 4G10; 1/750 dilution of the anti-p190RhoGAP Ab or anti-phosphotyrosine Ab), and thereafter washed three times for 15 min in PBS supplemented with 0.2% Tween 20. The membranes were subsequently incubated in Western blotting

Cell lysates were clarified by centrifugation, and the supernatants were immunoprecipitated. This was performed by exposure to the anti-phosphotyrosine Ab (3 μg/ml) or anti-p120RasGAP antiserum (3 μg/ml) for 1 h, and then to 40 μl of a 50% slurry of protein A-Sepharose for 45 min. The beads were subsequently collected by centrifugation and washed three times in a wash buffer (50 mM HEPES (pH 7.4), 1% Triton X-100, 0.1%, SDS, 150 mM NaCl, and 1 mM Na3VO4). The beads were then resuspended in 2× concentrated Laemmli sample buffer and boiled under reducing conditions for 5 min. The immunoprecipitated proteins were subjected to electrophoresis on 7.5% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) transfer membranes. The membranes were blocked in PBS supplemented with 0.2% Tween 20 and 3% BSA, incubated for 1 h with a primary Ab (1/5,000 dilution of the anti-phosphotyrosine Ab 4G10; 1/750 dilution of the anti-p190RhoGAP Ab or anti-phosphotyrosine Ab), and thereafter washed three times for 15 min in PBS supplemented with 0.2% Tween 20. The membranes were subsequently incubated in 1% peroxidase-conjugated anti-mouse IgGs (1/10,000) in PBS supplemented with 0.2% Tween 20 and 3% BSA. The blots were washed, and Ab binding was visualized by ECL.

Measurement of guanyl nucleotides bound to RhoA

Neutrophils were incubated for 2 h in a calcium- and phosphate-free medium (136 mM NaCl, 5.9 mM KCl, 1.2 mM MgSO4, 5.0 mM NaHCO3, 5.5 mM glucose, and 20 mM HEPES) containing the anti-RhoA antiserum (4 μg/ml) and anti-CD18 Ab IB4 for different periods of time. The cells were then lysed in an ice-cold lysis buffer (50 mM HEPES (pH 7.4), 1% Triton X-100, 0.5% deoxycholate, 0.05% SDS, 500 mM NaCl, 15 mM MgCl2, 1 mM EGTA, 10 mM benzamidine, 1 mM NaN3VO4, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM Pefabloc, and 1 μg/ml of pepstatin and antipain containing the anti-RhoA antisera (4 μg/ml)). The lysates were clarified by centrifugation, after which the supernatants were added to the tubes containing the supernatants were added to the tubes containing the supernatant, and then rotated in a cold room for 1 h. Thereafter, 40 μl of a 50% slurry of protein A-agarose was added, and the incubation was continued for an additional 45 min. The collected immune complexes were washed three times in ice-cold wash buffer (50 mM HEPES (pH 7.4), 500 mM NaCl, 0.1% Triton X-100, 0.005% SDS, and 5 mM MgCl2). The nucleotides bound to these immune complexes were eluted at 68°C for 20 min using a medium consisting of 5 mM DTT, 5 mM EGTA, 0.2% SDS, 0.5 mM GTP, and 0.5 mM GMP. The radiolabeled nucleotides bound to RhoA were resolved by TLC using 78 g/L ammonium formate and 9.6% (v/v) concentrated HCl
Measurement of RhoGAP activity

Neutrophils were plated on a surface coated with the anti-CD18 Ab IB4 for different periods of time and then lysed as described above. The p190RhoGAP molecules in each sample were immunoprecipitated (see above) with an anti-p190RhoGAP Ab (3 µg/ml) for 2 h at 4°C. The immune complexes were captured on protein G-agarose beads and washed three times with ice-cold wash buffer (50 mM Tris-HCl (pH 7.5), 1 mg/ml BSA, 10 mM MgCl₂, and 1 mM DTT). Simultaneously, purified RhoA (30 ng) was loaded with [γ-32P]GTP by incubation in a buffer (50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM EDTA, 1 mg/ml BSA, 1 mM DTT, and 1 µM GTP (10 µCi of [γ-32P]GTP) for 10 min at 37°C. The immunoprecipitates were then resuspended in 100 µl of wash buffer and incubated for 10 min at 25°C with 30 ng of RhoA protein (preloaded with [γ-32P]GTP). This reaction was stopped by adding 0.4 µl of ice-cold stop buffer (500 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, and 1 mM DTT). The samples were centrifuged (2000 × g, 10 s), and aliquots (0.25 ml) of the supernatant were filtered through nitrocellulose filters (0.45 µm). The filters were subsequently washed five times with the stop buffer described above. The retained radioactivity was determined in a liquid scintillation counter.

