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Rhodocytin Induces Platelet Aggregation by Interacting with Glycoprotein Ia/IIa (GPIa/IIa, Integrin $\alpha_2\beta_1$)

INVOLVEMENT OF GPIa/IIa-ASSOCIATED Src AND PROTEIN TYROSINE PHOSPHORYLATION*

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Katsue Suzuki-Inoue‡, Yukio Ozaki‡§, Mie Kainoh¶, Yongchol Shin‖, Yi Wu‡, Yutaka Yatomi‡, Tukasa Ohmori‡, Toshiaki Tanaka¶, Kaneo Satoh‡, and Takashi Morita‖

From the ‡Department of Clinical and Laboratory Medicine, Yamanashi Medical University, Yamanashi 409-3898, ¶Pharmaceutical Research Laboratories, Toray Industries, Inc., 1111 Tebiro, Kamakura, Kanagawa 248-8555, and the ‖Department of Biochemistry, Meiji Pharmaceutical University, Tokyo 204-8588, Japan

Although glycoprotein Ia/IIa (GPIa/IIa, integrin $\alpha_2\beta_1$) has established its role as a collagen receptor, it remains unclear whether GPIa/IIa mediates activation signals. In this study, we show that rhodocytin, purified from the *Calloselasma rhodostoma* venom, induces platelet aggregation, which can be blocked by anti-GPIa monoclonal antibodies. Studies with rhodocytin-coupled beads and liposomes loaded with recombinant GPIa/IIa demonstrated that rhodocytin directly binds to GPIa/IIa independently of divalent cations. *In vitro* kinase assays and Western blotting of GPIa immunoprecipitates revealed that Src and Lyn constitutively associate with GPIa/IIa and that Src activity increases transiently after rhodocytin stimulation. Src specifically associates with p130 Crk-associated substrate (Cas) in a manner dependent upon Cas phosphorylation, suggesting that Src is responsible for Cas tyrosine phosphorylation. While all these phenomena occur early after rhodocytin stimulation in a cAMP-resistant manner, tyrosine phosphorylation of Syk and phospholipase Cγ2, intracellular Ca^{2+} mobilization, and platelet aggregation occur later in a cAMP-sensitive manner. Cytochalasin D, which interferes with actin polymerization and blocks receptor clustering, inhibits all the rhodocytin-mediated signals we examined in this study. We suggest that rhodocytin, by clustering GPIa/IIa, activates GPIa/IIa-associated Src, which then mediates downstream activation signals.

Platelets adhere to collagen fibers exposed at sites of damage to the endothelial lining and become activated through specific membrane receptors for collagen. Many candidates have been proposed for putative collagen receptors on platelet membranes. Of these, GP Ia/IIa[1] and GPVI have now established their roles as collagen receptors (1). It has been reported that human blood platelets that showed no response to collagen lacked the surface expression of GPIa/IIa or GPVI, indicating that GPIa/IIa and GPVI are collagen receptors (2–8). Collagen-related peptide (CRP) containing repeats of the Gly-Pro-Hyp sequence (9–11), and convulxin, which is a C-type lectin obtained from a tropical rattlesnake venom, are reported to be platelet agonists acting on GPVI (12, 13). These proteins have been very useful for investigating the signal transduction pathways mediated by GPVI. Cross-linking of GPVI results in phosphorylation of the Fc receptor γ (Fc$R\gamma$)-chain by the Src family kinases, Fyn and Lyn, then the binding of Syk to the γ-chain, with resultant Syk activation (14–17). Syk activation appears to lie upstream of the tyrosine phosphorylation of PLCγ2, which releases intracellular calcium (18).

On the other hand, relatively little has been elucidated regarding the GPIa/IIa-mediated signal transduction system. Although the role of GPIa/IIa as a collagen receptor is well established, whether GPIa/IIa also mediates activation signals has remained to be elucidated. Only recently, Kehrel *et al.* (10) has demonstrated that GPVI-deficient platelets showed increased fibrinogen binding in response to collagen but not to CRP. Since collagen but not CRP contains the binding sites for GPIa/IIa, it is suggested that GPIa/IIa also mediates certain activation signals leading to GPIIb/IIIa activation (10). Studies with inhibitors have also suggested that GPIa/IIa can transduce activation signals in platelets; tyrosine phosphorylation of Syk or PLCγ2 induced by collagen was attenuated by pretreatment of anti-GPIa/IIa inhibitory antibodies or integrin β_1 (GPIIa)-cleaving metalloproteinase, Jararhagin (19–21).

In contrast to GPVI-mediated platelet activation, there have been few GPIa/IIa agonists appropriate for investigating the GPIa/IIa-related signal transduction pathway. Aggretin, purified from snake venom, is reported to induce platelet aggregation acting as a GPIa/IIa agonist, but its functional property has not been fully elucidated (22). JBS2, an anti-α_2 integrin monoclonal antibody (mAb) that promotes collagen binding to T-cells, stimulated tyrosine phosphorylation of PLCγ2 in human platelets. However, when Fcγ receptor II (FcγRII) was blocked by an anti-FcγRII mAb, IV.3, JBS2 did not induce PLCγ2 phosphorylation, suggesting that platelet activation induced by this anti-α2 mAb occurs mainly through FcγRII (20). Thus, although it has been well established that GPIa/IIa plays a major role in platelet adhesion to collagen (1, 23, 24), it remains unclear what signals GPIa/IIa mediates that finally lead to platelet activation.

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§ To whom correspondence should be addressed: Dept. of Clinical and Laboratory Medicine, Yamanashi Medical University, Shimokato 1110, Tamaho, Nakakoma, Yamanashi 409-3898, Japan. Tel.: 81 55 273 9884; Fax: 81 55 273 6713; E-mail: yozaki@res.yamanashi-med.ac.jp.

[1] The abbreviations used are: GPIa/IIa, glycoprotein Ia/IIa; Cas, p130 Crk-associated substrate; FAK, focal adhesion kinase; ASA, acetylsalicylic acid; PGE_1, prostaglandin E_1; PLCγ2, phospholipase C γ2; CRP, Collagen-related peptide; FcγRII, Fcγ receptor II; mAb, monoclonal antibody; pAb, polyclonal antibody; TXA_2, thromboxane A_2; PYK2, proline-rich tyrosine kinase 2; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; PBS, phosphate-buffered saline; PGI_2, prostaglandin I_2; PRP, platelet-rich plasma;

Recently, we have isolated and characterized a functionally novel platelet agonist, designated as rhodocytin, from the *Calloselasma rhodostoma* venom (25). We have suggested that rhodocytin belongs to the heterodimeric C-type lectin-like proteins and appears to induce platelet aggregation by interacting with GPIa/IIa (25, 26). We found that the GPIa/IIa-mediated signal induces Syk and PLCγ2 activation, similar to GPVI-mediated signals. However, distinct from GPVI-mediated signals, tyrosine phosphorylation of Syk and PLCγ2 induced by GPIa/IIa stimulation is potently inhibited by acetylsalicylic acid (ASA) or cytochalasin D treatment. These findings suggest that Syk and PLCγ2 activation requires actin polymerization and that tyrosine phosphorylation of Syk and PLCγ2 is facilitated by the thromboxane A_2 (TXA_2)-producing system in the GPIa/IIa-mediated signal transduction pathway. Susceptibility to ASA or cytochalasin D is reminiscent of collagen-induced platelet activation.

In this study, we present several lines of evidence to suggest that rhodocytin directly and specifically binds to GPIa/IIa and that this association is independent of divalent cations distinct from other integrin-ligand bindings. Our findings suggest that, upon rhodocytin stimulation, Src that constitutively associate with GPIa/IIa become activated, and several signaling molecules including Cas, Syk, and PLCγ2 undergo tyrosine phosphorylation. It is suggested that clustering of GPIa/IIa is necessary for the GPIa/IIa-mediated signal transduction, since all of these phenomena related to tyrosine phosphorylation are sensitive to cytochalasin D, which blocks actin polymerization.

EXPERIMENTAL PROCEDURES

Materials—Rhodocytin was purified from the venom of *C. rhodostoma* as described previously (25). Anti-GPIa mAbs, 7E10B and 3C8A, were generated by one of us. Briefly, human platelets were used for immunizing 5-week-old Balb/c mice. The hybridomas obtained were screened by the reactivity to human GPIa-transfected Chinese hamster ovarian cells and human osteosarcoma-derived cell line, MG63, which expresses GPIa. Immunoprecipitation study using MG63 confirmed that clones 7E10B and 3C8A react with GPIa. Clone 7E10B is an inhibitory antibody, and clone 3C8A is a nonfunctional antibody, as determined by adhesion assays of platelets to collagen. Liposomes carrying recombinant fragments of GPIa/IIa (rGPIa/IIa liposome) were made by the methods of Staatz *et al.* (5). Sepharose beads conjugated with 7E10B were used to purify the GPIa/IIa incorporated liposome. We have confirmed that rGPIa/IIa liposome has more than 90% purity by SDS-PAGE. Anti-GPIa mAb (6F1), anti-CD9 mAb, anti-GPIb mAb (WGA3), and collagen-related peptide (CRP) were kindly donated by Dr. B. S. Coller (Mt. Sinai Medical Center, New York), Dr. S. Nomura (Kansai Medical University, Osaka, Japan), Dr. M. Handa (Keio University, Tolyo, Japan) and Drs. M. Moroi and Y. Miura (Institute of Life Science, Kurume University, Fukuoka, Japan), respectively. The FcRγ-chain-deficient C57BL/6 (B6) mice and control B6 mice were generously provided by Dr. T. Takai (Tohoku University, Sendai, Japan).

