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Contribution of Protease-activated Receptors 1 and 4 and Glycoprotein Ib-IX-V in the G_i-independent Activation of Platelet Rap1B by Thrombin*

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Thrombin activates human platelets through three different membrane receptors, the protease-activated receptors PAR-1 and PAR-4 and the glycoprotein Ib (GPIb)-IX-V complex. We investigated the contribution of these three receptors to thrombin-induced activation of the small GTPase Rap1B. We found that, similarly to thrombin, selective stimulation of either PAR-1 or PAR-4 by specific activating peptides caused accumulation of GTP-bound Rap1B in a dose-dependent manner. By contrast, in PAR-1- and PAR-4-desensitized platelets, thrombin failed to activate Rap1B. Thrombin, PAR-1-, or PAR-4-activating peptides also induced the increase of intracellular Ca^{2+} concentration and the release of serotonin in a dose-dependent manner. We found that activation of Rap1B by selected doses of agonists able to elicit comparable intracellular Ca^{2+} increase and serotonin release was differently dependent on secreted ADP. In the presence of the ADP scavengers apyrase or phosphocreatine-phosphocreatine kinase, activation of Rap1B induced by stimulation of either PAR-1 or PAR-4 was totally inhibited. By contrast, thrombin-induced activation of Rap1B was only minimally affected by neutralization of secreted ADP. Concomitant stimulation of both PAR-1 and PAR-4 in the presence of ADP scavengers still resulted in a strongly reduced activation of Rap1B. A similar effect was also observed upon blockade of the P2Y12 receptor for ADP, as well as in P2Y12 receptor-deficient human platelets, but not after blockade of the P2Y1 receptor. Activation of Rap1B induced by thrombin was not affected by preincubation of platelets with the anti-GPIbα monoclonal antibody AK2 in the absence of ADP scavengers or a P2Y12 antagonist but was totally abolished when secreted ADP was neutralized or after blockade of the P2Y12 receptor. Similarly, cleavage of the extracellular portion of GPIbα by the cobra venom mocarhagin totally prevented Rap1B activation induced by thrombin in the presence of apyrase and in P2Y12 receptor-deficient platelets. By contrast, inhibition of MAP kinases or p160ROCK, which have been shown to be activated upon thrombin binding to GPIb-IX-V, did not affect agonist-induced activation of Rap1B in the presence of ADP scavengers. These results indicate that although both PAR-1 and PAR-4 signal Rap1B activation, the ability of thrombin to activate this GTPase independently of secreted ADP involves co-stimulation of both receptors as well as binding to GPIb-IX-V.

The small GTPase Rap1 is involved in the regulation of integrin activation and cell adhesion (1). Like other members of the Ras family of small GTPases, Rap1 is activated by binding of GTP and inactivated by hydrolysis of bound GTP to GDP. Constitutive active Rap1 increases adhesion mediated by β_1 and β_2 integrins in transfected cells, whereas expression of dominant negative mutants of Rap1, as well as overexpression of Rap1GAP, blocks integrin-mediated adhesion (2–6). In mouse megakaryocytes, agonist-induced ligand binding to integrin $\alpha_{IIb}\beta_3$ is augmented by a constitutive active form of Rap1B and is reduced by a dominant negative mutant of the protein (7).

Human platelets express very high levels of the Rap1 protein Rap1B, which may be involved in the regulation of cell-cell contacts leading to thrombus formation (8). Indeed, all of the platelet agonists able to stimulate fibrinogen binding to integrin $\alpha_{IIb}\beta_3$ and platelet aggregation have been shown to stimulate the rapid activation of endogenous Rap1B (9–14). Intracellular Ca^{2+} and protein kinase C have been proposed to mediate agonist-induced Rap1B activation in platelets (10, 11). However, more recently it has been reported that platelet Rap1B can also be activated by stimulation of members of the G_i family of heterotrimeric G-proteins in the absence of a Ca^{2+} increase or protein kinase C stimulation (12–15). For instance, the α_{2A}-adrenergic receptor, which couples to $G\alpha_z$ in platelets, mediates epinephrine-induced activation of Rap1B (12, 13). ADP stimulates platelets by binding to two different purinergic receptors on the platelet surface, P2Y1 and P2Y12 coupled to $G\alpha_q$ and $G\alpha_i$, respectively (16–18). Studies with selective pharmacological inhibitors and with platelets from $G\alpha_i$-knockout mice clearly have demonstrated a predominant contribution of the P2Y12 receptor in ADP-induced Rap1B activation (12–14). Other platelet agonists, such as the thromboxane A_2 analogue

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U46619 and the glycoprotein VI (GPVI)-selective[1] snake venom convulxin, largely rely on binding of secreted ADP to the P2Y12 receptor to promote Rap1B activation (12, 14). Because it has been shown that stimulation of a G_i-dependent pathway is generally necessary to support integrin $\alpha_{IIb}\beta_3$-dependent platelet aggregation (19–21), it is possible that activation of Rap1B by G_i participates in integrin activation in platelets.

Unlike many other platelet agonists, thrombin-induced activation of Rap1B has been shown to occur in the absence of G_i stimulation (10, 12). In contrast to what has been generally accepted for years, it has been shown recently that thrombin receptors do not couple directly to G_i (22). Moreover, thrombin-induced activation of Rap1B was found to be only minimally dependent on the binding of secreted ADP to the G_i-coupled P2Y12 receptor (10, 12). In this context, the evidence that thrombin can induce platelet aggregation independently of G_i (22) is consistent with a role of Rap1B in regulating integrin $\alpha_{IIb}\beta_3$ activation. Thrombin is believed to activate platelets by binding to two G-protein-coupled protease-activated receptors, PAR-1 and PAR-4, in human platelets (23, 24). Affinity for thrombin is higher for PAR-1 than PAR-4, and thus it is thought that platelet activation by low doses of thrombin is predominantly mediated by PAR-1 (23, 24). PAR-4 has been suggested to sustain prolonged platelet activation by high doses of thrombin (24–26). Both PAR-1 and PAR-4 are cleaved by thrombin at specific sites in the extracellular domain to unmask a new N-terminal sequence that binds to the body of the receptor and initiates transmembrane signaling (27). Specific peptides reproducing the sequence of the new N terminus of activated thrombin receptors are potent and selective activators of PAR-1 and PAR-4 and have been shown to trigger all of the platelet responses elicited by thrombin (24, 28). However, several findings indicate that, in contrast to thrombin, thrombin receptor-activating peptides are not full agonists for platelet activation and that the support of secreted ADP is necessary to achieve complete platelet response (29, 30). For instance, maximal doses of PAR-1-activating peptide (TRAP-1) require secreted ADP to trigger irreversible platelet aggregation, whereas ADP scavengers inhibit platelet aggregation only at low, but not high, doses of thrombin (29, 30). Similarly, it has also been shown that the late activation of phosphatidylinositol 3-kinase induced by TRAP-1 is dependent on secreted ADP (30).

