A G_i-dependent Pathway Is Required for Activation of the Small GTPase Rap1B in Human Platelets*

Paolo Lova‡§, Simona Paganini‡, Fabiola Sinigaglia§, Cesare Balduini‡, and Mauro Torti‡¶

From the ‡Department of Biochemistry, University of Pavia, via Bassi 21, 27100 Pavia, Italy and the §Department of Medical Sciences, University "A. Avogadro," via Solaroli 17, 28100 Novara, Italy

Stimulation of human platelets by cross-linking of the low affinity receptor for immunoglobulin, FcγRIIA, caused the rapid activation of the small GTPase Rap1B, as monitored by accumulation of the GTP-bound form of the protein. This process was totally dependent on the action of secreted ADP since it was completely prevented in the presence of either apyrase or creatine phosphate and creatine phosphokinase. Dose-dependent experiments revealed that the inhibitory effect of ADP scavengers was not related to the reduced increase of cytosolic Ca^{2+} concentration in stimulated platelets. Activation of Rap1B induced by clustering of FcγRIIA was totally suppressed by AR-C69931MX, a specific antagonist of the G_i-coupled ADP receptor P2Y12, but was not affected by blockade of the G_q-coupled receptor, P2Y1. Similarly, direct stimulation of platelets with ADP induced the rapid activation of Rap1B. Pharmacological blockade of the P2Y1 receptor totally prevented ADP-induced Ca^{2+} mobilization but did not affect activation of Rap1B. By contrast, prevention of ADP binding to the P2Y12 receptor totally suppressed activation of Rap1B without affecting Ca^{2+} signaling. In platelets stimulated by cross-linking of FcγRIIA, inhibition of Rap1B activation by ADP scavengers could be overcome by the simultaneous recruitment of the G_i-coupled α_{2A}-adrenergic receptor by epinephrine. By contrast, serotonin, which binds to a G_q-coupled receptor, could not restore activation of Rap1B. When tested alone, epinephrine was found to be able to induce GTP binding to Rap1B, whereas serotonin produced only a slight effect. Finally, activation of Rap1B induced by stimulation of the G_q-coupled thromboxane A_2 receptor by U46619 was completely inhibited by ADP scavengers under conditions in which intracellular Ca^{2+} mobilization was unaffected. Inhibition of U46619-induced Rap1B activation was also observed upon blockade of the P2Y12 but not of the P2Y1 receptor for ADP. These results demonstrate that stimulation of a G_i-dependent signaling pathway by either ADP of epinephrine is necessary and sufficient to activate the small GTPase Rap1B.

Rap1 proteins are members of a family of small GTPases highly related to the product of the ras protooncogene. Two isoforms of Rap1 are known, Rap1A and Rap1B: they share more than 90% sequence homology but are differently expressed in different cell types. For instance, in human platelets, expression of Rap1B is particularly high as it accounts for about 0.1% of the total cellular proteins, whereas Rap1A is almost undetectable (1, 2). For this reason, human platelets represent an excellent experimental model to study the biological and functional properties of Rap1B. In resting platelets, Rap1B is mainly located at the membrane as a consequence of post-translational modifications, including isoprenylation, proteolysis, and carboxylmethylation (3). Upon stimulation of platelets with extracellular agonists, Rap1B associates with the actin-based cytoskeleton (4), whereas upon platelet treatment with antagonists, such as prostacyclin, Rap1B undergoes cAMP-dependent phosphorylation and translocates from the membrane to the cytosol (5). Although Rap1B has been suggested to be involved in a number of cellular processes, its exact function in human platelets is still poorly understood. Like other GTPases, Rap1B is activated by binding of GTP. Several factors able to stimulate the exchange of GDP for GTP on Rap1 proteins have been discovered (6). These exchange factors can be activated by different intracellular messengers, including Ca^{2+}, cAMP, protein kinase C, and tyrosine kinases (6). In human platelets, Rap1B is rapidly activated by thrombin, the most potent extracellular agonist (7, 8). Experiments with cell-permeable Ca^{2+}-chelating agents and with Ca^{2+} ionophores have suggested that the rapid activation of Rap1B induced by thrombin is mediated by the increase of cytosolic Ca^{2+} concentration (8). Moreover, a second, delayed phase of Rap1B activation has been observed and found to be regulated by protein kinase C (9). Therefore, multiple pathways for Rap1B activation clearly exist in human platelets. In addition to thrombin, a number of other strong and weak platelet agonists, including ADP, collagen, and PAF, have been described to induce binding of GTP to Rap1B (8), but the biochemical mechanism underlying their action has not been characterized as yet.

In the most recent studies, a mounting body of evidence indicates that platelet aggregation induced by many different agonists results from concomitant signaling through both G_q- and G_i-coupled receptors. This concept has been initially developed upon studies on ADP-induced platelet activation. ADP binds to two different membrane receptors coupled to heterotrimeric G-proteins: the P2Y1 receptor, coupled to G_q (10, 11), and the recently cloned P2Y12 receptor, coupled to G_i (12, 13). Binding of ADP to the P2Y1 receptor induces phospholipase C activation, Ca^{2+} mobilization, and platelet shape change (10, 11) but is unable to trigger aggregation unless the G_i-coupled P2Y12 receptor is concomitantly activated (14). Interestingly, when the P2Y12 receptor is blocked by selective antagonists, full platelet response to ADP can be restored by the simultaneous activation of the G_i-coupled α_{2A}-adrenergic receptor by epinephrine (14). The critical role of G_i activation is also sup-

¶ To whom correspondence should be addressed: Dept. of Biochemistry, University of Pavia, via Bassi 21, 27100 Pavia, Italy. Tel.: 39-0382-507238; Fax: 39-0382-507240; E-mail: mtorti@unipv.it.

ported by many other findings. The P2Y12 receptor has been recently cloned and recognized as the target of antithrombotic drugs, such as clopidogrel and ticlopidine (12, 15), as well as a number of ATP analogues of the AR-C series (16). Moreover, this receptor is defective in patients with a selective congenital impaired response to ADP (17, 18). Finally, in platelets from G$_q$ knockout mice, high concentrations of ADP can still induce partial aggregation by binding to the P2Y12 receptor (19).