Determination of the translocation of RhoA, p190RhoGAP, and p120RasGAP

Neutrophils were stimulated as described in the figure legends and then directly disrupted by nitrogen cavitation. Briefly, cells were suspended (in 100 mM Tris-HCl (pH 7.5); 5 mM EDTA; 5 mM EGTA; 50 mM NaCl; 5 mM NaF; 1 mM Na₃VO₄; 20 µg/ml aprotinin; 1 µg/ml each of pepstatin, leupeptin, and antipain; 2.5 mM benzamidine; and 2 mM Pefabloc), placed in a cell disruption bomb at 4°C (27), equilibrated at 1000 l/min for 10 min, and then rapidly released. The suspension was collected, and nuclei, heavy membrane fractions, and undisrupted cells were sedimented by centrifugation at 10,000 × g for 10 min. The resulting supernatant was centrifuged at 100,000 × g for 1 h. The obtained pellet contained a crude membrane fraction, which was resuspended in the disruption buffer described above. The protein content was determined (28), and aliquots of the suspension were mixed with the Laemmli buffer supplemented with 50 mM DTT and boiled. The proteins were separated on either 7.5 or 12% SDS-polyacrylamide gels and transferred to nitrocellulose (0.45 µm). The membranes were stained with Ponceau red and analyzed by densitometry. Bands were quantified with ImageJ software (http://rsb.info.nih.gov/ij/).

Results

β₂ integrin engagement-induced activation of RhoA

Engagement of leukocyte β₂ integrins is known to be associated with profound alteration of the cytoskeleton. Moreover, small GTPases have been strongly implicated in cytoskeletal changes brought about by integrins (30). Therefore, we examined the possibility that clustering of neutrophil β₂ integrins regulates the activity of the small GTPase RhoA (the dominating Rho protein in neutrophils). We evaluated RhoA activity in two ways. First, we measured accumulation of [32P]GTP bound to RhoA in neutrophils that had been labeled with [32P]Pi and subjected to engagement of β₂ integrins (Fig. 1). We found both [γ-32P]GTP and [α-32P]GDP bound to RhoA in nonadherent control cells. There was a 20-fold greater amount of [α-32P]GDP bound to RhoA vs [γ-32P]GTP bound to RhoA in resting cells (Fig. 1A). Engagement of β₂ integrins, achieved by plating neutrophils on a surface coated with anti-CD18 Abs, induced a time-dependent increase in both [γ-32P]GTP and [α-32P]GDP bound to RhoA (Fig. 1A). The fold increases in [γ-32P]GTP bound to RhoA vs nonadherent control cells were 4.3 ± 1.0, 5.0 ± 1.8, and 7.9 ± 3.9 in cells plated for 10, 20, and 30 min, respectively (n = 4). The fold increases in [α-32P]GDP bound to RhoA vs nonadherent control cells were 1.26 ± 0.09, 1.48 ± 0.19, and 1.86 ± 0.26 in cells plated for 10, 20, and 30 min, respectively (n = 4). We refrained from examining accumulation of [32P]-labeled guanine nucleotides bound to RhoA for longer than 30 min due to dilution of the labeled GDP/GTP pool. We calculated for each time point of the kinetic the ratio of GDP:GTP + GDP recovered on RhoA, an index of RhoA activation. As shown in Fig. 1B, there was a 2.3-fold increase in the GDP:GTP + GDP ratio recovered on RhoA in cells that were plated for 10 min on an IB4-coated surface compared with nonadherent control cells. However, plating neutrophils on this surface for >10 min did not induce any further significant increase in the GDP:GTP + GDP ratio recovered on RhoA. In contrast, plating neutrophils on a surface coated with a control IgG2a Ab did not result in any increase in the accumulation of either [γ-32P]GTP or [α-32P]GDP bound RhoA compared with nonadherent control cells (Fig. 1A). In addition, there was a significant increase in the GDP:GTP + GDP ratio recovered on RhoA in neutrophils in suspension that had their β₂ integrins engaged (0.086) compared with control cells in suspension (0.018; duplicate experiment). We also evaluated RhoA activity by determining the effect of β₂ integrin engagement on the translocation of RhoA to a membrane-enriched fraction (Fig. 1, C and D). These experiments were based on the well-known fact that translocation of RhoA and other small GTP-binding proteins to the plasma membrane is related to the activation and function of these molecules (13, 14). Plating neutrophils for 30 min on a surface coated with the IB4 Ab resulted in a 2-fold increase in accumulation of RhoA in the membrane fraction (Fig. 1C), and a similar increase was seen upon engagement of the β₂ integrins on nonadherent neutrophils (Fig. 1D). In parallel, there was a slight decrease in RhoA in the cytosol (data not shown).