The following materials were obtained from the indicated suppliers: anti-GPIa mAb (Gi9), Immnotech, Marseille, France; anti-Syk mAb and leupeptin, Wako Pure Chemical Industries, Ltd., Tokyo, Japan; anti-PLCγ2 polyclonal antibody (pAb), Santa Cruz, CA; the hybridoma of FcγRII mAb (clone IV.3), American Type Culture Collection, Manassas, VA; anti-phosphotyrosine (PY20) mAb, anti-Cas mAb, and anti-GPIIa mAb (clone 18), Transduction Laboratories, Lexington, KY; anti-GPIa mAb (A2-IIE10), anti-Src mAb (327), and anti-Lyn pAb, Upstate Biotechnology, Lake Placid, NY; type I collagen, Nycomed Pharma GMBH, Munich, Germany; the peptide Gly-Arg-Gly-Asp-Ser (GRGDS), Peptide Institute, Osaka, Japan; bovine serum albumin (BSA), prostaglandin I_2 (PGI_2), phenylmethylsulfonyl fluoride, sodium orthovanadate, Triton X-100, tetramethylrhodamine isothiocyanate-conjugated phalloidin, and enolase, Sigma; protein A-Sepharose and CNBr-activated Sepharose 4B beads, Amersham Pharmacia Biotech; peroxidase-conjugated goat anti-mouse IgG, Cappel Organ Teknika Co.; PGE_1, Funakoshi, Tokyo, Japan; Fura2-AM, Dojindo Laboratories, Kumamoto, Japan; PP1, Biomol Research Laboratories, Inc., Plymouth Meeting, PA; PP2, Calbiochem; [γ-^{32}P]ATP, PerkinElmer Life Sciences.

Platelet Preparation—Venous blood from healthy drug-free volunteers was collected into a tube containing acid/citrate/dextrose (ACD). Platelet-rich plasma (PRP) was obtained after centrifugation of whole blood at 160 $\times$ *g* for 10 min. When indicated, PRP was incubated with 1 mM ASA for 30 min to exclude the secondary effects of TXA. The platelets were washed twice with 15% ACD and 100 nM PGI_2 and resuspended in HEPES buffer containing 138 mM NaCl, 3.3 mM $NaHPO_4$, 2.9 mM KCl, 1.0 mM MgCl, 1 mg/ml glucose, and 20 mM HEPES, pH 7.4, at a concentration of 10^9 cells/ml. Thirty minutes before experiments, the platelet suspension was supplemented with 1 mM CaCl.

FcR γ-Chain-deficient Mice and Preparation of Murine Platelets—From FcRγ-chain-deficient C57BL/6 (B6) mice and control B6 mice, blood (500–900 μl) was taken into a 1-ml plastic syringe containing 0.1 ml of ACD by cardiac puncture immediately after the mice died from deep ethyl ether anesthesia. PRP was obtained after centrifugation of whole blood at 160 $\times$ *g* for 10 min. Residual blood after PRP had been removed was further diluted by adding 200 μl of Tyrodes-HEPES buffer (134 mM NaCl, 2.9 mM KCl, 0.34 mM Na_2HPO_4, 12 mM $NaHCO_3$, 20 mM HEPES, 1 mM $MgCl_2$, pH 6.6). The mixture was centrifuged again, and the supernatant was obtained for the maximum recovery of platelets. After repeating this step several times, PRP was centrifuged at 1,000 $\times$ *g* for 5 min in the presence of 100 nM PGI_2 and 15% ACD. The platelets were resuspended in Tyrodes-HEPES buffer, pH 7.3, at a concentration of 1.3 $\times$ 10^8 cells/ml.

Platelet Aggregation—Washed human platelets at a concentration of 2.0 $\times$ 10^8/ml or murine platelets at a concentration of 1.3 $\times$ 10^8 cells/ml were activated by the indicated concentrations of agonists under continuous stirring at 1,000 rpm in a PA-100 platelet aggregation analyzer (Kowa, Tokyo, Japan). When indicated, platelets were pretreated with the indicated concentration of anti-GPIa mAbs, 1 μM PGE_1, 5 μM PP1 or 5 μM PP2 at 37 °C for 5 min. The instrument was calibrated with a platelet suspension for zero light transmission and with buffer for 100% transmission.

Platelet Proteins That Bind to Rhodocytin—Thirty five μg of purified rhodocytin was covalently coupled to 50 mg of CNBr-activated Sepharose 4B beads according to the manufacturer's instructions. One ml of platelet lysates (3.5 $\times$ 10^9 cells) lysed by an equal volume of 2$\times$ ice-cold lysis buffer(2% Triton X-100, 100 mM Tris/HCl, pH 7.5, 5 mM EGTA, 2 mM vanadate, 1 mM phenylmethylsulfonyl fluoride, and 100 μg/ml of leupeptin) was clarified by centrifugation at 16,000 $\times$ *g* for 5 min. The supernatant was precleared by 100 μl of Sepharose 4B (50% slurry) for 1 h and then was split into two portions. One-half was incubated with 80 μl of rhodocytin-bound Sepharose 4B, and the other half was incubated as the control with 80 μl of Sepharose 4B for overnight. The beads were washed 5 times in 1$\times$ lysis buffer, and proteins were eluted from the beads with 40 μl of SDS reducing buffer (19) and were boiled for 5 min.

Adhesion of rGPIa/IIa Liposomes or Platelets to Rhodocytin- or Collagen-coated Plates—For the preparation of microtiter plates coated with rhodocytin or collagen, 100 μl of the indicated concentrations of rhodocytin or collagen was added to each well of a 96-well flat-bottomed plate and was left to stand overnight at room temperature. After two cycles of washing with PBS, each well was blocked with 1% BSA in PBS for 30 min at room temperature. Before use, 1% BSA-containing PBS was heated at 80 °C for 10 min and sterilized by filtration. One hundred μl of rGPIa/IIa liposomes (2 μg/ml) or washed platelet (1 $\times$ 10^4 cells/ml) was then added to each well and incubated at 30 °C for 3 h or 20 min, respectively. With rGPIa/IIa liposome, wells were incubated with biotinylated anti-GPIa mAb (3C8A) after removing unbound rGPIa/IIa liposome. rGPIa/IIa liposome binding was detected by horseradish peroxidase-conjugated avidin and visualized with *o*-phenylenediamine. The optical density was measured by a microplate reader (NOVAPATH, Bio-Rad). When platelet adhesion was measured, after removing unbound platelet, wells were incubated with 3% paraformaldehyde for 30 min at room temperature. After platelet membranes were lysed with 0.2% Triton X-100, they were stained by 0.1 μg/ml tetramethylrhodamine isothiocyanate-conjugated phalloidin. Cells were viewed under a BH microscope (Olympus, Tokyo, Japan) and photographed on Fujicolor super G400 (Fujifilm, Tokyo, Japan). The number of adhered platelet was calculated on the photographs.

Immunoprecipitation and Western Blotting—After platelets were activated with rhodocytin for the indicated time intervals, reactions were terminated with an equal volume of ice-cold lysis buffer. The lysate was sonicated and centrifuged at 16,000 $\times$ *g* for 5 min. The supernatant was precleared with protein A-Sepharose beads for 30 min at 4 °C and then mixed with the indicated antibodies. The mixture was rotated for 1–2 h at 4 °C and, after the addition of protein A-Sepharose beads, was further rotated for 1 h. The Sepharose beads were washed three times with 1$\times$ lysis buffer. SDS reducing buffer was then added to the beads,

followed by boiling for 3 min. The proteins were separated by 8% SDS-PAGE and electrophoretically transferred onto Clear Blot Membrane-P. The membranes were blocked with 1% BSA in PBS. After extensive washing with PBS containing 0.1% Tween 80, the immunoblots were incubated for 2 h with the indicated antibody. Antibody binding was detected by using peroxidase-conjugated goat anti-mouse IgG or anti-rabbit IgG and visualized with ECL chemiluminescence reaction reagents (Amersham Pharmacia Biotech) and Konica x-ray film (JX 8 × 10, Konica Co., Tokyo, Japan). Where indicated, levels of tyrosine phosphorylation were quantified using PDI1400oe Scanner and Quantity One 2.5a software for Macintosh.

