Rap1B and Rap2B Translocation to the Cytoskeleton by von Willebrand Factor Involves FcyRII Receptor-mediated Protein Tyrosine Phosphorylation*

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Stimulation of human platelets with von Willebrand factor (vWF) induced the translocation of the small GTPases Rap1B and Rap2B to the cytoskeleton. This effect was specifically prevented by an anti-glycoprotein Ib monoclonal antibody or by the omission of stirring, but was not affected by the peptide RGDS, which antagonizes binding of adhesive proteins to platelet integrins. Association of Rap2B with the cytoskeleton was very rapid, while translocation of Rap1B was observed in a later phase of platelet activation and was totally inhibited by cytochalasin D. vWF also induced the rapid tyrosine phosphorylation of several proteins that was prevented by the tyrosine kinases inhibitor genistein and by cAMP-increasing agents. Under these conditions, also the association of Rap1B and Rap2B with the cytoskeleton was prevented. Translocation of Rap proteins to the cytoskeleton induced by vWF, but not by thrombin, was inhibited by a monoclonal antibody against the FcyRII receptor. The same antibody inhibited vWF-induced tyrosine phosphorylation of selected substrates with molecular masses of about 75, 95, and 150 kDa. Three of these substrates were identified as the tyrosine kinase pp72Src, the phospholipase C gamma 2, and the inositol 5-phosphatase SHIP. Our results indicate that translocation of Rap1B and Rap2B to the cytoskeleton is regulated by tyrosine kinases and suggest a novel role for the FcyRII receptor in the mechanism of platelet activation by vWF.

Rap proteins are low molecular weight GTP-binding proteins that share about 50% sequence homology with the product of the ras protooncogene. Human platelets express two members of the Rap family of proteins, Rap1B and Rap2B, which are located at the membrane as a consequence of post-translational modifications, including isoform conversion, proteolysis, and carboxymethylation (1). The role of Rap1B and Rap2B in platelet function is still poorly understood. Rap1B is phosphorylated by the cAMP-dependent protein kinase A (PKA)1 (2) and is rapidly activated upon platelet stimulation with extracellular agonists (3, 4). By contrast, Rap2B is not a substrate for PKA (5), and its activation upon cell stimulation has not been reported.

In thrombin-treated platelets both Rap1B and Rap2B translocate to the cytoskeleton (6, 7). This actin-based structure is not only responsible for the morphological changes of activated platelets, but also represents a network connecting several molecules involved in signal transduction processes, including protein-tyrosine kinases, lipid metabolizing enzymes, and membrane glycoproteins (8). Translocation of Rap2B to the cytoskeleton in thrombin-stimulated platelets requires cell aggregation and is promoted by secondary signals generated by binding of fibrinogen to the membrane glycoprotein IIb-IIIa (GP IIb-IIIa) (9). Similarly, fibrinogen binding to GP IIb-IIIa strongly supports Rap1B translocation to the cytoskeleton, although a small amount of this protein associates with the actin-based structures, even in activated cells in the absence of aggregation and ligand binding to GP IIb-IIIa (10, 11). The production of secondary signals from GP IIb-IIIa, generated during platelet aggregation and required for the translocation of Rap proteins to the cytoskeleton, hampers the possibility to elucidate the molecular mechanisms underlying this process. In fact, many of the most common agents able to interfere with specific signal transduction pathways, such as inhibitors of protein kinases, Ca2+ chelating and cAMP-increasing agents, prevent the agonist-induced conformational change of glycoprotein IIb-IIIa required for the expression of the fibrinogen binding sites (12). Therefore, their possible effects on Rap proteins translocation to the cytoskeleton in thrombin-stimulated platelets may not be direct, but may be a consequence of the prevention of platelet aggregation.