In addition to PAR-1 and PAR-4, thrombin can also bind to platelet GPIb-IX-V (31). This represents a high affinity receptor for thrombin, and a mounting body of evidence indicates that it may contribute to the activation of platelets (32–37). It has been known for years that platelets from patients affected by the Bernard-Soulier syndrome, which lack GPIb-IX-V, show impaired response to thrombin (38). Moreover, platelet activation by a low concentration of thrombin is affected by antibodies against GPIb-IX-V. More recently, it has been reported that binding of thrombin to GPIb-IX-V may initiate a new pathway for platelet aggregation that does not involve PARs and is supported by polymerized fibrin (36, 37). Finally, stimulation of platelets with thrombin upon desensitization of both PAR-1 and PAR-4 still promotes phosphorylation of MAPK and activation of the Rho-dependent kinase p160ROCK (37), suggesting an active and direct role of GPIb-IX-V in thrombin-induced transmembrane signaling. In addition, it has been shown that GPIb-IX-V may contribute to platelet activation by increasing the rate of hydrolysis of PAR-1 (39).

In this work we have investigated the relative contribution of PAR-1, PAR-4, and GPIb-IX-V in thrombin-induced activation of Rap1B. We have found that selective stimulation of either PAR-1 or PAR-4, but not GPIb-IX-V, by thrombin generates signals for Rap1B activation. However, in contrast to thrombin, PAR-1- and PAR-4-promoted activation of Rap1B required the support of secreted ADP. We also found that the ability of thrombin to stimulate activation of Rap1B in an ADP-independent manner is conferred by co-stimulation of PAR-1 and PAR-4, as well as by binding to GPIb-IX-V.

EXPERIMENTAL PROCEDURES

Materials—Sepharose CL-2B, GSH-Sepharose 2B, [^{14}C]serotonin (57 mCi/mmol), and the enhanced chemiluminescence substrate were from Amersham Biosciences. Thrombin, apyrase (type VII), creatine phosphate, creatine phosphokinase, MRS2179, and mouse IgG were from Sigma. Fura-2/AM and Y-27632 were from Calbiochem. The thrombin receptor-activating peptide for PAR-1, SFLLRN (TRAP-1), and that for PAR-4, AYPGKF (TRAP-4), were custom synthesized by PRIMM (Milan, Italy). The rabbit polyclonal antiserum against Rap1B was from Santa Cruz Biotechnology Inc. Anti-GPIbα monoclonal antibody AK2 was from Serotec Ltd. PD98059 was from Alexis Biochemicals. Peroxidase-conjugated goat anti-rabbit IgG was from Bio-Rad. AR-C69931MX was a generous gift from AstraZeneca R&D (Charnwood, UK). Mocarhagin was a generous gift from Dr. M. Berndt (Monash University, Clayton, Australia). The cDNA for the Rap binding domain (RBD) of RalGDS was kindly provided by Dr. J. L. Bos (University of Utrecht, The Netherlands).

Platelet Isolation and Stimulation—Blood was collected from healthy volunteers using citric acid/citrate/dextrose (152 mM sodium citrate, 130 mM citric acid, 112 mM glucose) as anticoagulant and centrifuged at 120 × *g* for 10 min at room temperature to obtain the platelet-rich plasma. Platelets were then recovered by centrifugation of the platelet-rich plasma at 300 × *g* for 10 min and resuspended in a small volume (0.5 ml) of autologous plasma. Platelets were then isolated by gel filtration on a 10-ml column of 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). The platelet count was adjusted to 0.35×10^9 cells/ml with the same buffer for the Rap1B GTP binding assay. Human platelets deficient in the P2Y12 receptor were obtained from a previously characterized patient (40) and prepared by a washing procedure as described by Cattaneo *et al.* (41). In parallel, washed platelets were also prepared from healthy donors and used as an appropriate control. Platelet samples (0.5 ml) were incubated at 37 °C in an aggregometer under constant stirring and typically stimulated for 1 min with 0.1 unit/ml thrombin, 10 μM TRAP-1, or 1 mM TRAP-4 in the presence of 1 mM $CaCl_2$ unless otherwise stated. Neutralization of secreted ADP was achieved by incubating platelet samples with either 2 units/ml apyrase or 5 mM creatine phosphate and 40 units/ml creatine phosphokinase for 2 min before stimulation. The P2Y12 receptor was blocked by incubating platelets with 1 μM AR-C69931MX for 2 min before stimulation, while blockade of the P2Y1 receptor was achieved by incubating platelets with 200 μM MRS2179. In some experiments, platelets were incubated with 5 μg/ml anti-GPIbα monoclonal antibody AK2 or an identical concentration of unrelated mouse immunoglobulins for 2 min or were treated for 15 min with 15 μg/ml the GPIbα-cleaving protease mocarhagin before stimulation. For PAR-1 and PAR-4 desensitization, gel-filtered platelets were incubated for 60 min with 10 μM TRAP-1 and 1 mM TRAP-4 in the presence of 0.02 unit/ml apyrase with no stirring.

Rap1B Activation Assay—Active GTP-bound Rap1B was precipitated from resting and stimulated platelets essentially as described by Franke *et al.* (10) using the GST-tagged Rap binding domain of RalGDS (GST-RBD). 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. Platelet stimulation was stopped by addition of an equal volume of ice-cold modified 2× radioimmune precipitation assay 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_3VO_4). Upon incubation on ice for 10 min, lysates were clarified by centrifugation at 18,000 × *g* in an Eppendorf microcentrifuge for 10 min at 4 °C. Aliquots of the cleared supernatant (typically 50 μl) were withdrawn for subsequent analysis of the total amount of Rap1B in the lysates. 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,

[1] The abbreviations used are: GP, glycoprotein; PAR, protease-activated receptor; TRAP, thrombin receptor-activating peptide; MAPK, mitogen-activated protein kinase; RBD, Rap binding domain; GST, glutathione *S*-transferase.