Several findings also indicate that the requirement for a G$_i$ pathway for full platelet activation is not restricted to ADP but is a general feature of many platelet agonists. For instance, the thromboxane A$_2$ analogue U46619 binds to a specific receptor on the platelet surface that is coupled to G$_q$ (20). U46619-induced platelet aggregation has been found to rely on the simultaneous stimulation of a G$_i$-dependent pathway by either secreted ADP or epinephrine (21). Similarly, platelet aggregation induced by the thrombin receptor-activating peptide, TRAP, which is a much weaker agonist than thrombin, is reversed by ADP scavengers or by selective antagonists of the P2Y12 receptor (22). Finally, even when platelet stimulation is promoted by the recruitment of receptors that are linked to tyrosine kinase-based signaling pathways rather than heterotrimeric G-proteins, such as in the case of FcγRIIA[1] cross-linking, platelet responses largely depend on the activation of the G$_i$-coupled receptor P2Y12 by secreted ADP (23). Although the essential role of a G$_i$-mediated signaling pathway in potentiating platelet activation by many agonists is very well documented, the exact mechanism of this effect is poorly understood. Several results indicate that inhibition of adenylyl cyclase by the G$_i$ α-subunit may not be relevant, and therefore, suggest the involvement of a still unidentified intracellular effector (24–26).

In this work, we have investigated the possible link between stimulation of G$_i$-dependent pathways and activation of the small GTPase Rap1B. We have found that under a number of the experimental models examined, including platelet stimulation with U46619, epinephrine, or ADP, or by clustering of the FcγRIIA, activation of Rap1B is absolutely dependent on stimulation of a membrane receptor coupled to G$_i$. This results reveal a new link between heterotrimeric G-proteins of the G$_i$ family and small GTPases of the Rap family and suggest a potential mechanism responsible for the potentiation of platelet activation by G$_i$-coupled receptors.

MATERIALS AND METHODS

Materials—Sepharose CL-2B, GSH-Sepharose 2B, and the enhanced chemiluminescence substrate were for Amersham Biosciences, Inc. The thromboxane A$_2$ analogue U46619, ADP, thrombin, epinephrine, sheep anti-mouse F(ab')$_2$ fragments, acetylsalicylic acid, and A3P5PS were from Sigma. AR-C69931MX was a generous gift from AstraZeneca R&D, Charnwood, UK. Fura-2/AM was from Calbiochem. The monoclonal antibody IV.3 against the FcγRIIA was obtained from Medarex. The rabbit polyclonal antiserum against Rap1B was described previously (3). The cDNA for the rap binding domain (RBD) of ralGDS was kindly provided by Dr. J. L. Bos (Department of Physiological Chemistry, University of Utrecht, The Netherlands). Peroxidase-conjugated goat anti-rabbit IgG were from Bio-Rad.

Platelet Isolation and Stimulation—Human platelets from healthy donors were prepared by gel filtration on Sepharose CL-2B and eluted with Hepes buffer (10 mM HEPES, 137 mM NaCl, 2.9 mM KCl, 12 mM NaHCO$_3$, pH 7.4) as described previously (27). Platelet concentration was adjusted to 0.35 × 10⁹ platelets/ml. Platelet samples (0.5 ml) were incubated at 37 °C in an aggregometer under constant stirring, and upon the addition of 1 mM CaCl$_2$, stimulated with the indicated ago-

nists. Cross-linking of FcγRIIA was obtained by incubation of platelets with 2 μg/ml monoclonal antibody IV.3 for 2 min followed by the addition of 30 μg/ml sheep anti-mouse F(ab')$_2$ fragments. Other platelet samples were stimulated with ADP (10 μM), epinephrine (1 μM), serotonin (5 μM), thromboxane A$_2$ analogue U46619 (10 μM), or thrombin (0.6 units/ml). Platelet stimulation was typically performed for 1 min unless otherwise stated. When indicated, 1 unit/ml apyrase, 5 mM CP, 40 units/ml CPK, 500 μM A3P5PS, or 100 nM AR-C69931MX were added to the platelet samples 2 min before stimulation.

Rap1B Activation Assay—Measurement of Rap1B activation was performed essentially as described by Franke *et al.* (8), exploiting the specific and selective ability of the GST-tagged rap binding domain (GST·RBD) of ralGDS immobilized on GSH-Sepharose to bind and precipitate the active, GTP-bound form of Rap1B from a platelet lysate. Platelet stimulation was stopped by the addition of an equal volume of ice-cold modified 2× RIPA buffer (100 mM Tris/HCl, pH 7.4, 400 mM NaCl, 5 mM MgCl$_2$, 2% Nonidet P-40, 20% glycerol, 2 mM phenylmethylsulfonyl fluoride, 2 μM leupeptin, 0.2 μM aprotinin, 0.2 mM Na$_3$VO$_4$). Cell lysis was performed on ice for 10 min. Lysates were clarified by centrifugation at 13,000 rpm in an Eppendorf microcentrifuge for 10 min at 4 °C. Recombinant purified GST·RBD was coupled to GSH-Sepharose by incubating 200 μg of the protein with 100 μl of GSH-Sepharose (75% slurry) for 2 h at room temperature under constant tumbling. In preliminary experiments, we determined that under these conditions, all the added GST·RBD was immobilized on the resin. GSH-Sepharose-coupled GST·RBD was added to the cleared platelet lysates (20 μg of GST·RBD/sample), and precipitation of GTP-bound Rap1B was performed by incubation at 4 °C for 45 min. The precipitates were collected by brief centrifugation, and the beads were washed three times with modified 1× RIPA buffer and finally resuspended with 25 μl of SDS sample buffer (25 mM Tris, 192 mM glycine, pH 8.3, 4% SDS, 1% dithiothreitol, 20% glycerol, and 0.02% bromphenol blue). Precipitated Rap1B was separated by SDS-PAGE on 10–20% acrylamide gradient gels and transferred to nitrocellulose. The presence of active Rap1B in precipitates with GST·RBD was evaluated by staining the nitrocellulose filters with a specific polyclonal antiserum directed against Rap1B, used at a final dilution of 1:1000. Reactive proteins were detected by enhanced chemiluminescence reaction. All the presented figures are representative of at least three separate experiments.