β₂ integrin-induced modulation of the RhoGAP activity of p190RhoGAP

The rapid hydrolysis of GTP-bound RhoA that occurs following engagement of neutrophil β₂ integrins may be due to an increase in overall RhoGAP activity in the cells. To explore that hypothesis, anti-p190RhoGAP immunoprecipitates were tested for their ability to enhance the GTPase activity of RhoA in vitro. Neutrophils were plated for 0–40 min on a surface coated with the IB4 Ab. Thereafter, the cells were lysed, and anti-p190RhoGAP immunoprecipitates were obtained from the different samples. The ability of the immunoprecipitates to promote GTP hydrolysis was determined by adding purified, bacterially expressed RhoA preloaded with [γ-32P]GTP. In this assay an increase in RhoGAP activity is detected as a decrease in γ-32P label bound to RhoA, and we expressed RhoGAP activity of anti-p190RhoGAP immunoprecipitates as a percentage of the total [γ-32P]GTP hydrolyzed during the 10-min incubation period. We found a time-dependent increase in RhoGAP activity in the immunoprecipitates when neutrophils were plated on an IB4-coated surface. The maximum RhoGAP activity was found in anti-p190RhoGAP immunoprecipitates from cells that were plated for 30–40 min (~2.5-fold increase over nonadherent control cells; Fig. 2A). As indicated in Fig. 3, plating neutrophils on a surface coated with anti-CD18 Ab did not affect the amount of p190RhoGAP immunoprecipitated.

In Fig. 2B, we tested how pretreatment of neutrophils with PP1, a selective inhibitor of Src family tyrosine kinases (31), would affect β₂ integrin-induced activation of RhoGAP activity of p190RhoGAP. We found a significant (1.9-fold) increase in RhoGAP activity in the immunoprecipitates from neutrophils plated for 30 min on a surface coated with an anti-β₂ integrin Ab, but no rise in activity in immunoprecipitates from control neutrophils plated on a surface coated with an IgG2a Ab (Fig. 2B, lower
Furthermore, the $\beta_2$ integrin-induced increase in RhoGAP activity was abolished in cells pretreated with PP1 (Fig. 2B, lower panel). Control experiments revealed that the $[\gamma^{32}\text{P}]$GTP hydrolyzed in nonspecific immunoprecipitates represented only 2.1% of the total amount of RhoA-associated $[\gamma^{32}\text{P}]$GTP (data not shown). As shown in Fig. 2B (top panel), the amount of p190RhoGAP protein that was immunoprecipitated was not affected by the different experimental conditions. We also confirmed the ability of $\beta_2$ integrins to activate p190RhoGAP in neutrophils by plating them on FN, which has been shown to be a natural ligand for $\beta_2$ integrins in the presence of TNF-$\alpha$ (24) (Fig. 2C). Next, we tested whether activation of p190RhoGAP was a direct consequence of $\beta_2$ integrin signaling or was due to cell spreading. To this end, neutrophils were plated for 30 min on a surface coated with anti-CD59 Abs, after which RhoGAP activity in anti-p190RhoGAP immunoprecipitates was measured as described above. We found indications that more p190RhoGAP was immunoprecipitated from cells plated on an anti-CD59 Ab-coated surface. Therefore, we
have normalized the RhoGAP activity of p190RhoGAP immunoprecipitates in Fig. 2C to the amount of p190RhoGAP measured by Western blot analysis. It is clear that plating neutrophils on an FN-coated, but not on an anti-CD59-coated, surface induced activation of p190RhoGAP.

\[ \beta_2 \text{ integrin engagement did not induce tyrosine phosphorylation of p190RhoGAP} \]

After discovering that PP1 blocked the \( \beta_2 \) integrin-induced increase in p190RhoGAP activity, we addressed the question of whether engagement of \( \beta_2 \) integrins affects the tyrosine phosphorylation status of p190RhoGAP. Neutrophils were plated on an anti-CD18-coated surface for different periods of time and then lysed, and p190RhoGAP was subsequently immunoprecipitated with a specific Ab. Anti-phosphotyrosine immunoblotting of these immunoprecipitates indicated that the basal level of tyrosine phosphorylation of p190RhoGAP was not altered by clustering of \( \beta_2 \) integrins, and it was also clear that the basal phosphorylation level of p190RhoGAP was not affected by PP1 (Fig. 3A, top panel). In parallel, control experiments revealed that plating neutrophils on an anti-CD18-coated surface resulted in a time-dependent increase in the overall tyrosine phosphorylation of proteins, an effect that was totally blunted by pretreatment with PP1 (Fig. 3A, bottom panel).

