Aggretin, a Heterodimeric C-type Lectin from Calloselasma rhodostoma (Malayan Pit Viper), Stimulates Platelets by Binding to $\alpha_2\beta_1$ Integrin and Glycoprotein Ib, Activating Syk and Phospholipase C$\gamma_2$, but Does Not Involve the Glycoprotein VI/Fc Receptor $\gamma$ Chain Collagen Receptor

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Aggretin, a potent platelet activator, was isolated from Calloselasma rhodostoma venom, and 30-amino acid N-terminal sequences of both subunits were determined. Aggretin belongs to the heterodimeric snake C-type lectin family and is thought to activate platelets by binding to platelet glycoprotein $\alpha_2\beta_1$. We now show that binding to glycoprotein (GP) Ib is also required. Aggretin-induced platelet activation was inhibited by a monoclonal antibody to GPIb as well as by antibodies to $\alpha_2\beta_1$. Binding of both of these platelet receptors to aggretin was confirmed by affinity chromatography. No binding of other major platelet membrane glycoproteins, in particular GPVI, to aggretin was detected. Aggretin also activates platelets from Fc receptor $\gamma$ chain (Fc$\gamma$)-deficient mice to a greater extent than those from normal control mice, showing that it does not use the GPVI/Fc$\gamma$ pathway. Platelets from Fc$\gamma$-deficient mice expressed fibrinogen receptors normally in response to collagen, although they did not aggregate, indicating that these platelets may partly compensate via other receptors including $\alpha_2\beta_1$ or GPIb for the lack of the Fc$\gamma$ pathway. Signaling by aggretin involves a dose-dependent lag phase followed by rapid tyrosine phosphorylation of a number of proteins. Among these are p72$^{SYK}$, p125$^{FAP}$, and PLC$\gamma2$, whereas, in comparison with collagen and convulxin, the Fc$\gamma$ subunit neither is phosphorylated nor coprecipitates with p72$^{SYK}$. This supports an independent, GPIb- and integrin-based pathway for activation of p72$^{SYK}$ not involving the Fc$\gamma$ receptor.

Platelet-collagen interactions are integral to primary hemo-
stasis (1, 2). Resting platelets using several receptors adhering to subendothelium of damaged blood vessels are activated and spread to provide finally a new nonthrombogenic surface until vasculature regeneration occurs. Reversible binding between GPIb-V-IX$^{1}$ and von Willebrand factor, associated with collagen, is crucial to slow down the platelet (especially under high shear) so that it can bind more firmly to other receptors (3, 4). This mechanism strongly parallels that of the selectins in leukocyte adhesion (5). Another important receptor is the $\alpha_2\beta_1$ integrin, which is essential for anchoring the platelet to collagen in the subendothelium (6) and for linking to the platelet cytoskeleton to prevent the receptor being torn from the membrane by the forces that it has to withstand. Activation induces the release of storage granules, and the expression of new receptors on the platelet surface (7) as well as changes in other receptors such as the fibrinogen receptor, $\alpha_{I\beta_3}$, which is critical for spreading. Although GPIb-V-IX and $\alpha_{I\beta_3}$ also participate in signaling to the platelet interior (8, 9), recent studies, particularly in patients with platelet receptor deficiencies, have implicated GPVI/Fc$\gamma$ as a major collagen receptor for platelet activation (10–12). Patients with platelets lacking many of these one of these receptors (GPIb-V-IX, $\alpha_{I\beta_3}$, or GPVI/Fc$\gamma$) have increased bleeding times, and platelet adhesion to subendothelium or collagen is defective under flow conditions (13, 14).

Because adhesion is rapid, the function of the individual receptor classes is difficult to assess. Inhibition of any of them can prevent platelet activation but does not indicate which synergies provide the final activating signal. In analyzing the mechanisms of each of these steps, reagents that bind to and activate platelets via individual receptor types are important tools. Convulxin, a C-type lectin from the venom of Crotalus durissus terrificus, the tropical rattlesnake, activates platelets by binding to and clustering the GPVI/Fc$\gamma$ receptor (15). Several other snake venom proteins are potent platelet activators and may act via collagen-like mechanisms. Some of these, including trimucytin from Trimeresurus microsquamatus (16) and aggretin from Calloselasma rhodostoma (17), have been reported to involve $\alpha_2\beta_1$ on platelets as receptor. We isolated a protein from C. rhodostoma venom that is a powerful platelet activator. This is most likely aggretin as described by Huang et al. (17), based on the molecular mass, sequence of subunits (18) and properties in activating platelets. Like aggretin it also belongs to the snake C-type lectin family. A C-type lectin from

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1. The abbreviations used are: GP, glycoprotein; PLC$\gamma_2$, phospholipase C$\gamma_2$; MoAb, monoclonal antibody; Fc, Fc receptor $\gamma$ chain; FITC, fluorescein isothiocyanate; PVDF, polyvinylidene difluoride; PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography.
the same species, termed rhodocytin, was described later (19) with N-terminal sequences identical to those of aggretin, but it was reported to have some different properties. The mechanism of rhodocytin action on platelets was recently investigated (20, 21), showing that rhodocytin interacts with the αg-subunit of αgβ2, on the platelet surface. On the other hand, Eble et al. (22) have reported that rhodocytin does not bind to a recombinant αgβ2 complex. We now show that aggretin activates platelets via interaction with GPIb as well as αgβ2; however, GPVI/Fcγ is not required. This mechanism via GPIb and αgβ2 may also be relevant to activation of platelets via collagen.