Measurement of Cytosolic Ca^{2+} Concentration—Platelets were prepared as described above with slight modifications. Platelet-rich plasma was incubated with 3 μM Fura-2/AM for 30 min at 37 °C. Platelets were recovered by centrifugation at 300 × *g* for 10 min at room temperature and resuspended in a small volume (0.5–1 ml) of autologous plasma. Platelets were then isolated by gel filtration on Sepharose CL-2B and eluted with Hepes buffer containing 0.5% bovine serum albumin and 5.5 mM glucose. Platelet count was then adjusted to 2 × 10⁸ platelets/ml. Measurement of cytosolic Ca^{2+} was performed on 0.4-ml samples prewarmed at 37 °C under gentle stirring in a Perkin-Elmer LS3 spectrofluorimeter in the presence of either 1 mM CaCl$_2$ or 1 mM EGTA. The fluorescence excitation and emission wavelengths were 340 and 510 nm, respectively. Fura-2 fluorescence signals were calibrated according to the method of Pollock *et al.* (28). F_{max} was determined by the addition of 2% Triton X-100 and saturating concentrations of CaCl$_2$, whereas F_{min} was determined by the addition of 2 mM EGTA and 20 mM Tris base. All determinations were repeated at least three times with platelets from different donors.

RESULTS

Activation of Rap1B in Human Platelets Stimulated by Cross-linking of FcγRIIA—Samples of gel-filtered platelets were placed in an aggregometer under constant stirring and treated with 2 μg/ml anti-FcγRIIA mAb IV.3 and 30 μg/ml sheep anti-mouse F(ab')$_2$ fragments for increasing times. After cell lysis, the active, GTP-bound form of Rap1B was selectively precipitated with GST·RBD and identified by immunoblotting using a specific polyclonal antiserum. Fig. 1*A* shows that clustering of FcγRIIA caused a strong and rapid activation of Rap1B that was already maximal after 30 s of stimulation. The activation of Rap1B clearly preceded platelet aggregation. The amount of active Rap1B was found to decrease progressively after prolonged stimulation, in parallel with the progression of platelet aggregation (Fig. 1*A*). Although platelets were stimulated in the presence of 1 mM CaCl$_2$, activation of Rap1B was found to be independent of extracellular calcium since it was
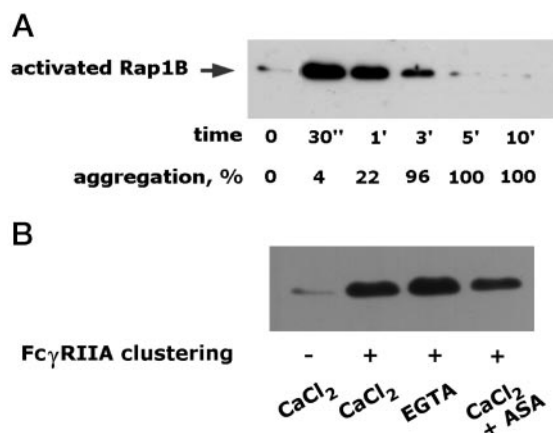

FIG. 1. **Activation of Rap1B by clustering of FcγRIIA.** *A*, Gel-filtered platelets were prewarmed at 37 °C in an aggregometer under constant stirring and stimulated by clustering of FcγRIIA with 2 µg/ml mAb IV.3 and 30 µg/ml sheep anti-mouse F(ab′)$_2$ fragments for the indicated times. Platelet aggregation was monitored continuously during stimulation. Platelet samples were then lysed, and activated Rap1B was precipitated with GST-tagged RBD immobilized on GSH-Sepharose. Precipitated, GTP-bound Rap1B was separated by SDS-PAGE on a 10–20% acrylamide gradient gel, transferred to nitrocellulose, and identified by immunoblotting with a specific polyclonal antiserum. *B*, Platelets were preincubated with either 1 mM acetylsalicylic acid (*ASA*) or buffer for 30 min at 37 °C and then stimulated by cross-linking of FcγRIIA for 1 min in the presence of either 1 mM CaCl$_2$ or 1 mM EGTA. Activated Rap1B was precipitated and detected by immunoblotting with a specific antiserum.
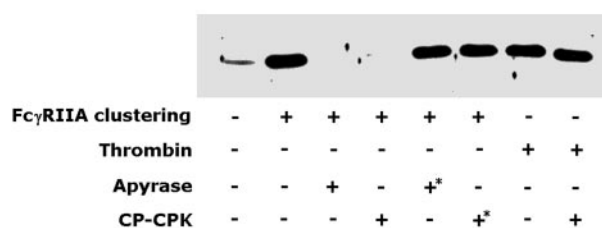

FIG. 2. **Inhibition of Rap1B activation by ADP scavengers in platelets stimulated through FcγRIIA cross-linking.** Gel-filtered platelets were incubated at 37 °C in the absence or presence of 1 unit/ml apyrase or 5 mM CP and 40 units/ml CPK for 2 min. Samples were then stimulated by clustering of FcγRIIA or by thrombin (0.6 units/ml) for 1 min. Platelets were then lysed, and activated Rap1B was isolated by binding to GST·RBD and detected by immunoblotting. When present, the *asterisks* identify samples in which apyrase or CP-CPK was added immediately after cell lysis rather than before stimulation.

TABLE I
Effect of ADP scavengers on FcγRIIA-mediated increase of cytosolic Ca^{2+} concentration

Fura-2-loaded platelets were prewarmed at 37 °C in the presence of 1 mM CaCl$_2$, and incubated without or with 1 unit/ml apyrase or 5 mM CP and 40 unit/ml CPK for 2 min. Platelets were then stimulated by clustering of FcγRIIA by addition of 2 µg/ml mAb IV.3 and 30 µg/ml sheep anti-mouse F(ab′)$_2$ fragments. The cytosolic concentration of Ca^{2+} was calculated as described under "Material and Methods."

Treatment	[Ca^{2+}]$_i$
	nM
None	45 ± 12 ($n = 13$)
FcγRIIA clustering	397 ± 58 ($n = 6$)
Apyrase + FcγRIIA clustering	335 ± 41 ($n = 3$)
CP-CPK + FcγRIIA clustering	159 ± 28 ($n = 4$)

also observed when 1 mM EGTA was present (Fig. 1*B*). Moreover, it did not require the production of thromboxane A$_2$ since it was only minimally affected by the treatment of platelets with acetylsalicylic (Fig. 1*B*).