To complement the results presented in Fig. 3A, we investigated tyrosine phosphorylation of p190RhoGAP following Ab-induced engagement of \( \beta_2 \) integrins on neutrophils in suspension (a situation mimicking adhesion-induced integrin clustering) and again found no increase in the indicated phosphorylation (Fig. 3B, top panel). In parallel, control experiments revealed that engagement of \( \beta_2 \) integrins on suspended cells resulted in an increase in the overall tyrosine phosphorylation of proteins, an effect that was totally blocked by pretreatment with PP1 (Fig. 3B, bottom panel).

\[ \beta_2 \text{ integrin engagement induced tyrosine phosphorylation of p190RhoGAP-associated proteins} \]

It is known that p190RhoGAP can associate with phosphoproteins such as p120RasGAP (18). In fact, p190RhoGAP was originally reported to be a tyrosyl-phosphorylated protein that coprecipitated with p120RasGAP (15). Accordingly, we performed experiments to determine whether increased tyrosine phosphorylation of a

\[ \text{FIGURE 2.} \]

\( \beta_2 \) integrin-induced modifications of the GAP activity of p190RhoGAP for RhoA. Neutrophils were incubated for the indicated periods of time on surfaces coated with an anti-CD18 Ab (IB4). Neutrophils were lysed, and clarified lysates were used for immunoprecipitation with an anti-p190RhoGAP Ab. The immunoprecipitates were incubated for 10 min with purified RhoA protein that was preloaded with [γ-32P]GTP.
p190RhoGAP-associated protein could explain the effect of PP1 on \( \beta_2 \) integrin-induced activation of p190RhoGAP. This was accomplished by analyzing anti-phosphotyrosine immunoprecipitates of neutrophils that had had their \( \beta_2 \) integrins engaged for different periods of time by plating on an anti-\( \beta_2 \)-integrin Ab-coated surface (Fig. 4) or by exposure to the Ab in suspension (Fig. 5). Immunoblotting of the anti-phosphotyrosine immunoprecipitates with an anti-p190RhoGAP Ab showed a single band at 190 kDa (Figs. 4A and 5A). A representative time course of the effects of \( \beta_2 \) integrins on the presence of p190RhoGAP in anti-phosphotyrosine immunoprecipitates is depicted in Fig. 4B. Den- sitometric analysis of such blots from several separate experiments revealed that the amount of p190RhoGAP in these immunoprecipitates reached a maximum after 30 min (3.3-fold increase over controls) and returned back to control levels after 40 min (Fig. 4B). Pretreatment of neutrophils with PP1 abolished the presence of p190RhoGAP in all immunoprecipitates (Fig. 4C). Similar results were found after clustering \( \beta_2 \) integrins (Fig. 4C).

\( \beta_2 \) integrin engagement induced tyrosine phosphorylation of p120RasGAP

It is not known whether a p120RasGAP/p190RhoGAP complex (15, 32, 33) exists in neutrophils. To examine that issue, we analyzed anti-p120RasGAP immunoprecipitates of control cells and cells with their \( \beta_2 \) integrins engaged in the absence or the presence of PP1. Western blotting of these immunoprecipitates with an anti-p190RhoGAP Ab demonstrated that the basal level of p190RhoGAP associated with p120RasGAP was not affected by either engagement of \( \beta_2 \) integrins or pretreatment with PP1 (Fig. 6A, top panel). To ensure that equal amounts of p120RasGAP had been immunoprecipitated, the blot was also analyzed with an anti-p120RasGAP Ab (Fig. 6A, middle panel). Reprobing and analysis of the immunoprecipitates with an anti-phosphotyrosine Ab showed that engagement of \( \beta_2 \) integrins significantly increased the low basal level of tyrosine phosphorylation of p120RasGAP and that this phosphorylation was totally blocked by PP1 (Fig. 6A, lower panel). Similarly, plating neutrophils on a surface coated with anti-CD18 Abs resulted in an identical increase in p120RasGAP tyrosine phosphorylation, which was totally blocked by a pretreatment with PP1 (Fig. 6B, lower panel).