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and the beads were washed three times with modified 1× radioimmune precipitation assay 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 antibody against Rap1B used at a final dilution of 1:1,000. In parallel, equal amounts of total cell lysates were separated on a different gel and similarly analyzed by immunoblotting with the anti-Rap1B antiserum to evaluate the amount of total Rap1B in the platelet lysates. The reactive proteins were detected by enhanced chemiluminescence reaction. The presented figures are representative of at least three different experiments, except for the analysis of P2Y12 receptor-deficient platelets, which is representative of two different experiments performed with platelets from the same patient. Analysis of the total amount of Rap1B in the platelet lysates never revealed significant differences, and therefore, the results have not been included in the figures. Quantification of the amount of active Rap1B in the different samples was performed by densitometric scanning of the immunoblots using a CAMAG TLC scanner II.

Measurement of Cytosolic Ca^{2+} Concentration—Platelets were prepared essentially as described above with slight modifications. Platelet-rich plasma was incubated with 3 μM Fura-2/AM at 37 °C for 30 min before further processing. Platelets were then eluted from the Sepharose CL-2B column with HEPES buffer containing 0.5% bovine serum albumin and 5.5 mM glucose. The platelet count was then adjusted to 2×10^8 platelets/ml. Measurement of cytosolic Ca^{2+} was performed on 0.4-ml samples prewarmed at 37 °C under gentle stirring in a PerkinElmer Life Sciences LS3 spectrofluorometer in the presence of 1 mM $CaCl_2$. 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.* (42). F_{max} was determined by addition of 2% Triton X-100 and saturating concentrations of $CaCl_2$, whereas F_{min} was determined by addition of 2 mM EGTA and 20 mM Tris base. All determinations were repeated at least three times with platelets from different donors.

Measurement of [^{14}C]Serotonin Secretion—Gel-filtered platelets at the concentration of 0.5×10^9 platelets/ml were labeled with 0.2 μCi/ml [^{14}C]serotonin at 37 °C for 30 min. Platelets were then collected by centrifugation at 300 × *g* for 10 min in the presence of 5 mM EGTA and finally resuspended in HEPES buffer, pH 7.4, at the final concentration of 10^9 cells/ml. [^{14}C]Serotonin-labeled platelets (0.1-ml samples) were stimulated with the appropriate agonist in the presence of 5 μM imipramine. A 10-μl aliquot was withdrawn before addition of the agonist to evaluate the total incorporated radioactivity. Stimulation was stopped by addition of 0.9 ml of 1.2% paraformaldehyde and 100 mM EDTA and by cooling on ice. Platelets were pelleted by centrifugation at 10,000 × *g* for 3 min, and the radioactivity of [^{14}C]serotonin released in the supernatant was determined by liquid scintillation counting. The release of [^{14}C]serotonin in the supernatant is reported as a percentage of the total incorporated radioactivity.

RESULTS

Stimulation of PAR-1 and PAR-4 Triggers Activation of Rap1B—It is known that platelet activation by thrombin is mediated by two different protease-activated receptors, PAR-1 and PAR-4 (23, 24). Because thrombin is a strong stimulator of GTP binding to Rap1B, we analyzed and compared the ability of each of these receptors to trigger Rap1B activation. Gel-filtered platelets were incubated at 37 °C and stimulated for 1 min with increasing concentrations of thrombin, PAR-1-activating peptide SFLLRN (TRAP-1), or PAR-4-activating peptide AYPGKF (TRAP-4). Active Rap1B was precipitated with the GST-conjugated RBD of RalGDS and detected by immunoblotting. Fig. 1 shows that in addition to thrombin, TRAP-1 and TRAP-4 also were able to stimulate GTP binding to Rap1B in a concentration-dependent manner. Activation of Rap1B by thrombin was already detectable at very low concentrations of the agonist (0.001 unit/ml) and was maximal at 0.025 unit/ml. TRAP-1 induced a substantial activation of Rap1B at 0.5 μM, and a maximal effect was observed at concentrations between 5 and 10 μM. Activation of Rap1B upon selective stimulation of PAR-4 was detected when platelets were treated with 0.1 mM TRAP-4, and the activation was maximal at 0.5 mM TRAP-4. These results indicated that stimulation of either PAR-1 or PAR-4 is sufficient to generate signals leading to Rap1B activation. Fig. 1 also shows that thrombin, TRAP-1, and TRAP-4 induced a dose-dependent increase of intracellular Ca^{2+} concentration and serotonin release that paralleled Rap1B activation. It can be noted that among the doses of agonists able to induce maximal activation of Rap1B, 0.1 unit/ml thrombin, 10 μM TRAP-1, and 1 mM TRAP-4 also elicited similar cellular responses in terms of serotonin release and Ca^{2+} mobilization.

Rap1B Activation Induced by PAR-stimulating Peptides but Not by Thrombin Is Dependent on Secreted ADP—It has been reported previously that Rap1B can be activated through a G_i-dependent pathway (12–14). Many platelet agonists, including U46619, antibodies to FcγRIIA, and the snake venom toxin convulxin, rely on stimulation of the G_i-coupled P2Y12 receptor by secreted ADP to promote Rap1B activation. By contrast, activation of Rap1B by thrombin appears to be largely independent of released ADP (10, 12). Therefore, we investigated the role of secreted ADP in Rap1B activation promoted by selective stimulation of PAR-1 or PAR-4. Fig. 2 shows that Rap1B activation induced by 0.1 unit/ml thrombin was only minimally affected in the presence of the ADP scavengers apyrase or creatine phosphate-creatine phosphokinase. By contrast, Rap1B activation induced by either TRAP-1 or TRAP-4 was almost totally prevented by ADP scavengers. Surprisingly, even when PAR-1 and PAR-4 were simultaneously stimulated by the concomitant addition of TRAP-1 and TRAP-4, activation of Rap1B still was largely dependent on secreted ADP, as it was strongly reduced, albeit not completely abolished, in the presence of apyrase or creatine phosphate-creatine phosphokinase.