Role of Secreted ADP in FcγRIIA-mediated Activation of Rap1B—It has been recently shown that FcγRIIA-mediated platelet activation is largely dependent on the action of secreted ADP (23, 29). To evaluate the impact of ADP on Rap1B activation, cross-linking of FcγRIIA was performed in the presence of two unrelated ADP scavengers, apyrase and CP-CPK. Fig. 2 shows that in the presence of these ADP scavengers, FcγRIIA-mediated activation of Rap1B was completely inhibited. The effect of apyrase and CP-CPK was found to be specific and selective since both scavengers were inactive when added immediately after cell lysis, and CP-CPK did not affect thrombin-induced activation of Rap1B (Fig. 2). Therefore, FcγRIIA-mediated activation of Rap1B was totally dependent on the action of secreted ADP.

In previous studies, activation of Rap1B has been reported to be mediated by an increase of the intracellular Ca^{2+} concentration (8). Moreover, the ADP scavenger CP-CPK has been found to inhibit Ca^{2+} mobilization from internal stores induced by clustering of FcγRIIA (23). Therefore, we verified whether inhibition of Rap1B activation by ADP scavengers was a consequence of the reduced cytosolic Ca^{2+} increase. Using Fura-2-loaded platelets, we actually confirmed that, even in the presence of extracellular CaCl$_2$, both apyrase and CP-CPK reduced the increase of Ca^{2+} concentration triggered by clustering of FcγRIIA (Table I). The two scavengers were constantly found to be differently effective since reduction of cytosolic Ca^{2+} increase was greater with CP-CPK (about 60%) than with apyrase (about 20%). Using ADP as agonist, we found that both apyrase and CP-CPK totally prevented cytosolic Ca^{2+} increase, indicating that they were equally effective in neutralizing ADP (data not shown). Moreover, the addition of P2Y1 and P2Y12 receptor antagonists to apyrase-treated platelets did not result in any further reduction of intracellular Ca^{2+} increase induced by clustering of FcγRIIA (data not shown),

indicating that the ADP scavenger is actually able to completely neutralize released ADP at the microenvironment of the platelet cell surface. These results indicate that, even when secreted ADP was neutralized, a residual albeit variable increase of cytosolic Ca^{2+} persisted in platelets stimulated by cross-linking of FcγRIIA. We thus treated platelets with decreasing concentrations of anti-FcγRIIA monoclonal antibody IV.3. Fig. 3*A* shows that treatment of platelets with 0.2 µg/ml IV.3 caused an increase of cytosolic Ca^{2+} similar to that observed in platelets stimulated with 2 µg/ml IV.3 in the presence of CP-CPK and still lower than that measured in the presence of apyrase. In similar dose-dependent studies, activation of Rap1B was found to occur at concentrations of IV.3 as low as 0.1 µg/ml (Fig. 3*B*). These results indicate that inhibition of FcγRIIA-induced activation of Rap1B by ADP scavengers is not due to the reduced increase of intracellular Ca^{2+}.

Activation of Rap1B Requires a G$_i$-dependent Pathway—ADP binds to two different purinergic receptors on the platelet surface, P2Y1 and P2Y12, which are coupled to G$_q$ and G$_i$, respectively (30). To investigate the relative contribution of each one of these receptors on activation of Rap1B induced by clustering of FcγRIIA, we performed experiments with selective antagonists. Fig. 4 shows that pretreatment of platelets with A3P5PS, a specific antagonist of the G$_q$-coupled P2Y1 receptor, did not significantly affect FcγRIIA-mediated activation of Rap1B. By contrast, AR-C69931MX, an antagonist of the P2Y12 receptor, totally suppressed activation of Rap1B induced by clustering of FcγRIIA. These results clearly indicate that the action of secreted ADP on Rap1B activation was totally mediated by its binding to the G$_i$-coupled receptor on the platelet surface.

To confirm these results, we investigated the activation of Rap1B triggered by exogenous ADP. When platelets were stimulated with 10 µM ADP, a rapid and sustained activation of Rap1B was observed (Fig. 5*A*). Experiments with selective
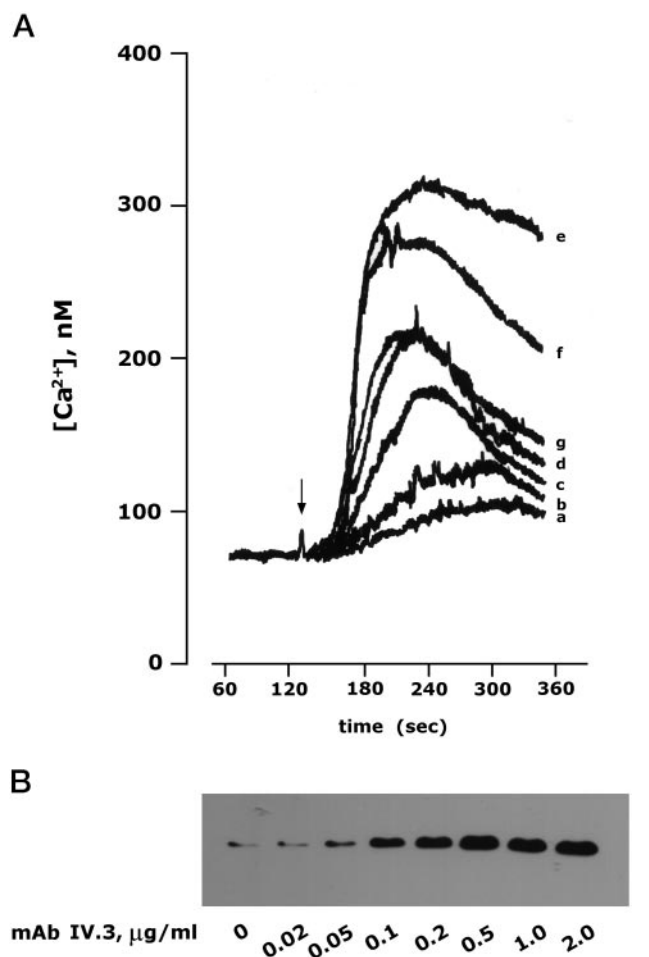
FIG. 3. **Dose-dependent Ca^{2+} mobilization and Rap1B activation in platelets stimulated by clustering of FcγRIIA.** *A*, Fura-2-loaded platelets prewarmed at 37 °C in the presence of 1 mM CaCl$_2$ and incubated for 2 min with increasing concentrations of anti-FcγRIIA mAb IV.3: 0.02 μg/ml (*a*), 0.05 μg/ml (*b*), 0.1 μg/ml (*c*), 0.2 μg/ml (*d*), 2 μg/ml (*e*). Cross-linking of FcγRIIA was then induced by the addition of 30 μg/ml sheep anti-mouse F(ab′)$_2$ fragments, as indicated by the *arrow*. In *curve f*, platelets were preincubated with 1 unit/ml apyrase, whereas in *curve g*, platelets were incubated with 5 mM CP and 40 units/ml CPK for 2 min before the addition of 2 μg/ml mAb IV.3 and 30 μg/ml sheep anti-mouse F(ab′)$_2$ fragments. Fura-2 fluorescence was monitored continuously. Traces show changes in the cytosolic concentration of Ca^{2+} and are representative of three or more experiments. *B*, Rap1B activation analyzed in platelets stimulated by the addition of increasing amounts of anti-FcγRIIA mAb IV.3 followed by the addition of constant amounts of sheep anti-mouse F(ab′)$_2$ fragments (30 μg/ml), by precipitation with GST·RBD, and by immunoblotting with a specific antiserum.
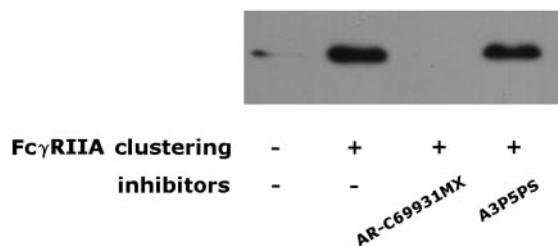