Searching for a more suitable model to investigate the mechanism regulating Rap proteins interaction with the cytoskeleton, we investigated the effect of a different platelet agonist, such as von Willebrand factor (vWF). vWF is a large glycoprotein synthesized by endothelial cells and megakaryocytes and plays an important role in platelet adhesion and thrombus formation (13). The main platelet receptor for vWF is the glycoprotein Ib-IX-V complex (GP Ib-IX-V complex), a member of the leucine-rich glycoprotein gene family (14). In activated platelets, vWF can also interact with GP IIb-IIIa through a RGD-containing sequence (15). Binding of soluble vWF to GP Ib-IX-V complex induces platelet activation associated with arachidonic acid release, Ca2+ mobilization, and protein tyrosine phosphorylation (16, 17). Moreover, it has been demonstrated that vWF stimulates the association of pp60src and...
phosphatidylinositol 3-kinase with the intracellular cytoskeleton. Interestingly, in thrombin-stimulated platelets, translocation of pp60^src and phosphatidylinositol 3-kinase to the cytoskeleton occurs by a mechanism similar to that of Rap proteins, since this process is regulated by fibrinogen binding to GP Ib-IIIa and aggregation (19, 20).

Herein we investigated the ability of vWF to induce Rap1B and Rap2B translocation to the cytoskeleton, and we found that this interaction is actually promoted by the interaction of the agonist with the GP Ib-IX-V complex. We also found that vWF-induced protein tyrosine phosphorylation is partially mediated by the recruitment of the FcγII receptor (FcγRII) on the cell surface, which is essential to promote Rap proteins translocation to the cytoskeleton.

### EXPERIMENTAL PROCEDURES

**Materials**—Sepharose CL-2B was from Amersham Pharmacia Biotech. Triton X-100 and bicinechonic acid (BCA) kit for protein determination were obtained from Pierce. Thrombin, ristocetin, cytochalasin D, acetylsalicylic acid, indomethacin, prostaglandin E1, (PGE1), RGDS peptide, protein A-Sepharose, and mouse IgG were from Sigma. Iloprost was from Schering. von Willebrand factor (Hemate P) was obtained from Behringwerke (Marburg, Germany). Peroxidase-conjugated anti-human IgG antiserum was from Dako. The monoclonal antibody M90 and the antisera against Rap1B and Rap2B were described previously (2, 21). Ascites of the anti-glycoprotein Ib monoclonal antibody AK2 were a gift of Dr. Patrizia Noris (IRCCS, Policlinico San Matteo, Pavia, Italy). The monoclonal antibody IV.3 against the FcγRII was obtained from Medarex. Anti-phosphotyrosine antibody was purchased from Upstate Biotechnology Inc. The polyclonal antisera against SHIP was a gift from Dr. C. Erneux (Interdisciplinary Research Institute IRRIBHN, Université Libre de Brussel, Brussels, Belgium). Polyclonal antibodies against pp72^FAK, PLCγ2, and pp125^FAK were from Santa Cruz Biotechnology.

**Platelet Preparation and Cytoskeleton Extraction—**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 NaHCO3, pH 7.4) as described previously (7). Platelet samples (0.4 ml, 10^9 platelets/ml) were incubated at 37 °C in an aggregometer (Chrono-Log) under constant stirring and then stimulated with 10 mM vWF and 0.5 mg/ml ristocetin or with 0.6 unit/ml thrombin. In some experiments, platelets were preincubated with 10 mM cytochalasin D, 1 mM RGDS, 20 μg/ml IV.3 monoclonal antibody or 20 μg/ml control IgG for 2 min before the addition of the agonists. Preincubation with 10 μg/ml iloprost, 10 μg/ml PGE1, 1 mM acetylsalicylic acid, 10 μg/ml indomethacin, 100 or 200 μg/ml genistein was performed for 30 min. Upon addition of the agonists, aggregation was monitored continuously. Except for the time course experiments, reaction was blocked after 5 min by addition of an equal volume of extraction buffer (HEPES buffer, containing 2% Triton X-100, 10 mM EGTA, 4 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 2 mM Na3VO4, 2% P-40, 0.5% sodium deoxycholate). Lysates were transferred to Eppendorf tubes, vigorously mixed, and placed on ice for 15 min. Triton X-100-insoluble material was recovered by centrifugation at 13,000 rpm for 5 min at 4 °C. The pellet was washed twice with 1 ml of extraction buffer diluted 1:1 with HEPES buffer and finally resuspended with 80 μl of 4% SDS. The protein concentration of the cytoskeleton samples was determined by the bicinchoninic acid assay. An equal volume of a mixture containing 1% dithiothreitol, 20% glycerol, and 0.02% bromphenol blue was added to the remaining of the samples and boiled for 5 min. For the analysis of protein tyrosine phosphorylation of total platelet lysate, samples of gel-filtered platelets were stimulated in the aggregometer as described above. In this case, reaction was stopped by adding an equal volume of SDS-sample buffer (25 mM Tris, 192 mM glycine, pH 8.3, 4% SDS, 1% dithiothreitol, 20% glycerol, and 0.02% bromphenol blue). Samples were boiled 5 min before being analyzed by SDS-PAGE.