It has been reported previously that secreted ADP contributes to Rap1B activation by binding to the G_i-coupled P2Y12 receptor (12–14). Therefore, we investigated the effect of a well characterized P2Y12 receptor antagonist, AR-C69931MX, on Rap1B activation induced by thrombin and TRAPs. As reported previously (12), AR-C69931MX did not significantly affect thrombin-induced activation of Rap1B. However, it strongly inhibited activation of Rap1B elicited by TRAP-1 or TRAP-4 (Fig. 3*A*). As observed with platelets preincubated with ADP scavengers, activation of Rap1B induced by the simultaneous addition of TRAP-1 and TRAP-4 was more sensitive to the blockade of P2Y12 receptor than that induced by thrombin, although inhibition was less evident than that observed upon stimulation with TRAP-1 or TRAP-4. To confirm the role of the G_i-coupled receptor for ADP in TRAP-1- and TRAP-4-induced activation of Rap1B, we analyzed platelets from a patient with congenital P2Y12 receptor deficiency. Fig. 3*B* shows that TRAP-1 was unable to induce Rap1B activation in P2Y12-deficient platelets and that activation induced by TRAP-4 was almost completely prevented. Again, in agreement with the results obtained with ADP scavengers and the P2Y12 antagonist, concomitant stimulation of both PARs with synthetic peptides still resulted in an evident activation of Rap1B in P2Y12-deficient platelets, although the amount of active Rap1B was lower than that observed in control cells. By contrast, as shown in Fig. 5*D*, thrombin caused identical activation of Rap1B both in control and P2Y12 receptor-deficient platelets. Fig. 3*B* also shows that preincubation of platelets with the P2Y1 receptor antagonist MRS2179 did not alter TRAPs-induced activation of Rap1B in control platelets and did not affect the residual activation of Rap1B in P2Y12 receptor-deficient platelets. These results, together with those reported in Fig. 2, indicate that the residual activation of Rap1B observed when the P2Y12 receptor is either blocked or not expressed reflects an ADP-independent pathway for Rap1B activation, which to different de-

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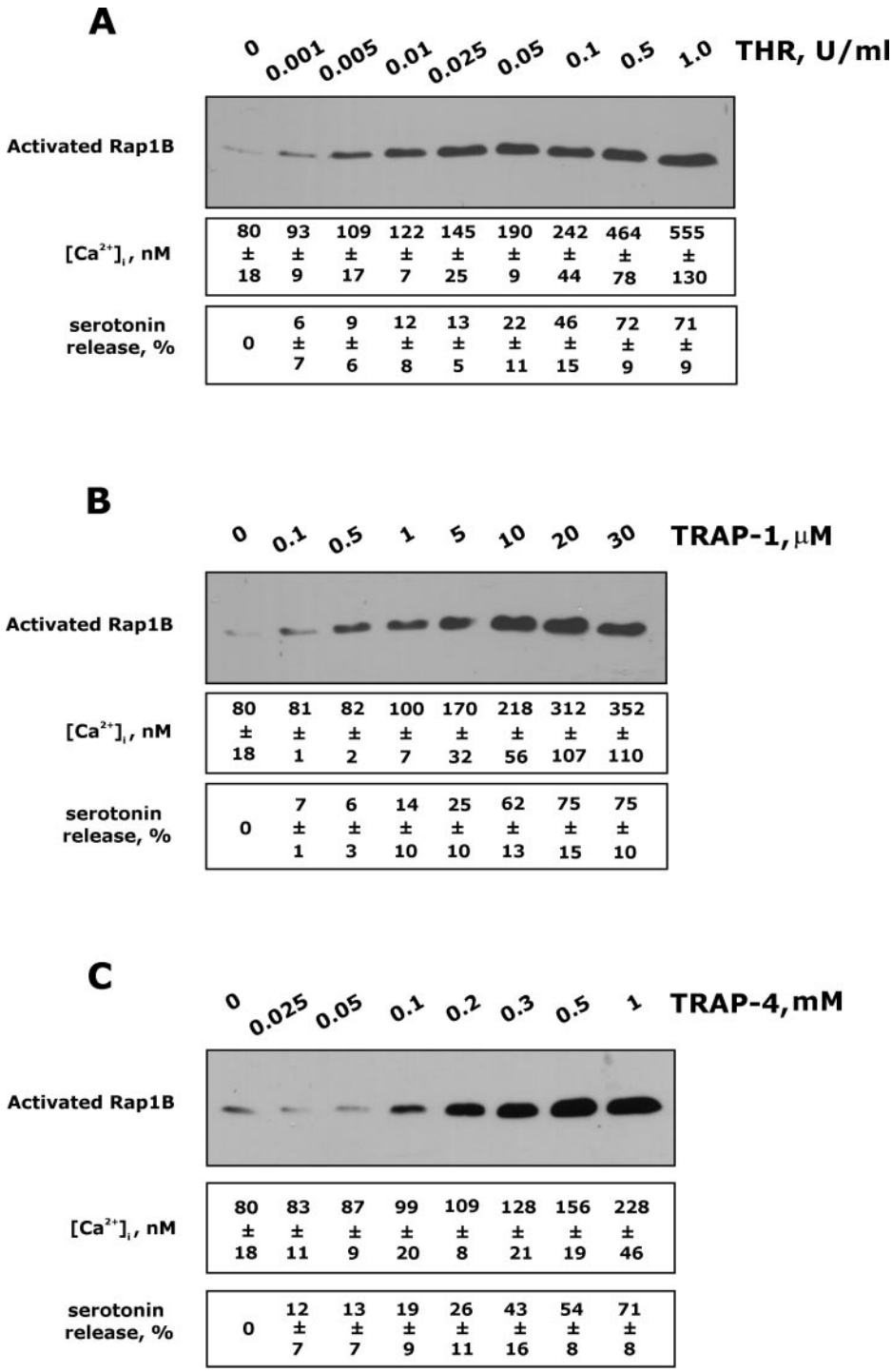

FIG. 1. **Dose-dependent activation of Rap1B, intracellular Ca^{2+} increase, and serotonin release induced by thrombin, TRAP-1, and TRAP-4.** Gel-filtered platelets were incubated at 37 °C and stimulated with the indicated doses of thrombin (*THR*) (*A*), TRAP-1 (*B*), or TRAP-4 (*C*) for 1 min with constant stirring. Platelets were lysed, and activated Rap1B was precipitated with the GST-tagged RBD of RalGDS bound to glutathione-Sepharose. Precipitated, GTP-bound Rap1B was separated by SDS-PAGE on 10–20% acrylamide gradient gels, transferred to nitrocellulose, and identified by immunoblotting with a specific polyclonal antibody. Intracellular Ca^{2+} concentration was evaluated using Fura-2-loaded platelets and calculated as described under "Experimental Procedures" on the basis of the maximal peak of fluorescence recorded after treatment of platelet samples with the indicated agonists. Data are the mean ± S.D. of 3–4 different determinations from separate experiments. Serotonin release was evaluated using [^{14}C]serotonin-labeled platelets and expressed as a percentage of the total incorporated radioactivity after subtraction of the radioactivity measured in the supernatant of resting platelets. Results are the mean ± S.D. of three different experiments.

grees contributes to the effect elicited by thrombin, TRAP-1, or TRAP-4.