FIG. 4. **Effect of ADP receptors antagonists on FcγRIIA-mediated Rap1B activation.** Platelets were incubated with buffer, 100 nM AR-C69931MX, or 500 μM A3P5PS for 2 min and then stimulated by clustering of FcγRIIA. Samples were lysed, and GTP-bound Rap1B was precipitated with GST·RBD and detected by immunoblotting.

inhibitors of P2Y1 and P2Y12 receptors revealed that the ability of exogenous ADP to activate Rap1B was exclusively mediated by agonist binding to the G$_i$-coupled receptor as it was
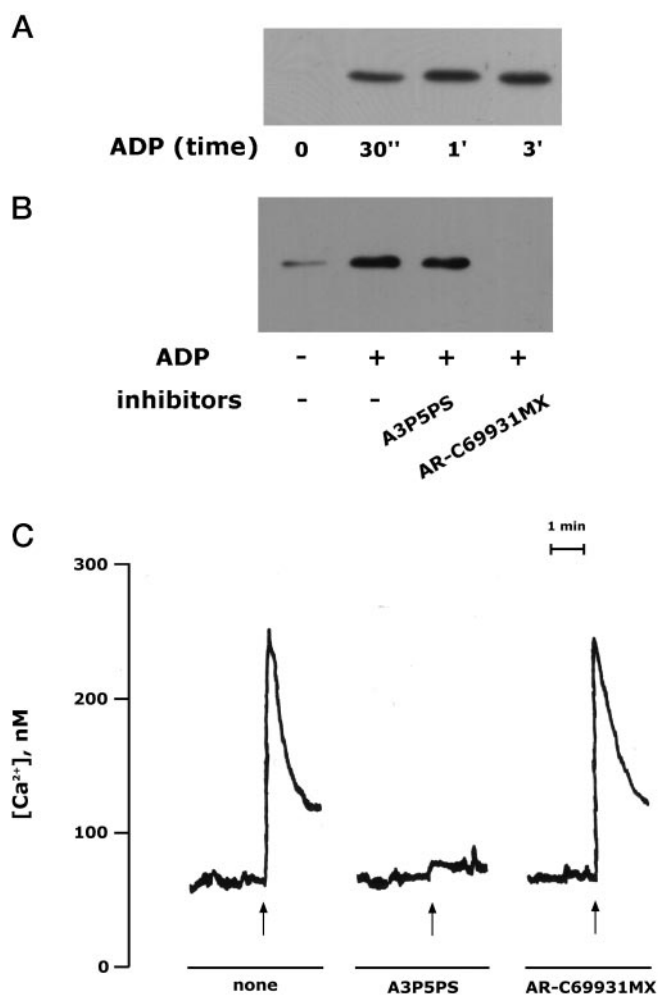

FIG. 5. **ADP-induced Rap1B activation.** *A*, Gel-filtered platelets were stimulated by the addition of 10 μM ADP for the indicated times. Samples were lysed, and activated Rap1B was precipitated with GST·RBD and visualized by immunoblotting. *B*, Platelets were preincubated with buffer, 500 μM A3P5PS, or 100 nM AR-C69931MX for 2 min before stimulation with 10 μM ADP. Analysis of Rap1B activation was performed by precipitation with GST·RBD and immunoblotting. *C*, Fura-2-loaded platelets were stimulated with 10 μM ADP in the absence or presence of 500 μM A3P5PS or 100 nM AR-C69931MX as indicated. The *arrows* indicate the addition of the agonist. Traces show changes in the cytosolic concentration of Ca^{2+} and are representative of four separate experiments.

completely inhibited by AR-C69931MX but unaffected by A3P5PS (Fig. 5*B*). Interestingly, using Fura-2-loaded platelets, we confirmed that, even under our experimental conditions, ADP-induced cytosolic Ca^{2+} increase was totally suppressed by the P2Y1 receptor antagonist A3P5PS but was unaffected by AR-C69931MX (Fig. 5*C*). Therefore, when ADP was allowed to bind exclusively to the P2Y12 receptor (*i.e.* in the presence of A3P5PS), Rap1B was activated, although cytosolic Ca^{2+} was not increased. By contrast, the sole binding of ADP to the P2Y1 receptor (*i.e.* in the presence of AR-C69931MX) did not result in Rap1B activation, although intracellular Ca^{2+} rose normally. These results indicate that a G$_i$-dependent pathway, rather than a cytosolic Ca^{2+} increase, is essential for activation of Rap1B.