**Immunoprecipitation—**Samples of gel-filtered platelets (0.25 ml, 10^9 platelets/ml), treated with buffer or stimulated with 10 μg/ml vWF and 0.5 mg/ml ristocetin for 5 min in the absence or in the presence of 20 μg/ml IV.3 monoclonal antibody, were lysed with an equal volume of immunoprecipitation buffer two times (100 mM Tris/HCl, pH 7.4, 200 mM NaCl, 2 mM EGTA, 20 μg/ml leupeptin, 20 μg/ml aprotinin, 2 mM phenylmethylsulfonyl fluoride, 2 mM Na3VO4, 2 mM NaF, 2% Nonidet P-40, 0.5% sodium deoxycholate). Lysates were preclared for 1 h at 4 °C with 100 μl of protein A-Sepharose (50 mg/ml stock solution). The cleared supernatants were incubated with 1 μg of anti-pp72^FAK, anti-PLCγ2, anti-pp125^FAK or 5 μl of anti-SHIP antisera for 2 h at 4 °C, and the immunocomplexes were precipitated by addition of 100 μl of protein A-Sepharose for 45 min. After brief centrifugation, immunoprecipitates were washed three times with 1 ml of lysis buffer one time and finally resuspended in 25 μl of SDS-sample buffer.

**Electrophoresis and Immunoblotting—**Proteins from cytoskeletal samples or total cell lysates, representing equal cell number (1 x 10^8 and 1 x 10^7, respectively), as well as immunoprecipitates, were separated on 7.5% linear or 5–15% and 10–20% gradient acrylamide gels and transferred to nitrocellulose membranes. Nitrocellulose membranes were blocked overnight at 4 °C with 6% bovine serum albumin in Tris-buffered saline (20 mM Tris/HCl, pH 7.5, 0.5 mM NaCl) and incubated with the appropriate antibodies for 2 h at room temperature. The monoclonal antibody M90, the Rap1B, Rap2B, and the SHIP antisera were used at 1:1,000 dilution, while the anti-phosphotyrosine antibody was diluted 1 μg/ml. Antibodies against pp72^FAK, PLCγ2 and pp125^FAK were diluted 1:200. Membranes were extensively washed with 50 mM Tris/HCl, pH 7.4, 0.2 mM NaCl, 1 mg/ml polyethylene glycol 20,000, 1% bovine serum albumin, 0.05% Tween 20, and incubated with peroxidase-conjugated secondary antibody (1:20,000 dilution) for 45 min. Upon extensive washing, reactive proteins were visualized with a chemiluminescence reaction. Washed nitrocellulose filters were stripped by incubation in 62.5 mM Tris/HCl, pH 6.8, 2% SDS, 100 mM β-mercaptoethanol at 50 °C for 30 min, blocked again with 6% bovine serum albumin, and reprobed with a different antibody. All the experiments presented are representative of at least three separated experiments.