Although the concentrations of thrombin, TRAP-1, and TRAP-4 used in these experiments elicited maximal activation of Rap1B and stimulated platelets with a similar potency as judged by the comparable increase of intracellular Ca^{2+} and serotonin release (Fig. 1), we considered that the contribution of secreted ADP to activation of Rap1B may be related to the dose of agonist used. In fact we observed that lower concentrations of thrombin, TRAP-1, or TRAP-4 (0.01 unit/ml, 1 μM, and 0.1 mM, respectively), which are able to elicit comparable increases of intracellular Ca^{2+} concentration and serotonin release, all caused a submaximal activation of Rap1B by a mechanism dependent on secreted ADP (Fig. 4). However, although the dependence of Rap1B activation on ADP was overcome by increasing the dose of thrombin, activation of Rap1B induced by higher concentrations of TRAP-1 or TRAP-4 was still prevented by neutralization of secreted ADP (Fig. 4). This indicates that although ADP clearly plays a role in reinforcing cellular response induced by low doses of agonists, it remains indispensable when platelets are stimulated with high doses of TRAPs but not thrombin.

Role of GPIb-IX-V in the ADP-independent Activation of Rap1B Induced by Thrombin—The previous results suggest that although both PAR-1 and PAR-4 can stimulate Rap1B activation, they do not completely account for the thrombin-induced activation of this GTPase by a mechanism independent of secreted ADP. In addition to PARs, thrombin can bind to

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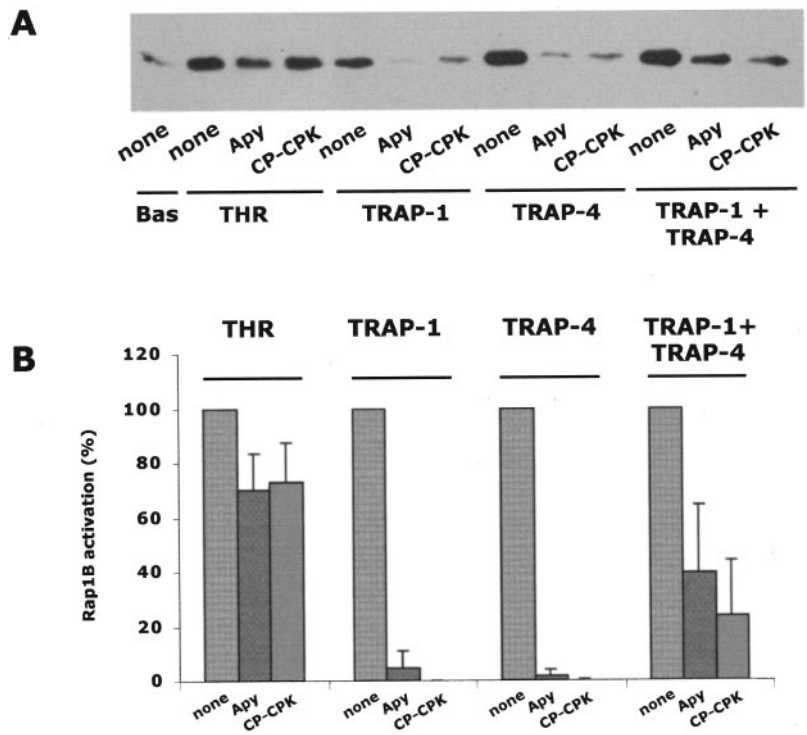

FIG. 2. **Role of secreted ADP in Rap1B activation induced by thrombin or thrombin receptor-activating peptides.** Gel-filtered platelets were incubated in the absence or presence of 2 units/ml apyrase (*Apy*) or 5 mM creatine phosphate and 40 units/ml creatine phosphokinase (*CP-CPK*) for 2 min and then stimulated with buffer (*Bas*), thrombin (*THR*) (0.1 unit/ml), TRAP-1 (10 μM), TRAP-4 (1 mM), or a mixture of TRAP-1 and TRAP-4 (10 μM TRAP-1 and 1 mM TRAP-4) for 1 min. Upon platelet lysis, active Rap1B was precipitated and identified by immunoblotting with a specific polyclonal antibody. *A*, typical immunoblots are shown. *B*, quantitative analysis of Rap1B activation obtained by densitometric analysis of several separate experiments. Results are expressed as a percentage of Rap1B activation, considering 100% to be the activation induced by every single agonist analyzed in the absence of ADP scavengers. The results are the means ± S.D. of five different experiments for thrombin- and TRAP-4-stimulated platelets and six different experiments for TRAP-1-treated platelets and TRAP-1- + TRAP-4-treated platelets.

GPIb-IX-V on the platelet surface, and this interaction may contribute to platelet activation (33–38). We therefore examined the role of GPIb-IX-V in thrombin-induced activation of Rap1B. To prevent thrombin binding to GPIb-IX-V, platelets were preincubated with the monoclonal antibody AK2 against the GPIbα subunit. As shown in Fig. 5*A*, preincubation of platelets with AK2 did not alter the ability of thrombin to activate Rap1B. However, in the presence of the monoclonal antibody AK2, thrombin-induced activation of Rap1B was completely inhibited by the ADP scavenger apyrase. By contrast, apyrase did not affect thrombin-induced activation of Rap1B in the presence of unrelated IgG or in the absence of added antibodies. Similarly, the P2Y12 ADP receptor antagonist AR-C69931MX inhibited thrombin-induced activation of Rap1B in the presence, but not in the absence, of the anti-GPIbα monoclonal antibody AK2 (Fig. 5*B*).

To confirm these results we pretreated platelets with 15 μg/ml mocarhagin, a protease that specifically cleaves GPIbα. In preliminary immunoblotting with an anti-GPIbα monoclonal antibody we had verified that this concentration of mocarhagin completely cleaved GPIbα on the platelet surface (data not shown). We also found that TRAP-1- and TRAP-4-induced activation of Rap1B occurred normally in mocarhagin-treated platelets (data not shown). Fig. 5*C* shows that thrombin was still able to induce activation of Rap1B in mocarhagin-treated platelets, although with a lower efficiency. However, in the presence of apyrase, thrombin-induced activation of Rap1B was totally abolished in mocarhagin-treated platelets.