**FIGURE 3.** Lack of tyrosine phosphorylation of p190RhoGAP following \( \beta_2 \) integrin engagement. Neutrophils were incubated for the indicated periods of time on surfaces coated with an anti-CD18 Ab (IB4; A), or engagement of \( \beta_2 \) integrins was achieved by incubating suspended neutrophils with mouse anti-human CD18 Ab and subsequently adding RAM IgG (B). These incubations were performed in the absence or the presence of PP1 (3 \( \mu \)M). Thereafter, neutrophils were lysed, and clarified lysates were used for immunoprecipitation with an anti-p190RhoGAP Ab (upper and middle panels in A and B). The immunoprecipitates were subjected to 7.5\% SDS-PAGE, transferred to a PVDF membrane, and then immunoblotted with an anti-phosphotyrosine Ab (indicated as blot PY) as described in Materials and Methods. The arrow indicates the position of p190RhoGAP. After stripping the blots, the membranes were reblotted with an anti-p190RhoGAP Ab (indicated as blot p190RhoGAP) to assess that equal amounts of p190RhoGAP were immunoprecipitated in the different conditions (middle panels in A and B). These blots are representative of eight (A) or 10 (B) experiments performed. Following densitometric analysis of all blots, the ratio of p190RhoGAP tyrosine phosphorylation vs that of total p190RhoGAP immunoprecipitated was calculated for each time point. A. The values are 0.9 for 0 min, 1.1 for 10 min, 1.0 for 20 min, 0.9 for 30 min, 1.0 for 40 min, and 1.0 for 50 min with PP1. B. The values are 1.2 for controls, 0.9 for cells in which \( \beta_2 \) integrins were engaged, and 0.9 for cells pretreated with PP1 before engaging the integrins. Whole lysate extracts were also subjected to 7.5\% SDS-PAGE, transferred to a PVDF membrane, and then immunoblotted with an anti-phosphotyrosine Ab as described above to assess overall tyrosine phosphorylation of proteins (bottom panels in A and B). The M( in kilodaltons) of proteins is indicated on the left.
integrin engagement induced relocalization of p190RhoGAP and p120RasGAP to a membrane fraction

It has been reported that modification of the activity of GAP proteins is associated with relocalization of these molecules (20, 34). Therefore, we investigated the effects of engagement of neutrophil β2 integrins on accumulation of p120RasGAP and p190RhoGAP in a membrane fraction. We found that engagement of the integrins led to an ~1.7-fold increase in p190RhoGAP in the membrane fraction, and this effect was blocked by PP1 (Fig. 7A); the same was seen for p120RasGAP (Fig. 7B). Similar results were obtained by plating the neutrophils on an anti-CD18 Ab-coated surface (data not shown). In contrast, PP1 did not affect the β2 integrin-induced translocation of RhoA to the membrane fraction (Fig. 7C). As shown in Fig. 7D, neither β2 integrin engagement nor PP1 treatment had an impact on the amount of the membrane marker HLA Cl-1 in the analyzed membrane fractions, which agrees well with previously published data (35).

**Effect of PP1 on β2 integrin-induced activation of RhoA in vivo**

Additional experiments were performed to determine whether the β2 integrin-induced effects on p190RhoGAP and p120RasGAP were related to regulation of RhoA activity in vivo. To this end, neutrophils were or were not pretreated with PP1 and then plated for 30 min on a surface coated with the IB4 Ab. Thereafter, we measured the level of radiolabeled guanine nucleotides bound to RhoA. As shown in Fig. 8, in the presence of PP1 the ratio of GTP·GDP on RhoA was increased by 28% (p < 0.01); albeit small, this increase is in the same range as that found by other investigators (36). Thus, activation and translocation of
We measured the amounts of $^{32}$P-labeled guanine nucleotides associated with immunoprecipitated RhoA, and our results demonstrate that both $[^{32}P]GDP$ and $[^{32}P]GTP$ were bound to RhoA in resting suspended neutrophils, with RhoA-$[^{32}P]GDP$ being the predominant guanine nucleotide-bound form. Engagement of $\beta_3$ integrins on neutrophils induced a time-dependent increase in both $[^{32}P]GTP$ and $[^{32}P]GDP$ bound to RhoA. The increase in RhoA-associated $[^{32}P]GTP$ agrees with our previous finding that $\beta_3$ integrin engagement causes phosphorylation of the exchange factor Vav (7) and also with the recent conclusion that Vav-2 is a GEF for the RhoA subfamily (37). Engagement of $\beta_2$ integrins induced a rapid activation of RhoA as reflected by the 2.5-fold increase in the GTP-GTP + GDP ratio recovered on RhoA in neutrophils that were plated for 10–30 min on an IB4-coated surface. The GTP:GTP + GDP ratio was maximum in neutrophils that had been plated for 10 min on an anti-CD18 Ab surface. Despite the fact that there was a gradual increase in GTP-bound RhoA over the time kinetic, we could not observe any further increase in this ratio when the neutrophils were plated for longer periods of time on an anti-CD18 Ab-coated surface.