### RESULTS

**Translocation of Rap1B and Rap2B to the Cytoskeleton in vWF-stimulated Platelets—**Stimulation of human platelets with 10 μg/ml vWF and 0.5 mg/ml ristocetin induced the incorporation of several proteins into the cytoskeleton, including actin-binding protein (250 kDa), myosin (200 kDa), and α-actinin (99 kDa), as revealed the Coomassie Blue-stained gel shown in Fig. 1A. Cytoskeleton reorganization induced by vWF was very similar to that induced by 0.6 unit/ml thrombin (Fig. 1A). The ability of vWF to promote the translocation of Rap proteins to the cytoskeleton was investigated by immunoblotting experiments, using both the monoclonal antibody M90, which recognizes all the members of the Rap family of proteins, and specific antisera against Rap1B and Rap2B. As shown in Fig. 1, B–D, both Rap1B and Rap2B translocated to the cytoskeleton upon stimulation with vWF, and the amount of cytoskeletal-associated Rap1B and Rap2B was comparable with that observed in thrombin-aggregated platelets. The translocation of Rap proteins to the cytoskeleton in vWF-stimulated platelets was not affected by the pretreatment of the cells with either acetylsalicylic acid or indomethacin, indicating that this effect was not mediated by the production of thromboxane A2 (data not shown).

To analyze the kinetics of vWF-induced translocation of Rap proteins to the cytoskeleton, platelets were stimulated in an aggregometer, and at different times the Triton X-100-insoluble materials were analyzed with the antisera against Rap1B and Rap2B. Fig. 2 shows that incorporation of Rap1B into the cytoskeleton was detected after 1 min of stimulation, when aggregation reached about 80%. By contrast, translocation of Rap2B was detectable after 15 s upon addition of the agonist, when only 15% of aggregation was measured. In both cases, incorporation of Rap1B and Rap2B into the cytoskeleton was maximal after 5 min and was parallelized by maximal platelet aggregation.

The role of agonist-induced actin polymerization in the translocation of Rap proteins to the cytoskeleton was investigated by treating platelets with 10 μg/ml cytochalasin D for 2 min before stimulation with vWF. As reported previously with thrombin-stimulated platelets (23), we found that cytochalasin D did not affect platelet aggregation induced by vWF (data not shown).
Gel-filtered platelets were placed in an aggregometer and stimulated with 10 μg/ml vWF and 0.5 mg/ml ristocetin (vWF) or 0.6 unit/ml thrombin (THR). A, proteins were separated by SDS-PAGE on a 5–15% acrylamide gradient gel and stained with Coomassie Brilliant Blue. B–D, proteins were separated by SDS-PAGE on 10–20% acrylamide gradient gels, transferred to nitrocellulose, and probed with the monoclonal antibody M90 (B), the Rap1B antiserum (C), or the Rap2B antiserum (D). The amount of cytoskeletal proteins applied to each gel lane was from 106 platelets.

Role of GP Ib-IX-V Complex and GP IIb-IIIa in the Translocation of Rap Proteins to the Cytoskeleton—In thrombin-stimulated platelets, translocation of Rap proteins to the cytoskeleton is reported in Fig. 5B. Immediately upon binding to the agonist, Rap1B and Rap2B do not associate with the cytoskeleton in vWF-stimulated platelets (data not shown). Immunoblotting with the specific antisera revealed that treatment of platelets with cytochalasin D completely prevented the association of Rap1B to the cytoskeleton and strongly reduced, but not totally inhibited, the translocation of Rap2B to the Triton X-100-insoluble material (Fig. 3).