Activation of Rap1B by Epinephrine—To confirm the essential role of a G$_i$ pathway for Rap1B activation, we investigated whether, upon clustering of FcγRIIA, the inhibitory effect of ADP scavengers could be overcome by the simultaneous stimulation of the G$_i$-coupled α_{2A}-adrenergic receptor by epinephrine. Fig. 6*A* actually shows that in the presence of apyrase, Rap1B activation triggered by FcγRIIA cross-linking could be
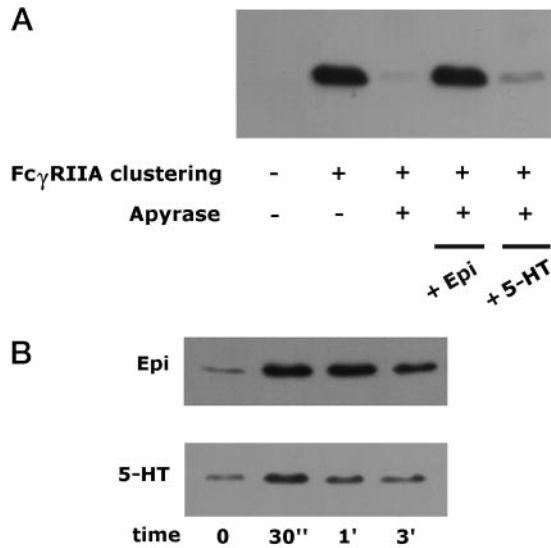
FIG. 6. **Inhibition of FcγRIIA-mediated activation of Rap1B by apyrase is reversed by epinephrine but not by serotonin.** *A*, Platelets were incubated without or with 1 unit/ml apyrase for 2 min, and clustering of FcγRIIA was induced by the addition of 2 μg/ml mAb IV.3 followed by 30 μg/ml sheep anti-mouse F(ab')$_2$ fragments. When indicated, 1 μM epinephrine (*Epi*) or 5 μM serotonin (*5-HT*) were added to the platelet samples concomitantly with sheep anti-mouse F(ab')$_2$ fragments. The immunoblot shows the accumulation of the activated Rap1B isolated by binding to GST·RBD. *B*, Platelets were stimulated with 1 μM epinephrine (*Epi*) or 5 μM serotonin (*5-HT*) alone for the indicated times, and activation of Rap1B was measured by precipitation with GST·RBD and immunoblotting.

restored by the simultaneous addition of epinephrine. By contrast, the addition of serotonin, which binds to a membrane G$_q$-coupled receptor, did not result in restoration of Rap1B activation. Our previous findings, indicating that stimulation of the G$_i$-coupled P2Y12 receptor by ADP is sufficient for activation of Rap1B, prompted us to investigate the ability of epinephrine alone to stimulate binding of GTP to Rap1B. As shown in Fig. 6*B*, treatment of platelets with 1 μM epinephrine caused a rapid and significant activation of Rap1B even in the absence of any other stimulus. Interestingly, stimulation of platelets with serotonin, which does not signal through G$_i$, produced only a slight activation of Rap1B (Fig. 6*B*). Using Fura-2-loaded platelets, we confirmed, in agreement with previous studies, that epinephrine did not cause any detectable Ca^{2+} movement (data not shown). By contrast, platelet stimulation with serotonin caused a small but significant increase of the intracellular concentration of Ca^{2+} (from 50 ± 9 nM to 88 ± 16 nM, *n* = 6). Once again, these results correlate Rap1B activation to a G$_i$-dependent signaling pathway rather than to cytosolic Ca^{2+} increase.

A G$_i$-dependent Pathway Mediates Rap1B Activation Induced by U46619—We further investigated the role of the G$_i$-dependent pathway on Rap1B activation by analyzing the effect of the thromboxane A$_2$ analogue U46619. It has been shown that U46619 binds to a G$_q$-coupled receptor, mobilizes intracellular Ca^{2+} through activation of phospholipase C, but totally relies on secreted ADP to trigger full platelet aggregation (20, 21). Fig. 7*A* shows that U46619 was a strong activator of Rap1B. However, activation of Rap1B was totally suppressed when stimulation was performed in the presence of the ADP scavengers apyrase (Fig. 7*A*) or CP-CPK (not shown). It has also been shown that cytosolic Ca^{2+} increase induced by U46619 was not reduced when secretion was prevented by inhibition of protein kinase C (21). In agreement with these results, we found that ADP scavengers did not significantly affect the rise of intracellular Ca^{2+} in platelets stimulated with
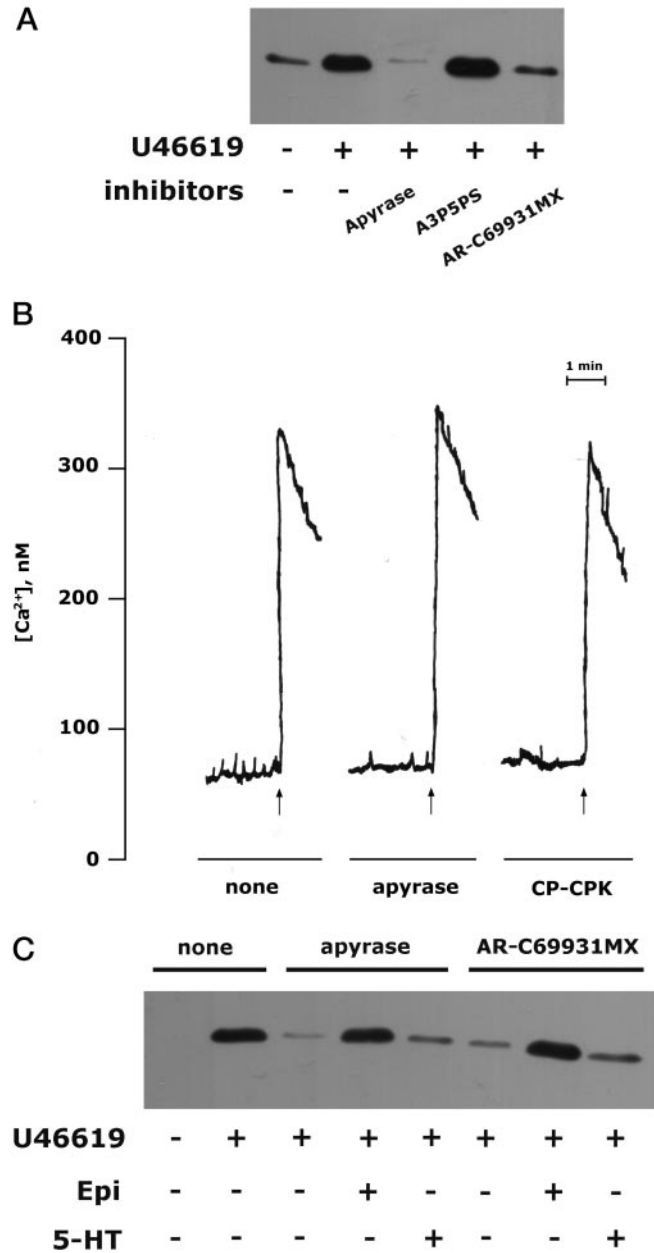
FIG. 7. **Activation of Rap1B induced by U46619.** *A*, Gel-filtered platelets were stimulated with 10 μM U46619 for 1 min in the absence or presence of the ADP scavenger apyrase (1 unit/ml), the P2Y1 receptor antagonist A3P5PS (500 μM), or the P2Y12 receptor antagonist AR-C69931MX (100 nM) as indicated. The reported immunoblot shows the accumulation of GTP-bound Rap1B isolated by precipitation with GST·RBD. *B*, Traces show the cytosolic Ca^{2+} increase induced by 10 μM U46619 in the absence or presence of the ADP scavengers apyrase and CP-CPK, as measured in Fura-2-loaded platelets. The *arrows* indicate the addition of U46619. *C*, Gel-filtered platelets were preincubated with apyrase or AR-C69931MX and stimulated with 10 μM U46619 alone or in combination with 1 μM epinephrine (*Epi*) or 5 μM serotonin (*5-HT*) as indicated. The immunoblot shows the accumulation of activated Rap1B.