In a resting cell most members of the Rho family are maintained as cytosolic complexes with Rho-GDP dissociation inhibitor protein (38, 39). Stimulation with an agonist causes these complexes to dissolve, whereupon Rho proteins are released and translocated to their site of action at the plasma membrane (13, 14, 40, 41). Linking of these molecules to the membrane is associated with an exchange of GDP for GTP; hence the Rho-membrane interaction may be controlled by membrane-associated GEFs (13, 14). It is also possible that recruitment of Rho to the plasma membrane involves other targets for activated Rho (42–46). If such movement of Rho does occur, it is considered to reflect activation of this GTPase regardless of what mechanism(s) is involved in the translocation of Rho-GTP-binding proteins to the membrane and despite the finding that such translocation is probably not required for formation of stress fibers (41, 47). In support of our previously mentioned data in this study we found that engagement of $\beta_3$ integrins in both adherent and nonadherent neutrophils led to increased accumulation of RhoA in the membrane fraction. In subsequent experiments, we investigated the mechanisms by which clustering of $\beta_3$ integrins could give rise to a rapid accumulation of RhoA-bound $[^{32}P]GDP$. We (present study) and others (48, 49) propose that this can be explained by the existence of an inducible intrinsic GTPase activity in leukocytes, whereby the exchange of GDP for GTP on RhoA is immediately counteracted by conversion of bound GTP to GDP. Accordingly, we were eager to determine whether a relationship exists between accumulation of $[^{32}P]GDP$-bound RhoA (which reflects a rapid turnover of guanine nucleotides on RhoA) and activation of p190RhoGAP, a GAP protein for RhoA (16, 17). We found that $\beta_2$ integrin engagement induced a rise in the RhoGAP activity of anti-p190RhoGAP immunoprecipitates, an effect that was totally blocked by pretreating cells with the selective Src family tyrosine kinase inhibitor PP1. In accordance with this, it has been shown that Src family tyrosine kinases, such as p59$^{61D}$, p58$^{c-fgr}$, and p53$^{56lyn}$, play essential roles in $\beta_2$ integrin signaling and regulation of neutrophil adherence and spreading (6, 9). Consequently, these tyrosine kinases are conceivable candidates for mediating the $\beta_2$ integrin-induced up-regulation of p190RhoGAP activity. However, in neutrophils plated on an anti-CD18 Ab surface, activation of p190RhoGAP was slow compared with the overall tyrosine phosphorylation of proteins. This raises the possibility that up-regulation of RhoGAP activity is not a direct consequence of $\beta_2$ integrin signaling, but perhaps is initiated by cytoskeletal rearrangements and cell spreading. However, we can rule out this possibility, since

p190RhoGAP to the membrane must contribute at least in part to $\beta_2$ integrin-induced deactivation of RhoA.

**Discussion**

The extensive changes in morphology exhibited by neutrophils during spreading and locomotion are associated with $\beta_2$ integrin-induced intracellular signals that, among other things, cause rearrangement of the actin-based cytoskeleton (10). In many cell types the small GTPases of the Rho subfamily have been implicated in cytoskeletal modulations generated by growth factors (11) and integrins (30). However, it is not yet known whether $\beta_2$ integrins actually participate in activation of the Rho family GTPases in neutrophils or how such involvement might occur.

**FIGURE 5.** Engagement of $\beta_2$ integrins on suspended neutrophils triggers tyrosine phosphorylation of p190RhoGAP-associated proteins. Engagement of $\beta_2$ integrins was accomplished by incubating suspended neutrophils with mouse anti-human CD18 Ab (IB4; 10 μg/ml) and subsequently adding RAM IgGs as described in Materials and Methods. This was done in the absence or the presence of the Src family specific tyrosine kinase inhibitor PP1 (3 μM). The neutrophils were lysed, and clarified lysates were used for immunoprecipitation with an anti-p190RhoGAP Ab. The immunoprecipitated proteins were subjected to densitometric analysis of the level of phosphotyrosine Ab. The immunoprecipitates, an effect that was totally blocked by pretreating the cells with the selective Src family specific tyrosine kinase inhibitor PP1. In accordance with this, it has been shown that Src family tyrosine kinases, such as p59$^{61D}$, p58$^{c-fgr}$, and p53$^{56lyn}$, play essential roles in $\beta_2$ integrin signaling and regulation of neutrophil adherence and spreading (6, 9). Consequently, these tyrosine kinases are conceivable candidates for mediating the $\beta_2$ integrin-induced up-regulation of p190RhoGAP activity. However, in neutrophils plated on an anti-CD18 Ab surface, activation of p190RhoGAP was slow compared with the overall tyrosine phosphorylation of proteins. This raises the possibility that up-regulation of RhoGAP activity is not a direct consequence of $\beta_2$ integrin signaling, but perhaps is initiated by cytoskeletal rearrangements and cell spreading. However, we can rule out this possibility, since