Protein Tyrosine Phosphorylation and the Translocation of Rap Proteins to the Cytoskeleton—In agonist-stimulated platelets, the association of Rap proteins to the cytoskeleton is regulated by fibrinogen binding to GP Ib-IIIa and aggregation (9, 11). Immunoblotting revealed that platelet aggregation induced by vWF could be prevented by the omission of stirring. Moreover, an antibody against the membrane glycoprotein Ib, AK2, also caused total inhibition of aggregation. By contrast, preincubation of platelets with 1 mM RGDS did not affect platelet aggregation induced by vWF (Fig. 4). The effect of the prevention of vWF interaction with GP Ib-IX-V complex and platelet aggregation on the translocation of Rap proteins to the cytoskeleton is reported in Fig. 5A. Preincubation of platelets with the antibody AK2 completely blocked the association of both Rap1B and Rap2B to the Triton X-100-insoluble material. Moreover, when binding of vWF to platelets was allowed, but aggregation was prevented by the omission of stirring, interaction of both Rap proteins with the cytoskeleton was not observed (Fig. 5A). Thus, GP Ib-IX-V complex-mediated aggregation of vWF-stimulated platelets is required for the interaction of Rap1B and Rap2B with the cytoskeleton. To investigate the role played by the receptor occupancy of GP Ib-IIIa, we compared the effect of 1 mM RGDS peptide on thrombin- and vWF-induced translocation of Rap proteins to the cytoskeleton. Fig. 5B shows that the ability of vWF to promote the interaction of both Rap1B and Rap2B with the cytoskeleton was not affected by the presence of the RGDS peptide. By contrast, Rap1B and Rap2B did not associate with the cytoskeleton in platelets stimulated with thrombin in the presence of the RGDS peptide.
analyzed with the antisera against Rap1B and Rap2B. As shown in Fig. 7B, inhibition of tyrosine kinases by genistein completely prevented the translocation of Rap1B to the cytoskeleton. By contrast, genistein, even at 200 μM, caused only a 50% reduction of the association of Rap2B with the cytoskeleton. Similar results were also obtained using different inhibitors of tyrosine kinases, including tyrphostin A47 and erbstatin (data not shown).

Increased levels of cAMP mediate the inhibition of platelet function (12). The effect of cAMP on vWF-induced translocation of Rap proteins to the cytoskeleton was investigated by treating platelets with the prostacyclin analogue iloprost or with PGE1, both of which are able to promote the activation of adenylate cyclase. Fig. 8A shows that pretreatment of platelets with iloprost or PGE1 totally prevented the ability of vWF to induce the translocation of Rap1B to the cytoskeleton and caused a strong, but not total, inhibition of the translocation of Rap2B.

To correlate the inhibitory effect of high levels of cAMP with that of the tyrosine kinases inhibitor genistein, the effect of iloprost and PGE1 on protein tyrosine phosphorylation was analyzed. Fig. 8B shows that increased cAMP levels completely antagonized vWF-stimulated protein tyrosine phosphorylation.

**Role of FcγRII in Platelet Activation by vWF**—The physical proximity between the vWF receptor, GP Ib-IX-V complex, and the FcγRII on the platelet surface has been reported recently (24). Therefore, it was interesting to know whether the FcγRII was related to the transmembrane signaling leading to Rap proteins interaction with the cytoskeleton. Platelets were treated with the anti-FcγRII monoclonal antibody IV.3, or with control IgG, and then stimulated with either vWF or thrombin. In both cases, we found that the presence of the IV.3 antibody did not affect platelet aggregation (data not shown). Cytoskeletal samples were then analyzed with the Rap1B and Rap2B antisera. Fig. 9 shows that vWF-induced translocation of both Rap1B and Rap2B was strongly inhibited by the preincubation
of platelets with the IV.3 antibody, but not with control IgG. Once again, while Rap1B was totally undetectable in the cytoskeleton from IV.3-treated, vWF-stimulated platelets, a reduced amount of Rap2B was still observed in the cytoskeleton from the same samples. Fig. 9 also shows that the ability of thrombin to induce Rap1B and Rap2B translocation to the cytoskeleton was not affected either by control IgG or by the IV.3 antibody.