Finally, we analyzed the ability of thrombin to induce Rap1B activation in P2Y12 receptor-deficient human platelets upon cleavage of GPIbα by mocarhagin. Fig. 5*D* shows that thrombin caused a comparable activation of Rap1B in both control and P2Y12 receptor-deficient platelets. However, in the absence of P2Y12 receptor, cleavage of GPIbα by mocarhagin completely prevented thrombin-induced activation of Rap1B. These results indicate that GPIb-IX-V significantly contributes to the ADP-independent activation of Rap1B by thrombin.

Recently, it has been proposed that thrombin interaction with GPIb-IX-V directly stimulates a signaling pathway for platelet activation and aggregation (36, 37). Therefore, we examined whether Rap1B could be activated independently of PAR stimulation upon binding of thrombin to GPIb-IX-V. For this purpose, we used platelets desensitized to both PAR-1 and PAR-4. Fig. 6*A* shows that as expected, neither TRAP-1 nor TRAP-4, alone or in combination, was able to stimulate Rap1B in desensitized platelets. Moreover, the same figure also shows that activation of Rap1B was not detected in desensitized platelets stimulated with 0.1 unit/ml thrombin, which occurred normally in response to the GPVI ligand convulxin. Binding of thrombin to GPIb-IX-V has been shown recently to initiate a signaling pathway leading to activation of MAP kinases and p160ROCK (37). Therefore, we investigated whether these effectors may contribute to the activation of Rap1B by GPIb-IX-V. Fig. 6*B* shows that incubation of platelets with the MAP kinase inhibitor PD98059 or with the p160ROCK inhibitor Y-27632 did not affect Rap1B activation induced by thrombin either in the absence or in the presence of apyrase. These results outline the absolute requirement of TRAPs for thrombin-induced Rap1B activation.

DISCUSSION

Two main conclusions can be drawn from the present work. First, stimulation of either PAR-1 or PAR-4 by thrombin is sufficient to trigger Rap1B activation in platelets. Second, neither PAR-1 nor PAR-4 alone can completely account for the ability of thrombin to induce activation of Rap1B independently of secreted ADP; rather, interaction with GPIb-IX-V is also required.

The ability of thrombin to promote activation of Rap1B was originally reported by Franke *et al.* (10) in 1997 and was ascribed to the agonist-mediated stimulation of TRAP-1, the only protease-activated receptor known to be present on the platelet surface at that time. The subsequent discovery that a second PAR, PAR-4, is expressed in human platelets and participates in platelet activation by thrombin (23, 24) raised the question of its contribution to Rap1B activation. In embryonic stem cell-derived megakaryocytes, PAR-4 has been shown to couple to Rap1B activation (43). However, PAR-4 is the only signal-

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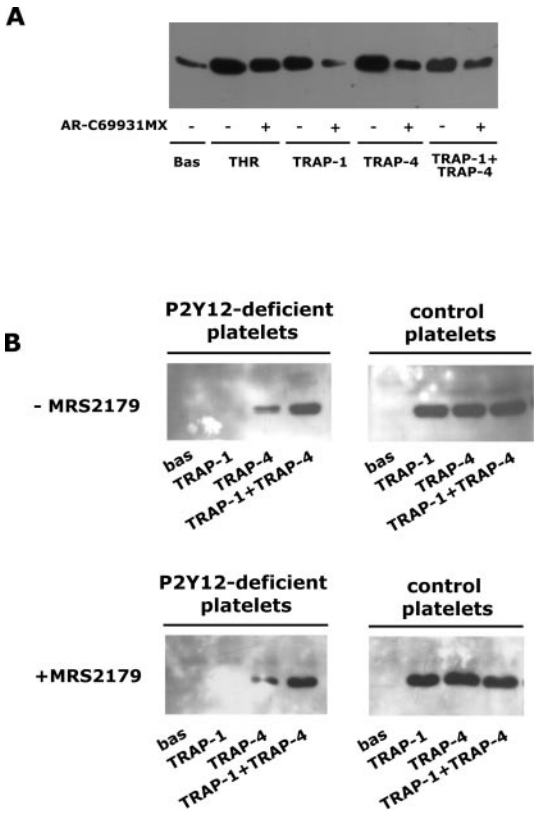

FIG. 3. **Involvement of the P2Y12 ADP receptor in Rap1B activation induced by thrombin and thrombin receptor-activating peptides.** *A*, gel-filtered platelets were preincubated at 37 °C in the absence or presence of 1 μM AR-C69931MX for 2 min and then stimulated with 0.1 unit/ml thrombin (*THR*), 10 μM TRAP-1, 1 mM TRAP-4, or a mixture of TRAP-1 plus TRAP-4 for 1 min as indicated. Active Rap1B was identified by immunoblotting upon precipitation with the GST-tagged RBD of RalGDS. *B*, analysis of Rap1B activation induced by 10 μM TRAP-1, 1 mM TRAP-4, or a mixture of TRAP-1 plus TRAP-4 was performed on washed platelets from healthy donors and from a patient congenitally deficient in the P2Y12 receptor. Blockade of the P2Y1 receptor was achieved by incubating platelets with 200 μM MRS2179 for 2 min before stimulation.

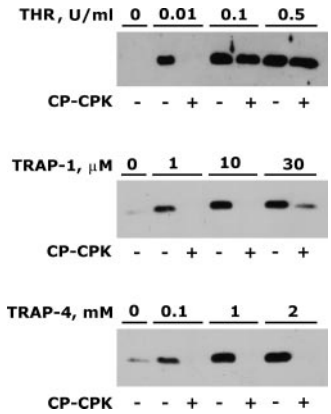

FIG. 4. **Role of ADP in the activation of Rap1B induced by different doses of thrombin, TRAP-1, and TRAP-4.** Gel-filtered platelets were stimulated with thrombin (*THR*) (0.01, 0.1, and 0.5 unit/ml), TRAP-1 (1, 10, and 30 μM), or TRAP-4 (0.1, 1, and 2 mM) for 1 min under constant stirring in the presence or absence of 5 mM creatine phosphate and 40 units/ml creatine phosphokinase (*CP-CPK*). Active Rap1B was precipitated and revealed by immunoblotting. In separate experiments, we determined that stimulation of platelets with 2 mM TRAP-4 increased the intracellular Ca^{2+} concentration from 80 ± 18 nM to 341 ± 51 nM and induced the release of 84.6% (±2.95%) of incorporated serotonin.

transducing receptor for thrombin in mice; PAR-1 is not expressed, and PAR-3 does not mediate transmembrane signaling (23, 44). In the present study we report that in human platelets, stimulation of either one of the PARs by specific peptides promotes the activation of Rap1B in a dose-dependent manner. Therefore, a dual receptor system regulates thrombin-induced activation of Rap1B. Quantitative analysis performed with maximally active concentrations of agonists revealed that activation of Rap1B in PAR-4-stimulated platelets was almost 2-fold higher than that observed in PAR-1-stimulated platelets (data not shown), suggesting that PAR-4 may predominantly contribute to thrombin-induced Rap1B activation.