U46619 (Fig. 7*B*). Therefore, when secreted ADP is neutralized, Rap1B activation is prevented, despite the normal rise of intracellular Ca^{2+}. By analyzing the effects of selective antagonists of membrane ADP receptors, we found that U46619-induced activation of Rap1B was dependent on stimulation of the G$_i$-coupled P2Y12 receptor (Fig. 7*A*). Finally, Fig. 7*C* shows that epinephrine, but not serotonin, could overcome the inhibitory effect of apyrase and AR-C69931MX on Rap1B activation induced by U46619.

DISCUSSION

Rap1 proteins are ubiquitously expressed small GTPases involved in several cellular processes, including cell activation, differentiation, and adhesion (6). Human platelets express very high levels of the Rap1 protein Rap1B, which is rapidly activated upon stimulation with several extracellular agonists (1, 7, 8). In the present work, we have investigated the mechanisms underlying Rap1B activation in human platelets, and we have demonstrated that this process is directly promoted by a signaling pathway initiated by members of the G$_i$ family of heterotrimeric G-proteins. In the most recent studies, it has emerged that activation of G$_i$ is a general requirement of almost all platelet agonists for completion and amplification of the platelet response. The activation of a G$_i$-dependent pathway may be achieved through different strategies. For instance, thrombin receptors are directly coupled to several heterotrimeric G-proteins, including G$_q$ and G$_i$ (31); ADP binds simultaneously to at least two different G-protein-associated surface receptors, one coupled to G$_q$ (P2Y1) and the other one coupled to G$_i$ (P2Y12) (10–13). In the case of agonists whose receptors are coupled exclusively to G$_q$, such as the thromboxane A$_2$ analogue U46619, or to tyrosine kinases, such as the FcγRIIA, recruitment of a G$_i$-coupled receptor is obtained through the action of secreted ADP (21, 23). Whatever the strategy, prevention of activation of G$_i$ severely compromises platelet aggregation in response to extracellular agonists. Despite this, it is very well known that the sole activation of a G$_i$-coupled receptor is not sufficient to promote platelet responses such as Ca^{2+} mobilization, secretion, and aggregation. In fact, none of these effects occur when platelets are stimulated with epinephrine, which binds to the G$_i$-coupled α_{2A}-adrenergic receptor, or with ADP in the presence of selective antagonists of the G$_q$-coupled P2Y1 receptor. Therefore, it is generally accepted that stimulation of G$_i$ leads to activation of one or more intracellular effectors, which are not sufficient *per se* to trigger platelet aggregation but are indispensable for the development of full platelet response. G$_i$ is known to inhibit adenylyl cyclase and to reduce the intracellular levels of cAMP. However, a mounting body of evidence suggests that inhibition of adenylyl cyclase is not responsible for the G$_i$-induced potentiation of platelet activation (24–26). Therefore, it is likely that a different effector, directly activated by G$_i$, is involved. The present study demonstrates that, differently from other previously studied parameters of platelet activation, such as Ca^{2+} mobilization, secretion, and aggregation, which are potentiated but not promoted by G$_i$, activation of the small GTPase Rap1B is directly triggered by the sole stimulation of a G$_i$-dependent pathway. In fact, we found that the sole binding of ADP to the G$_i$-coupled P2Y12 receptor is sufficient to promote Rap1B activation and that binding to the G$_q$-coupled P2Y1 receptor does not contribute to this process. Moreover, epinephrine, which binds exclusively to the G$_i$-coupled α_{2A}-adrenergic receptor, does efficiently activate Rap1B. Interestingly, epinephrine is not considered a real platelet agonist, but its ability to potentiate platelet aggregation induced by many agonists has been known for years (32–34). The mechanism underlying this effect of epinephrine is still unclear, but its ability to directly activate Rap1B, reported in the present work, suggests that this small GTPase may play an essential role. Finally, our study showed that stimulation of a G$_q$-mediated pathway, by means of U46619, or of a tyrosine kinase-based pathway, by means of cross-linking of FcγRIIA, is not sufficient to promote Rap1B activation unless a G$_i$-dependent pathway is concomitantly activated through binding of secreted ADP to the P2Y12 receptor. Unlike other agonists tested in this study, thrombin was found to induce activation of Rap1B through a pathway that is insensitive to ADP scavengers. Moreover, we have also found that the P2Y12 receptor antagonist AR-C69931MX did not significantly affect Rap1B activation induced by this agonist (data not shown). However, it is known that thrombin receptors on the platelet surface are coupled to both G$_q$ and G$_i$, and thus, it is possible that the direct stimulation of a G$_i$-dependent pathway by thrombin leads to activation of Rap1B.