To verify whether the vWF preparation used in this study could activate the FcγRII and lead to Rap proteins translocation to the cytoskeleton through contaminating immunoglobulins, 1 mg of vWF, along with known amounts of purified human IgGs, were analyzed by immunoblotting using an anti-IgG antiserum. Fig. 10A shows that the vWF preparation actually contained some contaminating immunoglobulins. However, by densitometric analysis, we calculated that contaminating IgGs represented about 2.4% of the vWF preparation. Thus, platelet samples stimulated with 10 μg/ml vWF were actually incubated with 0.24 μg/ml immunoglobulins. Such a low concentration, and the absence of secondary cross-linking antibodies, argues against a possible direct activation of the FcγRII. However, to completely rule out a possible role of activation of FcγRII by contaminating IgGs in the translocation of Rap proteins to the cytoskeleton, 0.5 ml of the vWF preparation was incubated with 10 mg of protein A-Sepharose, and the supernatant was analyzed by immunoblotting with anti-human IgG antibodies. As shown in Fig. 10A, this procedure completely removed contaminating IgGs. The IgG-free vWF was as effective as the crude vWF preparation in inducing platelet aggre-
vWF-induced Association of Rap Proteins with the Cytoskeleton

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FIG. 10. Translocation of Rap1B and Rap2B to the cytoskeleton is not mediated by vWF-contaminating immunoglobulins. A, 10 \( \mu l \) (1 mg) of vWF before (A) and after (B) incubation with protein A-Sepharose, together with 50, 100, and 200 ng of purified human immunoglobulins, were analyzed by immunoblotting using a peroxidase-conjugated goat anti-human IgG antiserum. B, cytoskeletal proteins from resting platelets (Bas) or platelets stimulated with 10 \( \mu g/ml \) vWF before (A) or after (B) treatment with protein A-Sepharose treated in combination with 0.5 mg/ml ristocetin were separated on a 10–20% acrylamide gel, transferred to nitrocellulose, and probed with the antisera against Rap1B and Rap2B as indicated.

FIG. 11. Inhibition of protein tyrosine phosphorylation by the IV.3 monoclonal antibody. Platelet samples were incubated and stimulated as described in the legend to Fig. 9. Reactions were stopped by the addition of SDS-sample buffer, and equal volumes of the total cell lysates were loaded on a 7.5% acrylamide gel. Proteins were transferred to nitrocellulose and probed with the anti-phosphotyrosine antibody. The position of the molecular mass markers are reported on the left.

FIG. 12. Effect of IV.3 monoclonal antibody on vWF-induced tyrosine phosphorylation of pp72\(^{FAK}\), PLC\(^{\gamma2}\), SHIP, and pp125\(^{FAK}\). Gel-filtered platelets were incubated with or without 20 \( \mu g/ml \) IV.3 monoclonal antibody for 2 min at 37 °C and then treated with buffer or 10 \( \mu g/ml \) vWF and 0.5 mg/ml ristocetin for 5 min. pp72\(^{FAK}\), PLC\(^{\gamma2}\), SHIP, and pp125\(^{FAK}\) were immunoprecipitated as indicated on the left and analyzed by immunoblotting with anti-phosphotyrosine antibodies (P-Tyr). Blots were then stripped and reprobed with the same antibody used for the immunoprecipitation, as indicated on the right.

DISCUSSION

vWF Causes the Translocation of Rap1B and Rap2B to the Cytoskeleton—Our present data demonstrate that stimulation of human platelets with vWF induced the translocation of the low molecular weight GTP-binding proteins Rap1B and Rap2B to the cytoskeleton. The amount of cytoskeletal-associated Rap1B and Rap2B in vWF-stimulated platelets was comparable with that measured in the cytoskeleton from thrombin-aggregated platelets. However, several differences between the effects of the two agonists were observed. Previous kinetic studies revealed that, in thrombin-stimulated platelets, translocation of Rap1B to the cytoskeleton preceded the translocation of Rap2B, which occurred only in a late phase of platelet aggregation (6, 7, 10, 11). Conversely, the interaction of Rap2B...
with the cytoskeleton in vWF-stimulated platelets was observed after 15 s, when only about 19% of aggregation was measured. Moreover, translocation of Rap1B occurred later on, when aggregation reached about 80%. It is likely that these kinetics are the expression of different mechanisms regulating the translocation of the two Rap proteins to the cytoskeleton. In this regard, it is interesting to note that pretreatment of platelets with cytochalasin D, that prevents agonist-induced actin polymerization, completely inhibited the vWF-induced translocation of Rap1B to the cytoskeleton, but only partially affected the translocation of Rap2B. This indicates that the direct or indirect interaction with the newly polymerized actin filaments totally mediates the incorporation of Rap1B into the cytoskeleton, but it is not sufficient to explain the incorporation of Rap2B. The cytochalasin D-insensitive interaction of Rap2B with the cytoskeleton may indicate either the binding of the protein to preformed actin filaments or the interaction with other structures, such as microtubules.