p190RhoGAP to the membrane must contribute at least in part to $\beta_2$ integrin-induced deactivation of RhoA.
there was no increase in RhoGAP activity in anti-p190RhoGAP immunoprecipitates from neutrophils spread on an anti-CD59 Ab-coated surface. Thus, a specific signal emanating from β2 integrins is responsible for the activation of p190RhoGAP.

Various reports have described increased tyrosine phosphorylation of p190RhoGAP in different types of cells stimulated with a variety of agonists (19, 20, 50, 51). Chang et al. (20) noticed a correlation between the cellular level of tyrosine-phosphorylated p190RhoGAP and actin stress fiber disassembly in K + cells overexpressing pp60 c-Src, and these investigators suggested that a covalent modification of p190RhoGAP may regulate the GAP activity of that protein toward Rho/Rac proteins. In contrast, although we did detect basal tyrosine phosphorylation of p190RhoGAP in neutrophils, we found no increase in p190RhoGAP phosphorylation following engagement of β2 integrins, and that was confirmed by measurements of p120RasGAP bound to p190RhoGAP. Indeed, formation of this complex is regulated predominantly by interaction between the phosphorylated tyrosine residue of p190RhoGAP and the SH2 domains of p120RasGAP (32, 33); thus the amount of the complex indirectly reflects the level of tyrosine phosphorylation of p190RhoGAP. We found that β2 integrin engagement did not affect the amount of p120RasGAP associated with p190RhoGAP in resting neutrophils. Accordingly, our results indicate that tyrosine phosphorylation of p190RhoGAP per se does not play a role in regulating β2 integrin-dependent up-regulation of p190RhoGAP activity. In agreement, Roof and coworkers (32) detected epidermal growth factor (EGF)-induced phosphorylation of p190RhoGAP only in 10T1/2 cells overexpressing EGF receptor and c-Src but not in 10T1/2 Neo control cells. The contrasting results of Schieffer and colleagues (50), who reported angiotensin II-induced phosphorylation of p190RhoGAP, is most likely explained by their approach to only isolate anti-phosphotyrosine immunoprecipitates to evaluate the phosphorylation statuses of p190RhoGAP.

As mentioned previously, p190RhoGAP is found associated with p120RasGAP, and this complex is predominantly cytosolic (52). Furthermore, p120RasGAP can form a complex with p62 (15) and SHC proteins (53). In fibroblasts, p120RasGAP has been shown to be tyrosine phosphorylated upon EGF stimulation and by overexpression of v-Src (15). Therefore, we investigated the possibility that β2 integrin-induced modification of p190RhoGAP activity is due to increased tyrosine phosphorylation of p120RasGAP. We immunoprecipitated tyrosine-phosphorylated proteins from a lysate extract using an anti-phosphotyrosine Ab and then blotted the immunoprecipitated fraction with an anti-p190RhoGAP Ab. This approach should reflect tyrosine phosphorylation of p190RhoGAP-associated proteins, because in our previous experiments it was obvious that clustering of β2 integrins did not cause increased tyrosine phosphorylation of p190RhoGAP. We found that engagement of β2 integrins transiently increased tyrosine phosphorylation of a p190RhoGAP-associated protein(s), an effect that was totally blocked by PP1. In parallel, clustering of the integrins induced relocalization of p190RhoGAP to a membrane fraction. Interestingly, although the integrin clustering did not increase tyrosine phosphorylation of p190RhoGAP, translocation of this RhoGAP to the membranes was blocked by PP1. Consequently, it is likely that the β2 integrin-induced relocalization of p190RhoGAP to the membrane is due to β2 integrin-induced tyrosine phosphorylation of a p190RhoGAP-associated protein(s).