Immunoprecipitation Kinase Assay—After preclearing, the sample was split into two portions. One-half was used for immunoblotting as described elsewhere, and the other half was processed further for an *in vitro* kinase assay. For *in vitro* kinase assay, the beads were washed once with HEPES buffer (10 mM HEPES/NaOH, 1 mM vanadate, pH 8.0) and then incubated with 25 μl of kinase reaction buffer (300 mM HEPES/NaOH, 15 mM $MnCl_2$, 150 mM $MgCl_2$, pH 8.0) with 10 μl of acid-treated enolase. The reaction was initiated by the addition of 2 μM ATP (10 μCi of [γ-^{32}P]ATP). After 10 min at 20 °C, reactions were stopped by the addition of Laemmli buffer and then subjected to boiling for 3 min. The proteins were separated under reducing conditions by 8% SDS-polyacrylamide gel electrophoresis (PAGE) and electrophoretically transferred onto Clear Blot Membrane-P (Atto, Tokyo, Japan). The membrane was treated with 1 M KOH for 60 min, dried, and visualized with a BAS-2000 PhosphorImager analyzer (Fuji Film, Japan). Where indicated, levels of radioactivity were quantified using PDI1400oe Scanner and Quantity One 2.5a software for Macintosh.

RESULTS

Effects of Anti-GPIa mAbs and an Anti-FcγRII mAb on Rhodocytin-induced Platelet Aggregation—We previously reported that echicetin, which is GPIb-blocking snake venom, does not inhibit rhodocytin-induced platelet aggregation, suggesting that rhodocytin induces platelet aggregation independently of GPIb (25). We also showed that rhodocytin induces platelet aggregation with a long lag time and that rhodocytin-induced platelet aggregation is susceptible to ASA and cytochalasin D treatment (26). Since all of these properties are reminiscent of collagen-induced platelet aggregation, we investigated the effect of antibodies directed against GPIa/IIa, which is one of the most important collagen receptors in platelets, on rhodocytin-induced platelet aggregation. A2-IIE10, a mAb directed against GPIa, potently blocked collagen-induced platelet aggregation, whereas Gi9, another mAb directed against GPIa, only partially inhibited collagen-induced platelet aggregation even at a concentration of 100 μg/ml (Fig. 1*A*). Rhodocytin (10 nM)-induced platelet aggregation was inhibited by 50 μg/ml of Gi9 and was completely inhibited by A2-IIE10 at 25 μg/ml, the concentration that also inhibits collagen-induced platelet aggregation (Fig. 1*A*). Relatively high concentrations of anti-GPIa mAbs required for inhibition of rhodocytin-induced platelet aggregation imply that rhodocytin binds to GPIa/IIa with high affinity. Performed as negative control experiments, none of these anti-GPIa antibodies inhibited platelet aggregation induced by CRP or thrombin (Fig. 1*A*). We confirmed that a blocking antibody against integrin-associated protein (B6H12), one of the platelet membrane proteins, had no effect on platelet aggregation induced by rhodocytin, even at a concentration of 100 μg/ml (26).

In contrast, IV.3 (anti-FcγRII blocking mAb), at a concentration that could completely inhibit platelet aggregation induced by a FcγRII-activating mAb (NNKY1–19), had virtually no effect on rhodocytin-induced platelet aggregation (Fig. 1*B*). These findings suggest that rhodocytin interacts with GPIa/IIa but not with GPIb or FcγRII.

We also examined the effects of an anti-GPIa antibody on the interaction between platelets and immobilized rhodocytin. Washed platelets incubated with A2-IIE10 or control mouse IgG were added to rhodocytin-coated wells. Platelet binding was detected by staining with rhodamine-conjugated phalloidin. This binding was reduced to 12.5% of the control in the presence of 25 μg/ml A2-IIE10 (data not shown). The fact that anti-GPIa antibody almost completely inhibits the association between platelets and rhodocytin indicates that GPIa/IIa is the major receptor responsible for the rhodocytin-platelet interaction.

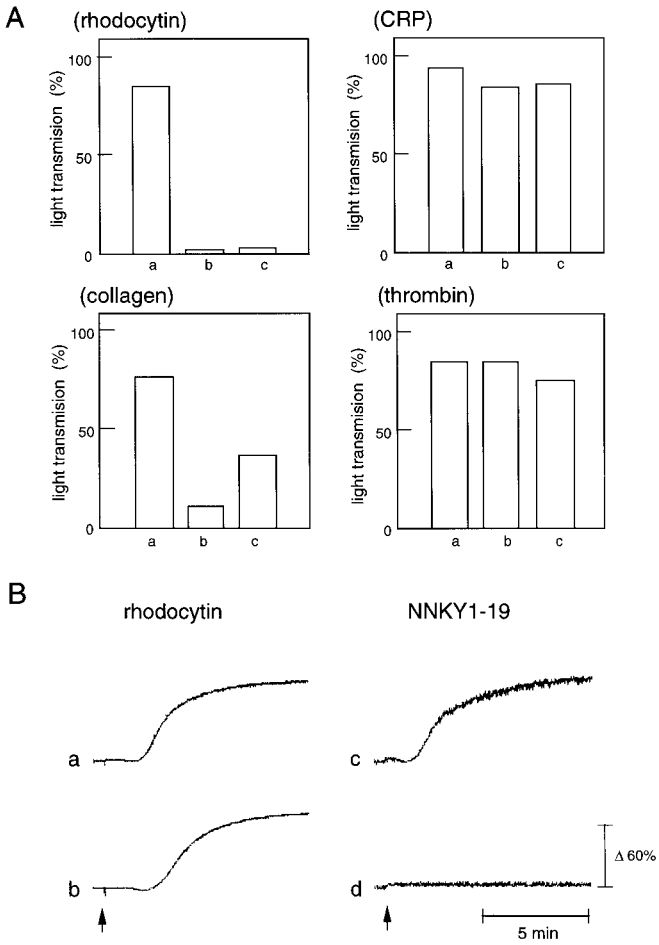

FIG. 1. **Effects of monoclonal antibodies against GPIa and FcγRII on platelet aggregation induced by rhodocytin or other agonists.** *A,* platelets were preincubated for 5 min with a vehicle solution (*a*), 25 μg/ml of A2-IIE10 (*b*), and 100 μg/ml of Gi9 (*c*). Platelets were then stimulated with 10 nM rhodocytin, 10 μg/ml of collagen, 0.5 μg/ml of CRP, or 0.2 units/ml of thrombin. Platelet aggregation was monitored by changes in light transmission. *B,* platelets were preincubated for 5 min with a vehicle solution (*a* and *c*) and 3 μg/ml FcγRII-blocking mAb (IV.3) (*b* and *d*). Platelets were then stimulated with 10 nM rhodocytin (*a* and *b*) and 100 μg/ml FcγRII-activating mAb (NNKY1-19) (*c* and *d*).

Platelet Aggregation in FcRγ-chain Knockout Mice Induced by Rhodocytin—We investigated whether rhodocytin-mediated platelet aggregation is mediated by GPVI, one of the major collagen receptors. FcRγ-chain, which associates with GPVI is necessary for GPVI-mediated signal transduction, and collagen-related peptide (CRP), which is a specific GPVI agonist, fails to induce aggregation of platelets obtained from GPVI-deficient patients or FcRγ-chain knockout mice (18). As shown in Fig. 2, 20 nM rhodocytin induced aggregation of platelets obtained from Fcγ −/− mice, whereas CRP up to the concentration of 1 μg/ml failed to do so. In contrast, both rhodocytin and CRP elicited platelet aggregation with Fcγ +/+ mice. These findings suggest that rhodocytin induces platelet aggregation independently of GPVI.

Precipitation of GPIa/IIa in Platelet Lysates with Rhodocytin-coupled Beads—Based on the effects of specific mAbs, we have already suggested that rhodocytin induces platelet aggre-

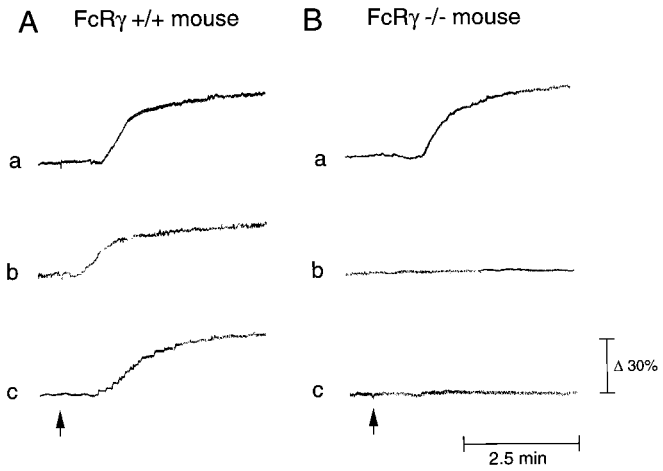

FIG. 2. **Effects of rhodocytin, collagen and CRP on platelets obtained from FcRγ-deficient mice.** Platelets obtained from control mice (*A*) or FcRγ-deficient mice (*B*) were stimulated with 20 nM rhodocytin (*a*), 10 μg/ml collagen (*b*), or 1 μg/ml of CRP (*c*). Platelet aggregation was monitored by changes in light transmission. The ordinate represents percent changes in light transmission. The data are representative of three experiments.