**GP Ib-IX-V Mediates the Translocation of Rap1B and Rap2B**—Our present information show that vWF-induced translocation of Rap proteins to the cytoskeleton required platelet aggregation triggered by the interaction of vWF with the GP Ib-IX-V complex. In thrombin-stimulated platelets, interaction of Rap proteins with the cytoskeleton is dependent on GP Ib-IIIa-mediated aggregation (9, 11). By contrast, vWF-induced translocation of Rap proteins to the cytoskeleton is not regulated by occupancy of the fibrinogen receptor, since no inhibitory effects of the RGDS peptide were observed. In this regard, Rap proteins behave much like pp60^src and phosphatidylsinositol 3-kinase, whose translocation to the cytoskeleton in vWF-stimulated platelets is independent of GP Ib-IIIa (18). We have also found that the antibody AK2, which prevents binding of vWF to the glycoprotein Ib-IX-V, and the omission of sample stirring, prevented vWF-induced translocation of Rap proteins to the cytoskeleton. This indicates that platelet aggregation mediated by interaction of vWF with the GP Ib-IX-V complex is sufficient to trigger this process.

**Protein Tyrosine Phosphorylation Is Involved in the Signaling of GP Ib-IX-V**—The evidence that, in vWF-stimulated platelets, interaction of Rap proteins with the cytoskeleton was independent of fibrinogen binding to GP Ib-IIIa allowed further investigations on the signal transduction pathways mediating these events. We successfully used an inhibitor of tyrosine kinases to demonstrate that protein tyrosine phosphorylation is associated to the translocation of Rap proteins to the cytoskeleton in vWF-stimulated platelets. vWF induced the rapid tyrosine phosphorylation of several substrates. By immunoprecipitation with specific antibodies we identified the tyrosine kinases pp72^tyr and pp125^FAK and the lipid-metabolizing enzymes PLCγ2 and SHIP as four substrates that are tyrosine-phosphorylated upon stimulation of human platelets with vWF. Tyrosine phosphorylation and activation of pp72^tyr induced by vWF has been demonstrated recently (25). In addition, our data provide the first evidence for a possible involvement of pp125^FAK, PLCγ2, and SHIP in the mechanism of platelet activation by this agonist. Protein tyrosine phosphorylation induced by vWF was completely blocked by genistein, which also totally prevented the interaction of Rap1B with the cytoskeleton and reduced the amount of cytoskeletal-associated Rap2B without affecting platelet aggregation. Once again, we noticed that translocation of Rap1B and Rap2B to the cytoskeleton was differentially sensitive to the inhibition of tyrosine kinases. This supports the hypothesis of the existence of two different mechanisms regulating Rap2B interaction with the cytoskeleton. The analysis of the effect of increased amount of cAMP levels strengthens this conclusion. In fact, both iloprost and PGE1, totally prevented the association of Rap1B, but only partially that of Rap2B. The inhibitory effect of cAMP on the translocation of Rap proteins to the cytoskeleton is unlikely to be related to the PKA-mediated phosphorylation of Rap1B, since previous works have demonstrated that both the phosphorylated and nonphosphorylated form of the protein can interact with the actin filaments (11). Moreover, we observed a partial inhibition of the association of Rap2B to the cytoskeleton, even if this protein is not a substrate for PKA (5). On the other hand, we have found that increased levels of cAMP prevented vWF-induced protein tyrosine phosphorylation. Therefore, we propose that the inhibition of Rap proteins translocation to the cytoskeleton by increased levels of cAMP is the consequence of its inhibitory effects on the activation of tyrosine kinases induced by vWF. Significantly, the different sensitivity of the translocation of Rap1B and Rap2B to the cytoskeleton to cAMP-increasing agents is similar to the effect of genistein. The mechanism of cAMP-mediated inhibition of protein tyrosine phosphorylation induced by vWF is still unclear, but the cross-talk between the two types of kinases shown herein is most interesting. The evidence that iloprost and PGE1 totally blocked this effect suggests that cAMP interferes with a very early event in the signal transduction pathway initiated by vWF. In this regard, it is interesting to note that one of the best characterized substrate for the cAMP-dependent kinase is the β-chain of glycoprotein Ib, an essential component of the vWF receptor on the platelet surface (26).