Recently, it has been shown that many platelet agonists, including the thromboxane A_2 analogue U46619, antibodies against FcγRIIA, or specific ligands for GPVI, largely depend on secreted ADP to activate Rap1B (12–14). The ability of ADP to promote GTP binding to Rap1B is mediated by the stimulation of the G_i-coupled P2Y12 receptor, whereas little contribution is provided by the G_q-coupled P2Y1 receptor. By contrast, thrombin-induced activation of Rap1B is largely independent of G_i stimulation by secreted ADP (10, 12). The recent finding that neither thrombin receptor directly couples to G_i indicates that thrombin operates a G_i-independent mechanism for platelet activation (22). However, it is also well known that the G_i independence of thrombin-induced platelet response is correlated to the concentration of the agonist, as ADP scavengers are able to affect platelet activation by low, but not high, doses of thrombin (22, 29). In this work we report that activation of Rap1B by 0.1 unit/ml thrombin is only minimally affected by preincubation with ADP scavengers apyrase or creatine phosphate-creatine phosphokinase. However, we observed that activation of Rap1B following stimulation of single PARs by synthetic peptides was almost totally prevented by ADP scavengers. In agreement with recent findings demonstrating a predominant role for the G_i-coupled P2Y12 receptor in ADP-dependent stimulation of Rap1B (12–14), we have also found that both TRAP-1- and TRAP-4-induced activation of Rap1B was inhibited by an antagonist of P2Y12 receptor and was hardly detectable in P2Y12 receptor-deficient human platelets. The ADP dependence of PAR-1-mediated activation of Rap1B is consistent with the notion that, unlike thrombin, TRAP-1 is only a partial platelet activator in the absence of secreted secondary agonists and that TRAP-1-induced aggregation is reverted by ADP scavengers (29, 30). In addition, we have found that also PAR-4-mediated activation of Rap1B is almost totally dependent on secreted ADP. Taken together, our results indicate that thrombin stimulation of individual PARs on the platelet surface is not able to justify the ADP independence of Rap1B activation. We have found that activation of Rap1B by very low doses of thrombin (below 0.01 unit/ml) significantly relies on secreted ADP, whereas at higher doses of thrombin (0.5 unit/ml) activation of Rap1B was absolutely unaffected by ADP scavengers. This clearly reflects a general role for ADP in reinforcing weak stimuli. By contrast, stimulation of single PARs was dependent on secreted ADP, even when higher doses of activating peptides were used. Moreover, when comparing the effect of ADP scavengers or agonists of ADP receptors on Rap1B activation induced by thrombin, TRAP-1, and TRAP-4, we have deliberately used a selected dose of thrombin able to elicit a platelet response comparable with that elicited by the analyzed concentrations of TRAP-1 or TRAP-4. In fact, under these selected experimental conditions, the intracellular calcium increase and the serotonin release induced by 0.1 unit/ml thrombin were comparable with those induced by 10 μM TRAP-1 or 1 mM TRAP-4. Despite this, Rap1B activation by TRAP-1 or TRAP-4 was totally dependent on secreted ADP, while ADP scavengers only reduced activation of Rap1B induced by thrombin by about 30%. This indicates that the ADP

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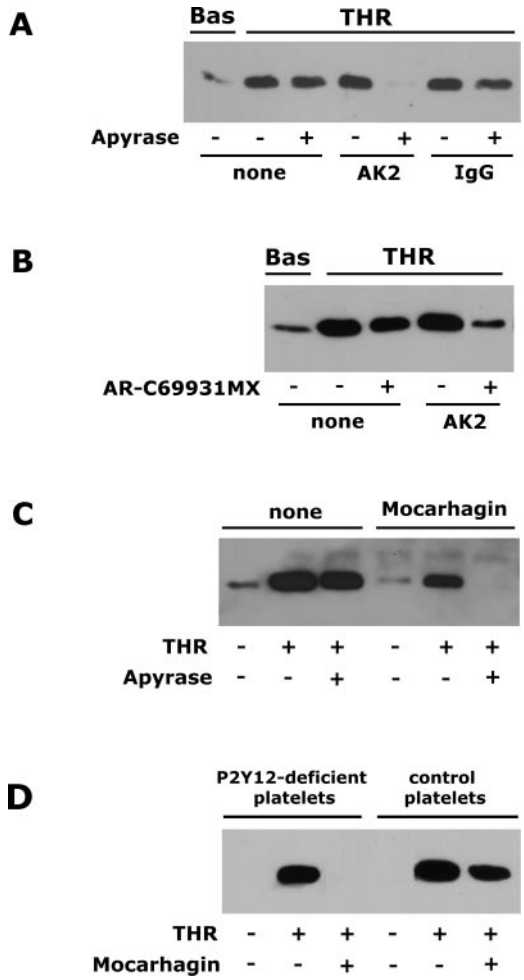

FIG. 5. **Role of GPIb-IX-V in thrombin-induced activation of Rap1B.** *A*, gel-filtered platelets were preincubated with 5 μg/ml anti-GPIb monoclonal antibody AK2 or with 5 μg/ml unrelated mouse IgG in the absence or presence of 2 units/ml apyrase and then stimulated with 0.1 unit/ml thrombin (*THR*) for 1 min. Active Rap1B was visualized by immunoblotting upon precipitation with the immobilized GST-tagged RBD of RalGDS. *B*, activation of Rap1B was evaluated in gel-filtered platelets preincubated with 5 μg/ml anti-GPIb monoclonal antibody AK2 and then stimulated with 0.1 unit/ml thrombin for 1 min in the absence or presence of the P2Y12 receptor antagonist AR-C69931MX. *C*, samples of gel-filtered platelets were incubated with buffer or with 15 μg/ml mocarhagin for 15 min and then stimulated with 0.1 unit/ml thrombin in the absence or presence of 2 units/ml apyrase. Active GTP-bound Rap1B was precipitated with GST-RBD and identified by immunoblotting with a specific polyclonal antibody. *D*, control and P2Y12-deficient human platelets were incubated in the absence or presence of 15 μg/ml mocarhagin for 15 min and then stimulated with 0.1 unit/ml thrombin. Active Rap1B was precipitated and visualized by immunoblotting.