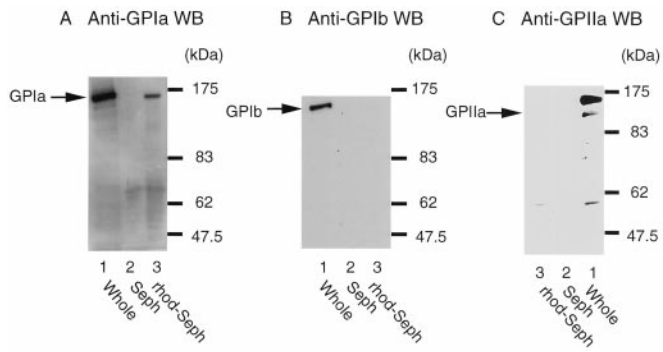

FIG. 3. **Affinity precipitation of GPIa with rhodocytin-coupled beads from platelet lysates.** Unstimulated washed platelets were lysed directly Triton X-100 lysis buffer. Whole cell lysates (*Whole, lane 1*) and proteins precipitated by affinity with Sepharose 4B alone (*Seph, lane 2*) or rhodocytin-coupled Sepharose 4B (*rhod-Seph, lane 3*) were resolved by 8% SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with anti-GPIa pAb (AB1936) (*A*), with anti-GPIb mAb (WGA3) (*B*), or with anti-GPIIa mAb (clone 18) (*C*). Molecular mass markers are indicated in kDa on the *right side* of panels. *WB*, Western blot.

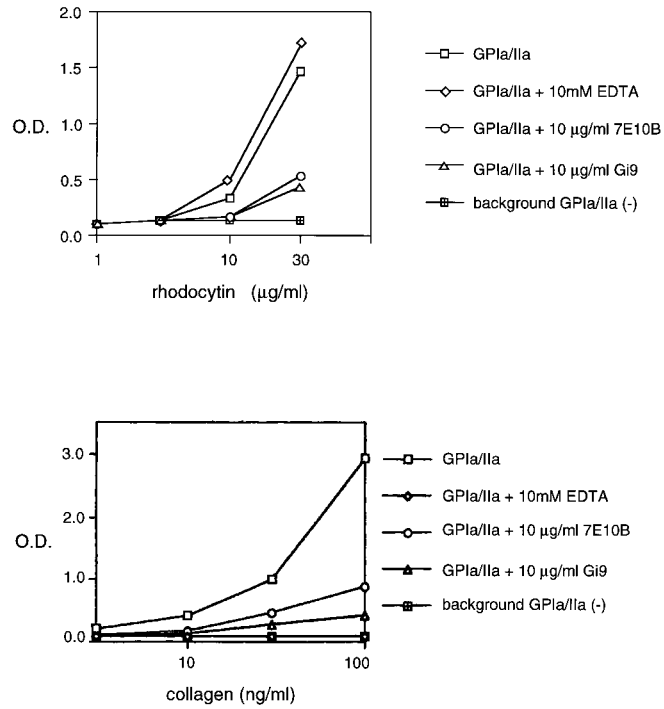

FIG. 4. **Adhesion of rGPIa/IIa liposomes on the rhodocytin-coated plate or the collagen-coated plate.** Plastic plates (96-well) were coated with the indicated concentrations of rhodocytin (*upper panel*) or collagen (*lower panel*) overnight at room temperature. Wells were blocked with 1% BSA for 30 min and washed twice with PBS. One hundred μl of rGPIa/IIa liposomes (2 μg/ml) was added to each well and incubated for 3 h at 30 °C. The amount of bound rGPIa/IIa liposome was detected by enzyme immunoassay as described under "Experimental Procedures." The *ordinate* represents the optical density. The data are representative of at least three experiments.

gation by interacting with GPIa/IIa, independently of GPIb (25, 26). To confirm this hypothesis, we sought to determine whether rhodocytin-coupled beads bound to GPIa/IIa. Sepharose 4B beads were covalently coupled with rhodocytin and added to platelet lysates. Immunoblot analysis showed that rhodocytin-coupled Sepharose 4B beads effectively precipitated GPIa, whereas there was no recovery of GPIa with Sepharose 4B control beads (Fig. 3*A*). On the other hand, neither rhodocytin-coupled Sepharose 4B beads nor Sepharose 4B beads as a control precipitated GPIb (Fig. 3*B*). We could not detect coprecipitation of GPIIa in rhodocytin-coupled Sepharose 4B beads, although an anti-GPIIa antibody detected the presence of GPIIa in whole cell lysates of platelets. These findings suggest that rhodocytin binds to GPIa but not to GPIIa or GPIb.

GPIa/IIa or Platelet Adhesion to Immobilized Rhodocytin and the Effects of EDTA—To investigate whether rhodocytin binds directly or indirectly to GPIa (for example, via an unidentified protein which associates with GPIa/IIa), we evaluated the binding of liposomes carrying recombinant fragments of GPIa/IIa (rGPIa/IIa liposomes) to immobilized rhodocytin. As shown in Fig. 4, rGPIa/IIa liposomes adhered to immobilized rhodocytin or collagen. This binding was potently blocked by 10 μg/ml anti-GPIa blocking mAbs, 7E10B and Gi9. It is well known that the binding of integrins to their ligands requires the presence of divalent cations. The adhesion of rGPIa/IIa liposomes to collagen was completely inhibited by 10 mM EDTA. To our surprise, EDTA did not inhibit the binding of rGPIa/IIa liposomes to rhodocytin at all. These findings suggest that rhodocytin specifically and directly interacts with GPIa/IIa, independently of divalent cations.

Platelet Aggregation, Intracellular Calcium Mobilization, Tyrosine Phosphorylation of Syk, and PLCγ2 Mediated by GPIa/IIa Are Completely Inhibited by PGE_1—We previously reported that rhodocytin-induced GPIa/IIa stimulation elicits tyrosine phosphorylation of Syk, subsequent PLCγ2 activation, and intracellular calcium mobilization, which finally leads to platelet aggregation and that these activation signals are inhibited by ASA treatment (26). We also confirmed that Syk and PLCγ2 were activated even when platelets were pretreated with GRGDS peptides and EGTA, suggesting that this activation is aggregation-independent and that this is not mediated through GPIIb/IIIa (data not shown). In this study, we extended our study to evaluate the effects of PGE_1, which increases the intracellular cAMP concentration. As shown in Fig. 5, platelet aggregation, intracellular calcium mobilization, and tyrosine phosphorylation of Syk and PLCγ2 were completely inhibited by 1 μM PGE_1. Since several parameters of collagen-induced platelet activation are resistant to the elevation of intracellular cAMP (27–29), we then sought to determine rhodocytin-induced activation signals, which were resistant to PGE_1.

Rhodocytin Stimulation Induces Tyrosine Phosphorylation of an Adapter Protein, Cas, Independent of PGE_1 Treatment—Recently, several groups reported that Cas (Crk-associated pro-