**Signaling by GP Ib-IX-V Is Mediated by FcγRII**—Although protein tyrosine phosphorylation in vWF-stimulated platelets has been reported previously, the mechanism coupling GP Ib-IX-V complex occupancy to the activation of tyrosine kinases has not been identified. Recent evidence was advanced for the association of the 14-3-3 signaling protein with the intracellular domain of glycoprotein Ib and its translocation to the cytoskeleton upon stimulation with vWF (27, 28). We now provide evidence that signaling initiated by binding of vWF to GP Ib-IX-V complex is partially mediated by the FcγRII. Blockade of the FcγRII by the monoclonal antibody IV.3 selectively prevented tyrosine phosphorylation of specific substrates in vWF-stimulated, but not in thrombin-stimulated, platelets. A previous work reported the physical proximity of GP Ib-IX-V complex with the FcγRII (24). Here it is demonstrated for the first time that FcγRII participates in platelet activation induced by vWF. In this respect the mechanism of action of vWF resembles that of collagen. In this case it has been shown that tyrosine phosphorylation of some proteins, including the tyrosine kinase pp72^tyr, is promoted by binding of collagen to glycoprotein VI and is mediated by the association with the Fc receptor γ chain (29). Moreover, a direct involvement of the FcγRII in collagen-induced platelet activation was suggested by Keely and Parise (30). It is interesting to note that both collagen and vWF are multimeric adhesive proteins expressing different binding sites for specific receptors and are able to promote the formation of glycoproteins clusters upon binding to the platelet surface. The inhibition of vWF-induced tyrosine phosphorylation by blockade of the FcγRII appeared to be limited to a selected number of substrates, which include pp72^tyr, PLCγ2, and SHIP, but not pp125^FAK. The exact mechanisms by which FcγRII participate in vWF-initiated signaling, as well as the possibility that FcγRII itself becomes tyrosine-phosphorylated, deserves further investigations. Interestingly, we found that blockade of the FcγRII by the IV.3 antibody totally inhibited the association of Rap1B to the cytoskeleton. In light of similar inhibitory effect observed in genistein-treated platelets, our results suggest that translocation of Rap1B to the cytoskeleton is mediated by signaling through tyrosine phosphorylation.
induced by vWF in a FcγRII-dependent mechanism. In this regard, it is interesting to note that the partial inhibition of the translocation of Rap2B to the cytoskeleton in genistein-treated platelets closely correlates with that observed in platelets incubated with the IV.3 antibody.

Conclusion—We have demonstrated that binding of vWF to the GP Ib-IX-V complex induced the translocation of both Rap1B and Rap2B to the platelet cytoskeleton through a GP IIb-IIIa-independent mechanism. The evidence shows a novel role of FcγRII in regulating both protein tyrosine phosphorylation and translocation of Rap proteins to the cytoskeleton. These results provide new insights into the mechanisms of platelet activation by vWF.

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