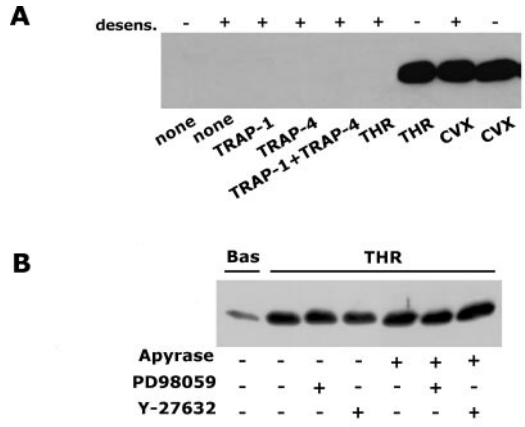

FIG. 6. **Activation of Rap1B in PAR-1- and PAR-4-desensitized platelets.** *A*, gel-filtered platelets were desensitized (*desens.*) by incubation with 10 μM TRAP-1 and 1 mM TRAP-4 for 60 min. Native or PAR-desensitized platelets were then stimulated with 10 μM TRAP-1, 1 mM TRAP-4, 10 μM TRAP-1 plus 1 mM TRAP-4, 0.1 unit/ml thrombin (*THR*), or 100 ng/ml convulxin (*CVX*) for 1 min at 37 °C under constant stirring. Active Rap1B was precipitated and revealed by immunoblotting with a specific polyclonal antibody. *B*, gel-filtered platelets were incubated with 20 μM PD98059 for 10 min, with 20 μM Y-27632 for 30 min, or with an equivalent volume of Me_2SO and then stimulated with 0.1 unit/ml thrombin for 1 min in the presence or absence of 2 units/ml apyrase. Active Rap1B was precipitated and identified by immunoblotting.

requirement for TRAP-1 and TRAP-4 to activate Rap1B is not due to the fact that these synthetic peptides are weaker agonists than thrombin itself. Interestingly, when both PAR-1 and PAR-4 were simultaneously stimulated by activating peptides in the attempt to mimic the effect of thrombin, activation of Rap1B was still significantly reduced, although not completely inhibited, both in the presence of ADP scavengers and, to a lesser extent, in the presence of an antagonist of the P2Y12 receptor or in platelets from a patient deficient in the P2Y12 receptor. This indicates that co-stimulation of PAR-1 and PAR-4 by thrombin is required to partially overcome the need of secreted ADP to induce activation of Rap1B. It is interesting to note that the reduced dependence on ADP for Rap1B activation in platelets stimulated with a mixture of TRAP-1 and TRAP-4 is not a consequence of stronger platelet stimulation or of an increased accumulation of active Rap1B. In fact, we found that the amount of GTP-bound Rap1B in platelets treated with both peptides was comparable with that accumulated in TRAP-4-treated cells (data not shown). It is also clear from our results that in the presence of ADP scavengers, thrombin is a stronger stimulator of Rap1B activation than a mixture of TRAP-1 and TRAP-4, indicating that even when added together, PAR-activating peptides cannot completely reproduce the effect of thrombin. Altogether, these findings suggest the involvement of an additional platelet receptor.

In this work we have provided evidence that the unique property of thrombin to stimulate ADP-independent activation of Rap1B requires not only the co-activation of PAR-1 and PAR-4 but also binding to GPIb-IX-V. Preventing thrombin interaction with GPIb-IX-V by preincubation of platelets with the blocking monoclonal antibody AK2 or by proteolytic cleavage of GPIbα with mocarhagin allows thrombin to activate Rap1B exclusively through stimulation of PAR-1 and PAR-4. Under these conditions, thrombin-induced GTP binding to Rap1B becomes dependent on secreted ADP. Moreover, cleavage of GPIbα by mocarhagin totally abolished the ability of thrombin to induce activation of Rap1B in P2Y12-deficient platelets. The complete inability of thrombin to activate Rap1B upon cleavage of GPIbα in the presence of apyrase or in P2Y12 receptor-deficient platelets is surprising, as a mixture of TRAP-1 and TRAP-4 can still elicit a response in the absence of the G_i-coupled receptor for ADP. Although we do not have a clear explanation for this finding, it may reflect an impaired thrombin stimulation of PARs in the absence of GPIb-IX-V. Several recent works have reevaluated a relevant specific role for GPIb-IX-V in platelet activation by thrombin, which had been originally suggested from the evidence that platelets from patients affected by Bernard-Soulier syndrome display impaired responses to thrombin (35–37). Two models have been proposed to explain the contribution of GPIb-IX-V in thrombin-induced platelet activation. The first model suggests that this complex binds and presents thrombin for efficient activation of PARs, a role similar to that played by PAR-3 in mouse platelets. In this regard, it has been demonstrated that the binding

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of thrombin to GPIb-IX-V actually increases the rate of PAR-1 hydrolysis (39). The second model proposes that binding of thrombin to GPIb-IX-V directly initiates transmembrane signals for platelet activation. In fact, activation of the Rho kinase p160ROCK and phosphorylation of MEK1 have been found to occur in thrombin-stimulated platelets upon desensitization of both PAR-1 and PAR-4 (37). Moreover, binding of thrombin to GPIb-IX-V has been shown to directly trigger fibrin-dependent platelet aggregation and clot retraction (36). Here we found that in PAR-desensitized platelets, thrombin was no longer able to induce activation of Rap1B. Moreover, Rap1B activation induced by thrombin remained independent of secreted ADP when MAPK or p160ROCK was inhibited. Altogether these results indicate that binding to GPIb-IX-V alone is not sufficient to trigger Rap1B activation and that stimulation of PARs is absolutely required. These results also indicate that the mechanism by which binding to GPIb-IX-V confers to thrombin the ability to activate Rap1B independently of secreted ADP does not involve activation of MAPK or p160ROCK. Therefore, it appears most likely that GPIb-IX-V contributes to thrombin-induced Rap1B activation by improving the performance of PARs. The exact mechanism by which binding of thrombin to GPIb-IX-V overcomes the need of secreted ADP for Rap1B activation remains to be elucidated. However, our results provide evidence that the optimization of thrombin-mediated PAR stimulation by binding to GPIb-IX-V results in a qualitative rather than quantitative gain of function and also support the concept that interaction with three different receptors is required for thrombin to behave like a complete platelet agonist.

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Contribution of Protease-activated Receptors 1 and 4 and Glycoprotein Ib-IX-V in the G_i-independent Activation of Platelet Rap1B by Thrombin

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