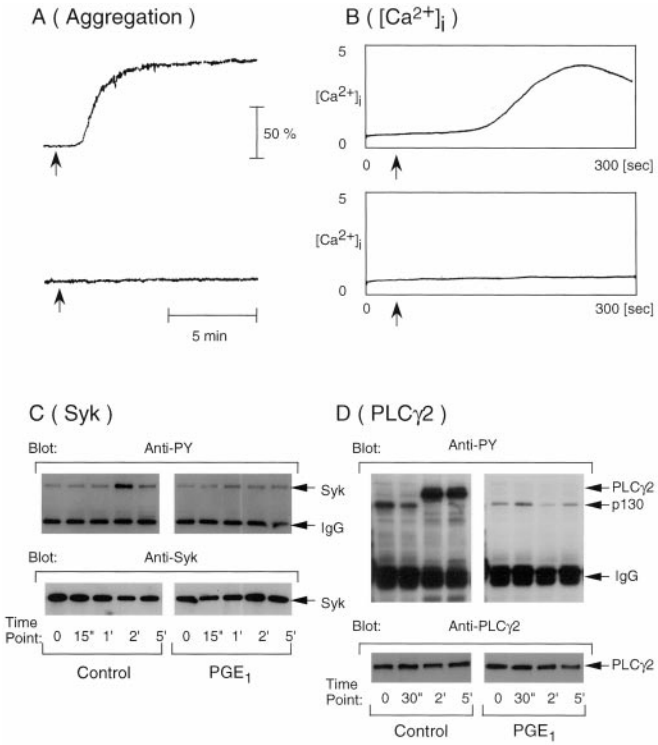

FIG. 5. **Platelet aggregation, intracellular calcium mobilization, Syk, and PLCγ2 tyrosine phosphorylation induced by GPIa/IIa stimulation were inhibited by PGE_1.** *A*, washed platelets were pretreated with vehicle solution (*upper panel*) or 1 μM PGE_1 (*lower panel*) for 5 min and then activated with 10 nM rhodocytin added at the time indicated by *arrows*. Platelet aggregation was monitored by the changes in light transmission. The *ordinate* represents percent changes in light transmission. *B*, platelets were incubated with vehicle solution (*upper panel*) or 1 μM PGE_1 (*lower panel*) for 5 min in buffer containing 1 mM $CaCl_2$ and then stimulated with 10 nM rhodocytin, added at the time indicated by *arrows*. $[Ca^{2+}]_i$ elevation was monitored as changes in fura-2 fluorescence for 300 s. The *ordinate* represents the ratio of fura-2 fluorescence. *C* and *D*, platelets were incubated with a vehicle solution or 1 μM PGE_1 for 5 min and then activated by 20 nM rhodocytin for the indicated times. Reactions were terminated with lysis buffer, and platelet proteins associated with $p72^{Syk}$ or PLCγ2 were immunoprecipitated with anti-$p72^{Syk}$ mAb (*C*) or anti-PLCγ2 pAb (*D*). The sample was then Western-blotted with anti-phosphotyrosine mAbs (4G10 *plus* PY20), anti-Syk mAb, or anti-PLCγ2 pAb. The data are representative of at least three experiments.

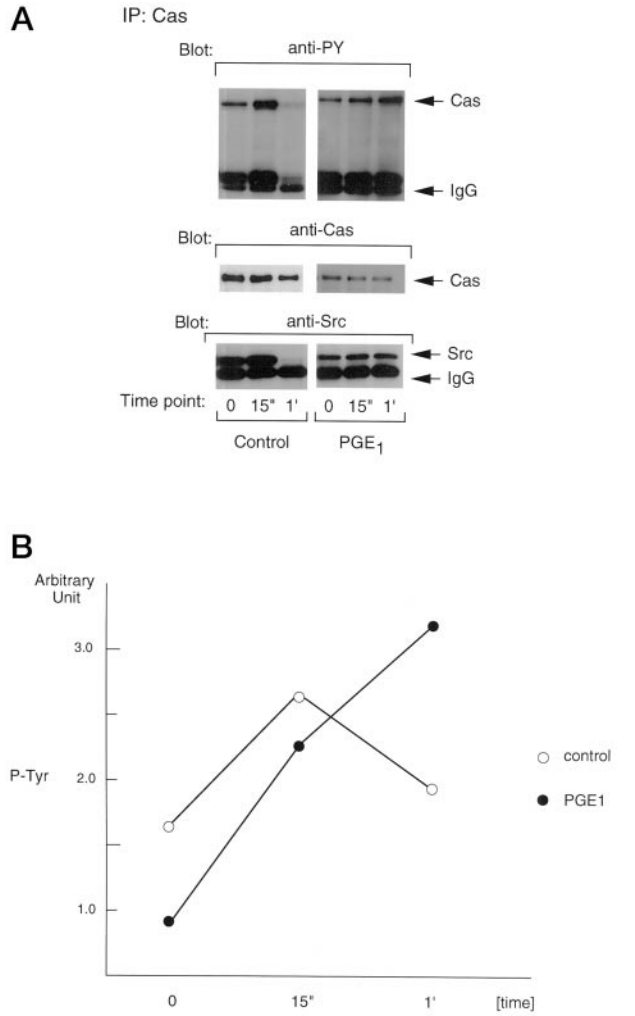

FIG. 6. **PGE_1-independent tyrosine phosphorylation of Cas induced by GPIa/IIa stimulation and Src associates with Cas in a manner dependent upon Cas tyrosine phosphorylation.** *A,* washed platelets were pretreated with vehicle solution or 1 μM PGE_1 for 5 min and then activated by 20 nM rhodocytin for the indicated times. Reactions were terminated with lysis buffer, and platelet proteins associated with Cas were immunoprecipitated (*IP*) with anti-Cas pAb. The sample was then Western blotted (*Blot*) with anti-phosphotyrosine mAbs, anti-Cas mAb and anti-Src mAb. The data are representative of at least three experiments. *B,* tyrosine phosphorylation of Cas was quantified with the image analyzing software, Quantity One. Cas tyrosine phosphorylation was quantified as relative intensity to Cas protein.

tein), an adapter protein of 130 kDa, and FAK are involved in cell activation induced by cross-linking of β_1 integrin or cell adhesion to extracellular matrix including collagen in other cells (30, 31, 39). Recently, we identified Cas in platelets (33) and found that it undergoes changes upon platelet activation (34). Therefore, we investigated tyrosine phosphorylation of Cas and FAK upon rhodocytin stimulation. FAK tyrosine phosphorylation occurred relatively late after stimulation and was dependent upon platelet aggregation (data not shown). On the other hand, Cas underwent tyrosine phosphorylation 15 s after stimulation, and the tyrosine-phosphorylated Cas was dephosphorylated after 1 min, as aggregation proceeded further (Fig. 6*A*). Tyrosine-phosphorylated Cas may be dephosphorylated by the increased phosphatase activity mediated through GPIIb/IIIa (34). We also confirmed that Cas tyrosine phosphorylation is independent of platelet aggregation, which is distinct from FAK tyrosine phosphorylation (34). The onset of Cas tyrosine phosphorylation was significantly earlier (15 s) than that of Syk or PLCγ2 (2 min after stimulation in both cases), which suggests that Cas tyrosine phosphorylation lies more proximal to GPIa/IIa than Syk or PLCγ2. Quantification of Cas tyrosine phosphorylation relative to Cas protein showed that PGE_1 pretreatment did not inhibit Cas tyrosine phosphorylation (Fig. 6*B*). Under PGE_1 pretreatment, which inhibited platelet aggregation, the level of tyrosine phosphorylation was kept elevated 1 min after stimulation, whereas the tyrosine-phosphorylated Cas was dephosphorylated after 1 min without PGE_1. This is in strong contrast to Syk and PLCγ2, the tyrosine phosphorylation of which was completely abrogated by PGE_1. Since Cas is an adapter protein without kinase activity, a tyrosine kinase must be activated before tyrosine phosphorylation of these adapter proteins occurs.

Src Associates with Cas in a Manner Dependent upon Cas Tyrosine Phosphorylation—Several studies have suggested that Src, FAK, PYK2, or Fyn play an important role in Cas tyrosine phosphorylation in other cells (30, 32, 40–43, 46, 48–52). Immunoprecipitation with an anti-Cas antibody, followed by Western blotting with an anti-Src mAb, revealed that Src associates with Cas upon GPIa/IIa stimulation (Fig. 6). The association between Src and Cas appears to be related to the

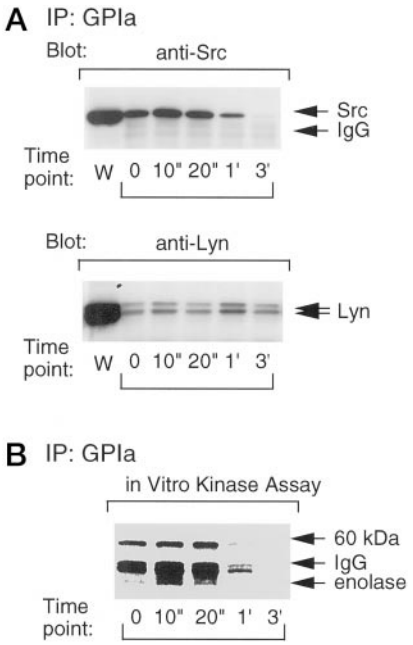

FIG. 7. **Src and Lyn constitutively associate GPIa/IIa and the kinase activity associated with GPIa/IIa is transiently activated upon rhodocytin stimulation.** Washed platelets were activated by 20 nM rhodocytin for the indicated times. Reactions were terminated with lysis buffer, and GPIa/IIa was isolated by immunoprecipitation with anti-GPIa mAb, Gi9. The samples were divided into two parts, one-half was used for *in vitro* kinase assay with enolase as exogenous substrate, and the other half was for Western blotting with anti-Src mAb (*A, upper panel*) and anti-Lyn pAb (*A, lower panel*). Radioactivity of GPIa/IIa-associated protein was visualized with a Bio-Imaging analyzer BAS2000 (*B*).