Simultaneous redistribution of p190RhoGAP and p120RasGAP into perinuclear concentric arcs has been described in C3H10T1/2 murine fibroblasts overexpressing c-Src after being stimulated with EGF (20) and in mouse fibroblasts during integrin-mediated interaction with a substrate (34). We found that engagement of β2 integrins induced tyrosine phosphorylation and translocation of p120RasGAP to a membrane-enriched fraction. Clearly, it is the phosphorylation of p120RasGAP that regulates translocation of the protein to the membrane, since both these events were completely blocked by PP1. This finding agrees with the work of Park and Jove (54) showing that one role of tyrosine phosphorylation of p120RasGAP is to increase the association of this RasGAP with the membrane. Thus, together these results suggest that the β2
integrin-induced tyrosine phosphorylation of p120RasGAP is, directly or indirectly, responsible for the \( \beta_2 \) integrin-induced activation and translocation of p190RhoGAP to a membrane fraction. In addition to blocking \( \beta_2 \) integrin-induced activation and translocation of p190RhoGAP to the membrane, PP1 also increased the ratio of GTP to GDP-GTP on RhoA from neutrophils with stimulated \( \beta_2 \) integrins. Thus, p190RhoGAP appears to be involved in the \( \beta_2 \) integrin-induced regulation of RhoA activity in neutrophils in vivo. In support of that, it has been suggested that EGF-induced relocation of p120RasGAP and p190RhoGAP in C3H10T1/2 murine fibroblasts participates in the regulation of Rho activity, since translocation of those proteins to the membrane was accompanied by EGF-induced disassembly and reassembly of actin stress fibers (20). Another important observation is that PP1 did not block \( \beta_2 \) integrin-induced relocation of RhoA to the membrane, which explains why the \( \beta_2 \) integrin-induced accumulation of Rho-bound GTP was not blunted by PP1. The latter finding suggests that the \( \beta_2 \) integrin-induced activation of RhoA guanine nucleotide exchange activity is regulated by a signaling pathway that does not depend on a Src family tyrosine kinase.

**FIGURE 7.** Engagement of \( \beta_2 \) integrins on suspended neutrophils triggers relocation of p120RasGAP and p190RhoGAP to the membrane. Engagement of \( \beta_2 \) integrins was accomplished in suspended neutrophils as described in the legend to Fig. 5. The neutrophils were subsequently disrupted by nitrogen cavitation, and membrane fractions were prepared. The membrane proteins were resolved on either 7.5 or 12% SDS-PAGE, transferred to PVDF membranes, and immunoblotted with anti-p190RhoGAP Ab (A), anti-p120RasGAP Ab (B), or anti-RhoA Ab (C). The top inserts in A–C depict representative Western blots; the arrows indicate the positions of p190RhoGAP (A), p120RasGAP (B), and RhoA (C). The lanes in the blots are in the same order as the bars in the corresponding panel. The bars show the results of densitometric analysis of each individual blot. Amounts of p120RasGAP, p190RhoGAP, and RhoA are expressed as a percentage of unstimulated control values and are the mean ± SEM of six (A), three (B), or 10 (C) experiments. D, The amount of a membrane marker HLA Cl-1 was measured by a mixed ELISA method (see Materials and Methods). The amounts of HLA Cl-1 protein in membrane fractions are expressed as a percentage of that in control cells and are the mean ± SEM of four separate experiments. Statistical significance vs control: **, \( p < 0.01; \) ***, \( p < 0.001 \) (by unpaired Student's t test).
FIGURE 8. Effect of the tyrosine kinase inhibitor PP1 on the ratio of GTP:GDP + GTP recovered on RhoA following β2 integrin engagement. Suspended neutrophils were incubated for 2 h with [32P]orthophosphate and then pretreated, or not, with PP1 (3 µM) for 10 min. Thereafter, cells were plated for 30 min on surfaces coated with an anti-CD18 Ab (IB4). Neutrophils were lysed, and clarified lysates were used for immunoprecipitation with an anti-RhoA antiseraum. Guanine nucleotides bound to RhoA were eluted and separated by TLC. The TLC plates were subjected to Phosphorimage analysis. The amounts of GTP and GDP were quantified as described in the legend to Fig. 1. The ratio of GTP:GDP + GTP recovered on immunoprecipitated RhoA was calculated. The data are the mean ± SEM of six separate experiments performed in duplicate. Statistical significance vs control: **, p < 0.01 (by paired Student’s t test).

Acknowledgments

We are grateful to Dr Bengt Hallberg for very helpful suggestions, to Dr. Niels Borregaard and Charlotte Horn for quantification of HLA C1-protein, and to Patty Ödman for linguistic revision of this manuscript.

References