ADP is known to be required for the stabilization of platelet aggregation induced by many extracellular agonists, and this effect is mediated by its binding to the P2Y12 receptor (21, 23). Moreover, platelets from patients with a congenital deficiency of the P2Y12 receptor have been shown to undergo reversible aggregation in response to ADP as well as other agonists (17, 18, 35). Platelet aggregation is a special kind of homotypic cell adhesion supported by fibrinogen binding to integrin $\alpha_{IIb}\beta_3$. Recently, Rap1 proteins have been implicated in the regulation of cell adhesion and in the modulation of integrin function (36–38). In the light of these considerations, our results suggest Rap1B as a possible intracellular effector linking activation of a G$_i$ pathway to the stabilization of integrin-mediated platelet aggregation.

The finding that Rap1B can be activated solely by stimulation of G$_i$ introduces a new mechanism regulating this small GTPase. Previous studies, based on the use of the cell-permeable Ca^{2+}-chelating agent BAPTA-AM as well as the use of Ca^{2+} ionophores, had shown that Rap1B can be activated by a Ca^{2+}-dependent mechanism in stimulated platelets (8). Using the Ca^{2+} ionophore A23187, we also found that an increase of intracellular Ca^{2+} concentration can actually lead to GTP binding to Rap1B (data not shown). However, in this study, we have provided several evidences that, in the experimental models analyzed, the contribution of the G$_i$-dependent pathway for Rap1B activation is more relevant than that of intracellular Ca^{2+}. For instance, in platelets stimulated with epinephrine or with ADP in the presence of the P2Y1 receptor antagonist A3P5PS, activation of Rap1B occurs even in the absence of a detectable increase of cytosolic Ca^{2+}. By contrast, serotonin induces an increase of Ca^{2+} in intact platelets, which promotes only a small activation of Rap1B. Moreover, in the presence of ADP scavengers, Rap1B activation induced by U46619 is totally prevented under conditions in which intracellular Ca^{2+} increase is not affected. Finally, when platelets are stimulated by cross-linking of FcγRIIA, ADP scavengers can actually reduce the intracellular concentration of free Ca^{2+}; however, dose-dependent experiments clearly showed that, even at this lower Ca^{2+} concentration, Rap1B can be activated as long as secreted ADP can bind to the P2Y12 receptor. Therefore, the previously reported Ca^{2+}-dependent pathway and the G$_i$-dependent pathway for Rap1B activation described here are most likely to represent separated events. The relevance of intracellular Ca^{2+} in agonist-induced activation of Rap1B came from the observed inhibitory effect of BAPTA-AM. In our hands, BAPTA-AM constantly caused a total prevention of Rap1B activation even when no Ca^{2+} movements were detected, such as in platelets stimulated with epinephrine or with ADP in the presence of A3P5PS (data not shown). These findings strongly question the suitability of the use of this inhibitor to investigate activation of Rap1B. Although we do not have a definitive explanation for the inhibitory effects of BAPTA-AM in the absence of Ca^{2+} mobilization, it must be held in due consideration that this compound may also sequester Mg^{2+}, which is essential for nucleotide binding to Rap1B. Alternatively, it is also possible that the low basal levels of Ca^{2+} in resting platelets, which are further lowered upon challenge with BAPTA-AM, are essential for the activity of some exchange factors for Rap1B. However, preincubation of BAPTA-AM-loaded plate-

lets with increasing concentrations of $CaCl_2$ did not restore activation of Rap1B induced by epinephrine (data not shown). Therefore, the exact mechanism responsible for the effect of BAPTA-AM remains to be elucidated.

How can activation of G_i lead to increased binding of GTP to Rap1B? Our preliminary results indicate that inhibition of adenylyl cyclase is not involved.[2] However, several different possibilities may be considered. Members of the G_i family of heterotrimeric G-proteins may directly regulate the activity of factors controlling the nucleotide binding to Rap1B, such as exchange factors or GAPs. In this regard, it is interesting to note that the ability of epinephrine to inhibit Rap1GAP activity in intact platelets has been reported (39). Moreover, a physical and functional association between $G_{\alpha z}$ and Rap1GAP has been described (40), and $G_{\alpha z}$ has been proposed to be associated to the α_{2A}-adrenergic receptor in platelets (41). Activation of Rap1B by a G_i-dependent pathway may also involve tyrosine kinases since an exchange factor for Rap1 proteins, C3G, is activated by tyrosine phosphorylation (6). Interestingly, abnormal protein tyrosine phosphorylation has been described in platelets from a patient with a congenital defect of ADP-induced platelet aggregation that could be due to P2Y12 deficiency (42). Moreover, it has been recently shown that G_i proteins can activate members of the Src family of tyrosine kinases (43), and association between $G_{\alpha i}$ and p60[src] has been described in platelets (44). Finally, a possible role of G_i-associated $\beta\gamma$ dimers, which might, directly or indirectly, regulate the activity of nucleotide exchange factors for Rap1B, must be considered. In this scenario, the elucidation of the regulatory pathways connecting G_i proteins and Rap1B awaits further investigations.

In conclusion, the present work describes a new, direct link between members of the G_i family of heterotrimeric G-proteins and the small GTPase Rap1B. Rap1B is emerging as a key regulator of integrin function, and our findings suggest the possibility that it could be involved in the G_i-dependent stabilization of integrin-dependent platelet aggregation.

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A G_i-dependent Pathway Is Required for Activation of the Small GTPase Rap1B in Human Platelets

Paolo Lova, Simona Paganini, Fabiola Sinigaglia, Cesare Balduini and Mauro Torti

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