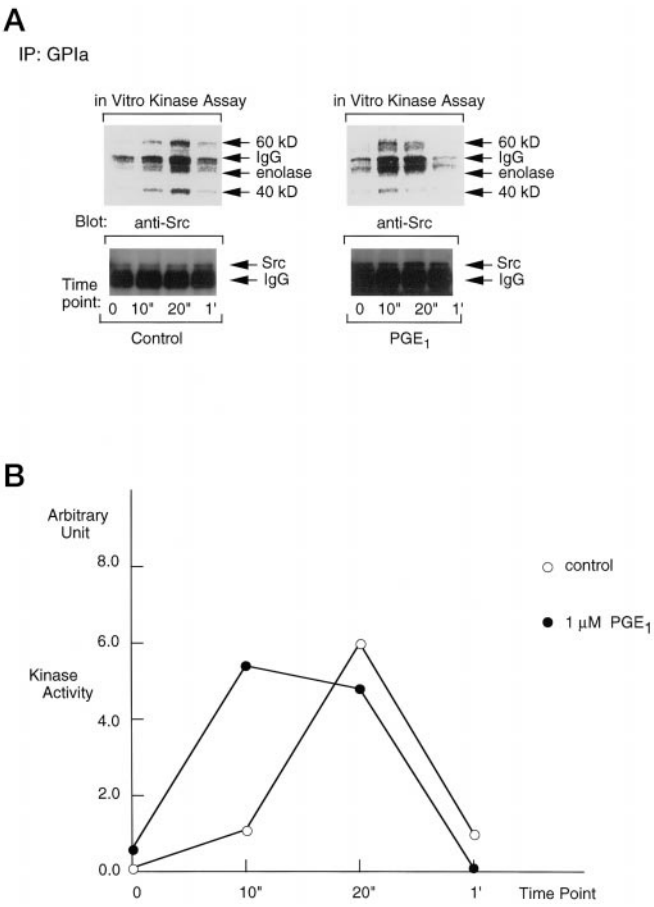

FIG. 8. **The GPIa/IIa-associated kinase is transiently activated upon rhodocytin stimulation, independent of PGE_1.** Washed platelets were pretreated with vehicle solution or 1 μM PGE_1 in buffer containing 500 μM GRGDS and 500 μM EGTA and then activated by 20 nM rhodocytin for the indicated times. Reactions were terminated with lysis buffer. GPIa/IIa was isolated by immunoprecipitation (IP) with anti-GPIa mAb, 6F1. The samples were divided into two parts. One-half was used for *in vitro* kinase assay, with enolase as exogenous substrate; the other half is for Western blotting (*Blot*) with anti-Src antibody (*A, lower panel*). Radioactivity of GPIa/IIa-associated protein was visualized with Bio-Imaging analyzer BAS2000 (*A, upper panel*) and quantified with the image analyzing software, Quantity One (*B*). Kinase activity toward enolase was calculated.

tyrosine-phosphorylated level of Cas. In contrast, we were unable to identify the co-presence of FAK, PYK2, Fyn, Lyn, or Syk in Cas immunoprecipitates (data not shown). These findings suggest that Src specifically associates with Cas and that Src but not FAK or PYK2 may phosphorylate Cas in platelets. We did not detect Cas in the immunoprecipitates of Src, probably because the total amount of Src in platelets far exceeds that of Cas in platelets (data not shown).

Src and Lyn Constitutively Associate with GPIa/IIa and the GPIa/IIa-associated Kinase Activity Rapidly Increases Upon GPIa/IIa Stimulation—Immunoprecipitation of GPIa/IIa with an anti-GPIa antibody, followed by Western blotting with antibodies against FAK, Fyn, phosphatidylinositol 3-kinase, Lck, Jak1, Jak2, PTP1D/SHP2, Cas, Cbl, 14-3-3, and Fc receptor γ-chain produced negative results, suggesting that these proteins do not associate with GPIa/IIa (data not shown). However, immunoprecipitates with anti-Src mAb or anti-Lyn pAb revealed that Src and Lyn constitutively associated with GPIa/IIa and that Src but not Lyn dissociated from GPIa/IIa as platelets formed concrete aggregates (Fig. 7*A*). Src and Lyn did not co-precipitate with control IgG (data not shown). *In vitro* kinase assays of GPIa/IIa immunoprecipitates revealed that the 60-kDa kinase activity increased 10 s after stimulation and completely disappeared 1 min after stimulation (Fig. 7*B*). Lyn constantly associated with GPIa/IIa, even when the kinase activity was totally absent at 3 min. On the other hand, Src almost completely dissociated from GPIa/IIa 3 min after stimulation. Thus, the presence of Src correlates well with the GPIa/IIa-associated kinase activity. Since the Western-blotted membrane was incubated with KOH, to minimize serine/threonine phosphorylation, the kinase activity most probably belongs to a tyrosine kinase. The molecular mass of this autophosphorylated kinase, 60 kDa, is the same as that of Src but distinct from that of Lyn (56/53 kDa). None of other tyrosine kinases that exist in platelets were detected in GPIa/IIa immunoprecipitates. These findings strongly suggest that the GPIa/IIa-associated kinase activity belongs to that of Src. When platelets were pretreated with EGTA and GRGDS to inhibit platelet aggregation, the association between GPIa/IIa and Src/Lyn remained constant, regardless of GPIa/IIa stimulation (Fig. 8*A, left panel*). Although the amount of GPIa/IIa-associated Src remained constant under GRGDS treatment, *in vitro* kinase assays of GPIa/IIa immunoprecipitates revealed that the 60-kDa kinase activity increased 10–20 s after stimulation and decreased after 1 min (Fig. 8*A*). We sometimes detected a yet unidentified 40-kDa phosphorylated protein. The pattern of the 60-kDa kinase activity was not greatly altered by PGE_1; it increased 10–20 s after stimulation and decreased after 1 min (Fig. 8*A, right panel*, and Fig. 8*B*). Taken together, we suggest that a change in the GPIa/IIa-associated Src activity is the most proximal step in the GPIa/IIa-mediated signal transduction.

PP1 Potently Inhibits Platelet Aggregation Induced by Rhodocytin—Since we found that GPIa/IIa-associated Src activity is the most proximal step in the GPIa/IIa stimulation, we

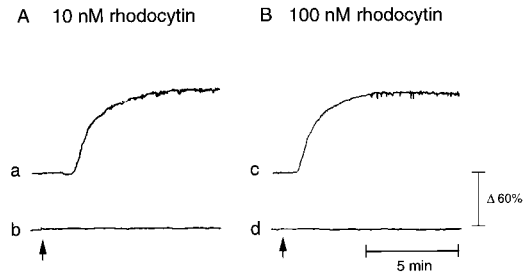

FIG. 9. **Inhibitory effects of PP1 on platelet aggregation induced by rhodocytin.** Washed platelets were pretreated with vehicle solution (*a*) or 5 μM PP1 (*b*) for 5 min, then activated with 10 nM (*A*) or 100 nM (*B*) rhodocytin added at the time indicated by arrows. Platelet aggregation was monitored by changes in light transmission. The ordinate represents % changes in light transmission.

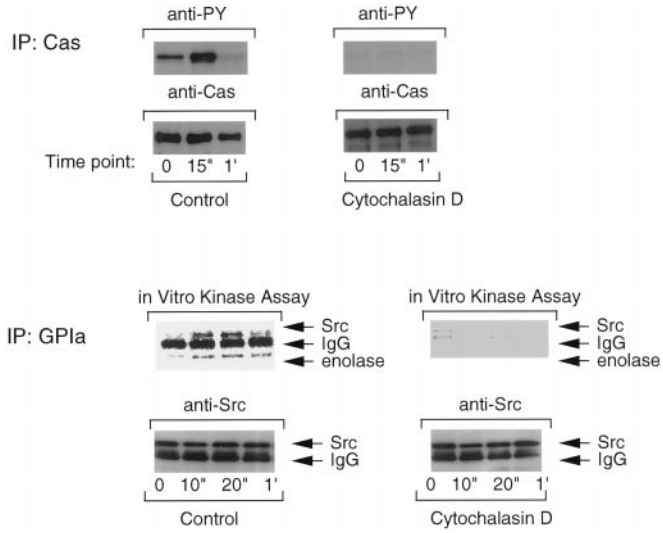

FIG. 10. **Inhibitory effects of cytochalasin D on rhodocytin-induced signals.** Washed platelets were pretreated with a vehicle solution or 10 μg/ml of cytochalasin D for 5 min, then activated by 20 nM rhodocytin for the indicated periods of time. In the *lower panel*, 500 μM GRGDS was also used to inhibit platelet aggregation. Reactions were terminated with lysis buffer, and platelet proteins associated with Cas and GPIa/IIa were immunoprecipitated with anti-Cas mAb (*upper panel*) and anti-GPIa mAb (*lower panel*), respectively. The samples were then Western blotted with the anti-phosphotyrosine mAbs, anti-Cas mAb and anti-Src mAb. In the *lower panel*, *in vitro* kinase assays were also performed, with enolase as exogenous substrate. The data are representative of at least three experiments.

next sought to investigate the effects of PP1, a specific inhibitor of Src kinase inhibitor, on platelet aggregation induced by rhodocytin. As shown in Fig. 9, 5 μM PP1 completely inhibited platelet aggregation induced by 10 nM rhodocytin, and this inhibitory effect was not overcome by the higher concentration of rhodocytin (100 nM). These findings suggest that Src is activated after rhodocytin stimulation and plays a functionally important role in rhodocytin-induced platelet aggregation.

Cytochalasin D Inhibits the Activation of GPIa/IIa-associated Kinase, Tyrosine Phosphorylation of Cas—Cytochalasin D, which disrupts actin filament organization, inhibits tyrosine kinase-mediated integrin signaling in several systems (44). We have already shown that cytochalasin D inhibits platelet aggregation, intracellular calcium mobilization, and Syk or PLCγ2 tyrosine phosphorylation induced by rhodocytin (26). In this study, we evaluated the effects of cytochalasin D on the signaling molecules more proximal to GPIa/IIa, which are the GPIa/IIa-associated kinase activity and Cas. As shown in Fig. 10, cytochalasin D potently inhibited the activation of GPIa/IIa-associated kinase and tyrosine phosphorylation of Cas. Thus, cytochalasin D inhibited all the activation signals evaluated in this study, implying that cytochalasin D inhibits the GPIa/IIa-mediated signals from the most proximal step. The potent inhibitory effect of cytochalasin D indicates that the clustering of GPIa/IIa is important for eliciting the downstream activation signals induced by rhodocytin.

DISCUSSION

Rhodocytin Induces Platelet Aggregation by Interacting with GPIa/IIa—In the present study, we sought to identify the membrane molecule that rhodocytin binds and the mechanism by which it elicits platelet activation. Several membrane glycoproteins have been identified, which induce activation signals upon ligand binding. They include GPIb, FcγRII, GPIa/IIa, and GPVI. In a previous study, we found that echicetin, GPIb-blocking snake venom, has no effect on rhodocytin-induced platelet aggregation (25). We found in this study that IV.3, an anti-FcγRII antibody, did not inhibit platelet aggregation induced by rhodocytin. It is well known that FcRγ-chain physically associates with GPVI and plays a crucial role in the GPVI-mediated signaling pathway. It is reported that CRP, a GPVI agonist, fails to induce aggregation of Fcγ-deficient platelets (18). In this study, aggregation of Fcγ-deficient platelets was induced by rhodocytin. These findings taken together suggest that rhodocytin induces platelet aggregation independently of GPIb, FcγRII, or GPVI. On the other hand, three anti-GPIa mAbs potently inhibited platelet aggregation induced by rhodocytin, suggesting that it interacts with GPIa/IIa, a collagen receptor on the platelet membrane. Furthermore, complete inhibition of platelet adhesion to rhodocytin by anti-GPIa mAbs indicates that the major binding site of rhodocytin on the platelet membrane is GPIa/IIa.

Immunoprecipitation study using rhodocytin-coupled beads showed that rhodocytin indeed interacts with GPIa/IIa. We could not detect the association of GPIIa with rhodocytin-coupled beads, suggesting that rhodocytin binds to GPIa but not to GPIIa. Failure of anti-GPIIa antibody (100 μg/ml of DE9) to inhibit rhodocytin (10 nM)-induced platelet aggregation also supports this hypothesis (data not shown). To determine whether rhodocytin directly binds to GPIa or indirectly via certain proteins on the platelet membrane, we performed binding studies of rGPIa/IIa liposomes to immobilized rhodocytin. We found that GPIa/IIa liposomes bound to immobilized rhodocytin in a dose-dependent manner and that this binding was totally inhibited by anti-GPIa mAbs. These findings clearly demonstrate that the rhodocytin binding to GPIa/IIa is direct and specific. Furthermore, the fact that the anti-GPIa antibodies inhibit rhodocytin-induced platelet aggregation suggests that the binding of rhodocytin to GPIa/IIa is functionally relevant.

To our surprise, EDTA did not inhibit the binding of rhodocytin to rGPIa/IIa liposomes at all. Most of the ligand-integrin interactions are dependent upon divalent ions and thus can be inhibited by EDTA (5). To the best of our knowledge, echovirus 1 binding to integrin $\alpha_2\beta_1$ (GPIa/IIa) is the only case reported that is independent of divalent cations (45, 47). The inhibitory effects of anti- α_2 (GPIa) or β_1 (GPIIa) antibodies on echovirus binding to integrin $\alpha_2\beta_1$ also had a profile distinct from collagen fiber binding to integrin $\alpha_2\beta_1$ (47). Later, they determined the binding site of echovirus 1 on the integrin α_2 I domain, which is distinct from the metal ion-dependent adhesion site residues essential for the divalent cation-dependent interaction with collagen (53). The binding site of rhodocytin on GPIa/IIa also appears to be different from that of collagen, and this may partly explain the several differences between rhodocytin-induced and collagen-induced signals. The binding site of rhodocytin on GPIa/IIa needs to be addressed in the future.

Relatively high concentrations of immobilized rhodocytin

were required for rGPIa/IIa liposomes binding, compared with those required for eliciting platelet aggregation. This may be attributed to the possible reduction in the binding ability of rhodocytin to GPIa/IIa during the immobilization procedure. Alternatively, it is also possible that rhodocytin induces conformational changes of GPIa/IIa on the platelet membrane, as in the case of ligand binding to other integrins. Conformational changes of GPIa/IIa on the platelet membrane may result in the increased binding capacity of GPIa/IIa to rhodocytin, whereas this apparently cannot happen with rGPIa/IIa liposomes. Further experiments are needed to address this issue.

The Most Proximal Step of the Rhodocytin-mediated Signal Transduction Pathway—We then investigated the activation signals most proximal to GPIa/IIa by the use of immunoprecipitation studies. Immunoprecipitation of GPIa/IIa, followed by Western blotting with anti-Src mAb or anti-Lyn pAb, revealed that Src and Lyn constitutively associated with GPIa/IIa. GPIa/IIa-associated kinase activity increased upon rhodocytin stimulation, which was only slightly modified by PGE_1 treatment. We assume that this kinase activity belongs to that of Src, since its molecular mass, 60 kDa, is similar to that of Src, and no association was detected between GPIa/IIa and other major kinases present in platelets including FAK, Fyn, phosphatidylinositol 3-kinase, Lck, Jak1, and Jak2.

Furthermore, we found that PP1, an inhibitor of Src family kinases, completely inhibited rhodocytin-induced platelet aggregation at the concentration of 5 μM. Higher concentrations of rhodocytin (up to 100 nM) could not overcome the inhibitory effect of PP1. We also examined effects of PP2, which is another inhibitor of Src family kinase, and we obtained results similar to that of PP1 (data not shown). Based on these findings, we assume that GPIa/IIa-associated Src is the most proximal step of the GPIa/IIa-mediated signal transduction pathway, although we cannot totally exclude the possibility that the Lyn kinase activity is also involved in rhodocytin-induced platelet activation via GPIa/IIa. Since Triton X-100 treatment does not completely disrupt the noncovalent bond between GPIa and GPIIa (data not shown), it is still premature to draw a conclusion as to whether Src associates with GPIa or GPIIa and which molecule plays a more important role in the GPIa/IIa-mediated signal transduction pathway.

From these findings described above, we suggest that rhodocytin mediates platelet activation signals through GPIa/IIa (integrin $\alpha_2\beta_1$). By analogy to integrin-mediated signaling pathways in other cells, in which FAK and Cas are involved, we evaluated the changes in FAK and Cas in rhodocytin-induce platelet activation. FAK tyrosine phosphorylation occurred relatively late and was dependent upon platelet aggregation. On the other hand we found that rhodocytin stimulation induced the level of Cas tyrosine phosphorylation 15 s after stimulation. Src but not FAK associated with Cas in a manner dependent upon the tyrosine phosphorylation of Cas. These findings suggest that, unlike other cells in which FAK phosphorylates Cas in an integrin-related activation, Src is responsible for Cas tyrosine phosphorylation in GPIa/IIa-related platelet activation. Since we previously found that ASA and PGE_1 potently inhibited platelet aggregation, intracellular calcium mobilization, and tyrosine phosphorylation of Syk and PLCγ2, we then examined the effects of these inhibitors on Cas tyrosine phosphorylation. Cas tyrosine phosphorylation was not inhibited by ASA (data not shown) or PGE_1. The GPIa/IIa-associated Src activity enhanced upon rhodocytin stimulation was also resistant to ASA (data not shown) or PGE_1 treatment. This is in marked contrast to Syk and PLCγ2 tyrosine phosphorylation, Ca^{2+} mobilization, and platelet aggregation, which are almost completely inhibited by these agents, suggesting that GPIa/IIa-associated Src activity and Cas tyrosine phosphorylation are located at the more proximal steps in the rhodocytin-mediated signaling pathway.

Based upon these findings, we propose the following steps for rhodocytin-induced platelet activation; the binding of rhodocytin to GPIa/IIa results in activation of Src, which constitutively binds to GPIa/IIa. Activated Src associated with GPIa/IIa then induces tyrosine phosphorylation of Cas. Several activation signals including Syk and PLCγ2 are also involved in rhodocytin-induced platelet activation.

The Similarities and Differences between Rhodocytin-induced and Collagen-induced Activation Signals—We suggest that rhodocytin induces platelet aggregation by interacting with GPIa/IIa, one of the most important collagen receptors. We found several similarities and differences between rhodocytin-induced and collagen-induced platelet activation, which may give insights into the platelet activation pathway related with collagen receptors.

1) Rhodocytin induces aggregation of Fcγ-deficient platelets, whereas collagen does not. The "two-site, two-step" theory has been proposed for collagen activation of platelets. Based on this notion, collagen first interacts with one type of collagen receptors, and then the second receptor is involved to induce full activation of platelets. The major role of GPIa/IIa for platelet adhesion and the lack of collagen-induced platelet aggregation in GPIa/IIa-deficient platelets have suggested that GPIa/IIa is the first receptor to react with collagen and plays the initial role in the collagen-platelet interaction. However, this notion was challenged by a recent report by Jung and Moroi (35) who suggest that GPVI activates GPIa/IIa through its inside-out activation pathway. Furthermore, CRP, which specifically interacts with GPVI, is a potent agonist of platelet activation in various aspects, whereas the peptides that specifically bind GPIa/IIa appear to induce no activation signal in platelets (64). These findings have provided evidence for the major role of GPVI in collagen-induced platelet activation. In this aspect, it is of interest how rhodocytin elicits platelet aggregation by interacting with GPIa/IIa but not with GPVI. It is possible that the mode of binding between rhodocytin and GPIa/IIa, which is distinct from that between collagen fibers and GPIa/IIa, elicits intracellular activation signals that cannot be observed with the binding between GPIa/IIa and its specific peptides. Alternatively, it is possible that cross-linking of GPIa/IIa by rhodocytin may suffice to produce intracellular activation signals without the involvement of GPVI. Collagen fails to induce platelet aggregation in GPIa/IIa-deficient platelets, suggesting that the interaction between collagen fiber and GPVI does not lead to full activation of platelets. On the other hand, CRP, which binds only to GPVI potently, induces platelet aggregation. Thus extensive cross-linking of one type of collagen receptors may suffice to activate platelets, and the same mode of activation may be applied to that of rhodocytin.

It may be also argued that rhodocytin binds to yet unidentified molecules other than GPIa/IIa, although the interaction between rhodocytin and GPIa/IIa is essential. In this study, we showed by several lines of evidence that rhodocytin binds to GPIa/IIa but not to GPVI or GPIb. However, we cannot totally exclude the possibility that rhodocytin may have binding sites other than GPIa/IIa on the platelet membrane, and this may contribute to the initiation of activation signals. We need to address this issue in the near future.

2) It is reported that Fyn and Lyn but not Src are involved in the GPVI-mediated signal transduction pathway (15, 17), whereas rhodocytin activates GPIa/IIa-associated Src. Ichinohe *et al.* (60) reported that collagen induces Src activation in GPVI-deficient platelets, and an anti-α_2 blocking antibody in-

hibited this activation. The Src activation is most probably mediated through GPIa/IIa, although the involvement of other collagen receptors cannot be excluded. Our findings that rhodocytin induces the GPIa/IIa-associated Src kinase activity appear to be in good agreement with their study using GPVI-deficient platelets. Taken together, we suggest that Src activation is involved in the GPIa/IIa-mediated signal transduction pathway, whereas Fyn and Lyn are involved in the GPVI-mediated signal transduction pathway.

3) Cytochalasin D totally inhibited all the rhodocytin-elicited signals we evaluated in a previous study and this one. GPIa/IIa-associated Src activation, Cas tyrosine phosphorylation, Syk and PLCγ2 activation, intracellular calcium mobilization, and platelet aggregation were completely blocked (26). It is reported that several collagen-induced activation signals are also susceptible to cytochalasins (29, 62, 63). On the other hand, cytochalasin D does not inhibit platelet aggregation, intracellular calcium mobilization, or tyrosine phosphorylation of Syk and PLCγ2 induced by CRP as we have previously reported (26). Therefore, the susceptibility to cytochalasin D of collagen-induced platelet activation is most likely derived from the GPIa/IIa-mediated signaling pathway. There have been a number of reports (54, 56–59) that suggest that integrin clustering, which is facilitated by cytoskeletal reorganization, activates tyrosine kinases associated with integrins. Since cytochalasin D interferes with cytoskeletal assembly, cell activation mediated by integrins is severely suppressed by cytochalasin D. Our findings that rhodocytin mediates tyrosine phosphorylation of several proteins via GPIa/IIa, which can be inhibited by cytochalasin D, fit well with this notion.

Susceptibility to cytochalasin D suggests that GPIa/IIa clustering is required to activate GPIa/IIa-associated Src. How does rhodocytin, which is a heterodimer of 18- and 15-kDa units (25), induce GPIa/IIa clustering? In a preliminary experiment, we estimated the molecular mass of rhodocytin to be 61 kDa in water by gel filtrating chromatography.[2] Wang *et al.* (55) also isolated and partially characterized a protein whose structural and functional property is identical with rhodocytin, although the complete sequence awaits to be determined. This protein probably has a quaternary structure consisting of at least two disulfide-linked dimers since it has a native molecular mass of about 66 kDa as determined by gel filtration. Thus, rhodocytin appears to form a multimer in water. This multivalency in water may induce platelet activation by clustering GPIa/IIa. Cytochalasin D is likely to disturb GPIa/IIa clustering induced by rhodocytin stimulation, resulting in inhibition of the GPIa/IIa-mediated signals from the most proximal step.

Alternatively, cytochalasin D may directly inhibit the signal transduction related to GPIa/IIa. It is known that GPIa/IIa is linked to the membrane cytoskeleton through association with actin-binding protein and short actin filaments. Therefore, disruption of actin polymerization may inhibit transducing GPIa/IIa-mediated activation signals.

4) Although we found that rhodocytin-induced tyrosine phosphorylation of Syk and PLCγ2 was potently inhibited by ASA or PGE_1, several reports (27–29, 61) suggest that tyrosine phosphorylation of several proteins induced by collagen is resistant to TXA_2 blocking or cAMP-elevating agents. These reports used high dose collagen (50–150 μg/ml) to prove the resistance of collagen-induced protein tyrosine phosphorylation to these inhibitors. It is well established that several parameters of platelet activation induced by low dose collagen are highly dependent on TXA_2 generation. In our work, 1 mM ASA or 1 μM PGE_1 apparently inhibited Syk and PLCγ2 tyrosine phosphorylation induced by 10 μg/ml collagen. The maximum level of PLCγ2 tyrosine phosphorylation induced by 10 μg/ml collagen was decreased to 60% of the control by ASA treatment, and the peak time was delayed from 30 s to 5 min.[3] Thus, the inhibitory profile of these agents on low dose collagen stimulation is similar to that of rhodocytin, which seems to activate platelets by binding to GPIa/IIa. On the other hand, similar to high dose collagen, Syk and PLCγ2 tyrosine phosphorylation induced by GPVI agonists is not dependent on TXA_2 generation (26) or cAMP (27). Taken together, we speculate that low concentrations of collagen mediate activation signals through GPIa/IIa and GPVI, whereas at high concentrations of collagen, signals from GPVI become predominant over those mediated by GPIa/IIa.

5) Rhodocytin, which binds to GPIa/IIa and not to GPVI, induces faint tyrosine phosphorylation of Syk, which typically peaks at 2 min after stimulation, whereas collagen induces marked phosphorylation of Syk in the early stage of activation. Others and we (26, 65) found that CRP that binds to GPVI also induces marked phosphorylation of Syk in the early stage of platelet activation, similar to collagen. Ichinohe *et al.* (60) reported that collagen-induced Syk tyrosine phosphorylation was severely compromised in platelets lacking GPVI. Our finding is in accordance with previous reports and confirms that GPVI is a main receptor that induces Syk tyrosine phosphorylation in collagen-induced platelet activation.

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Katsue Suzuki-Inoue, Yukio Ozaki, Mie Kainoh, Yongchol Shin, Yi Wu, Yutaka Yatomi, Tukasa Ohmori, Toshiaki Tanaka, Kaneo Satoh and Takashi Morita

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