**EXPERIMENTAL PROCEDURES**

**Materials—**Lyophilized *C. rhodostoma* venom was from ICN Biomedicals GmbH (Eschwege, Germany) and CV Herpafauna (Indonesia). Protein A-Sepharose, peroxidase-conjugated goat anti-mouse and anti-rabbit antibodies, bovine serum albumin, and biotinamidocaproate N,N-diethylmaleimide, and 2 m sodium orthovanadate. After centrifugation, platelet lysates precleared with protein A-Sepharose were stirred for 2 h with specific antibodies before adding 20 μl of protein A-Sepharose followed by 6 h of incubation.

**Preparation of Triton X-100 Platelet Lysates, Wheat Germ Agglutinin Affinity Chromatography, and Biotinylated Aggretin-Avidin-Sepharose Affinity Chromatography—**Human platelets were isolated from buffy coats as described above but in the presence of 10 μM Iloprost. Washed platelets were diluted with phosphate-buffered saline to 5 × 10^10/ml and solubilized in phosphate-buffered saline containing 1% Triton X-100, 1 mM phenylmethanesulfonyl fluoride, 2 mM EDTA, 2 mM N,N-diethylmaleimide, and 2 mM sodium orthovanadate with or without 5 mM EDTA. After centrifugation (40,000 × g, 1 h, 4 °C) the supernatant was applied to a column of wheat germ agglutinin-Sepharose 4B equilibrated with 130 mM NaCl, 10 mM Tris/HCl, pH 7.4 (buffer D). The column was washed thoroughly with buffer D containing 0.2% octanoyl-N,N,N-trimethylglucamide. Biotinylated aggretin A was added to the pooled fractions containing eluted membrane glycoproteins, and after 2 h of incubation avidin-Sepharose was added to the mixture. After further incubation avidin-Sepharose was washed thoroughly with buffer D containing 0.2% octanoyl-N,N,N-trimethylglucamide. The avidin-Sepharose with bound biotinylated aggretin A and platelet proteins was boiled for 1 min with buffer E containing 1% SDS. Eluted proteins were separated by electrophoresis and transferred to the PVDF membrane.

**Fcγ Chain-deficient Mice—**Fcγ chain-deficient C57BL/6 (B6) mice have been previously described (25) and were kindly provided by Dr. Ole Korsgren (26). Normal B6 mice were used as controls. Whole blood (250 μl) from Fcγ-deficient mice was mixed with phenobarbital by puncturing the inferior vena cava with heparinized syringes at a final concentration of 25 units of heparin/ml of blood.

**Flow Cytometric Analysis of Fibrinogen–FITC Binding to Aggretin, Convolxin, and Collagen-activated Mouse Platelets—**Mouse platelet-rich plasma was diluted to 2.5 × 10^10 platelets/ml with Tyrode’s solution, buffered to pH 7.4, to minimize the formation of platelet aggregates and was precleared for 3 min at room temperature with 150 μg/ml fibrinogen–FITC (saturating concentration). Platelet suspension (100 μl) was added to 20 μl of the platelet agonist solution (aggretin, convolxin, or collagen). Platelet activation was stopped after 120 s by fixing with formaldehyde in phosphate-buffered saline for 30 min. Platelets were washed and resuspended in 100 μl of phosphate-buffered saline, and 10^5 single platelets were analyzed by flow cytometry (FACSscan, Becton Dickinson, Heidelberg, Germany). Non-specific background labeling was determined using control platelets treated with 10 μM GRGDPS (N-vinylobead, Bad Soden, Germany) to prevent specific fibrinogen binding.

**RESULTS**

**Aggretin Is a Heterodimeric C-type Lectin—**Aggretin was purified from lyophilized *C. rhodostoma* venom by gel filtration and ion-exchange chromatography. The final product gave 60–, 28-, and 13–14-kDa bands under nonreduced conditions and bands at 14 and 12 kDa under reduced conditions, by SDS-PAGE/silver staining analysis (Fig. 1). Gel filtration under nondenaturing conditions gave a peak at 60 kDa, suggesting that a tetrameric form (dimer of heterodimer) is the principle native form. Reverse phase HPLC gave two forms, one of which was more hydrophobic and eluted later from the HPLC column. Reduced and S-pyridylethylated aggretin was separated by HPLC into αα and αβ subunits. Both the more hydrophobic forms of aggretin gave similar subunits, but these forms also showed differences in hydrophobicity. The α-subunits and the β-subunits, respectively, of the two forms had identical N-terminal sequences. The assignment to the α-subunit and the β-subunit was based upon the nomenclature used for the other heterodimeric snake C-type lectins where the
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No platelet receptors bound specifically to biotinylated aggretin/avidin-Sepharose or avidin-Sepharose as a control following affinity chromatography of platelet lysate prepared in the presence of EDTA. However, affinity chromatography of platelet lysate prepared in the absence of EDTA gave specific binding of α2β1 and GPIb to biotinylated aggretin/avidin-Sepharose. Avidin-Sepharose 4B alone did not bind any membrane proteins from the platelet lysate (Fig. 4).

**Aggretin Activates Mouse Platelets from the Fcγ-deficient Line and Therefore Does Not Act via GPVI/Fcγ**—GPVI requires Fcγ for platelet activation by collagen, and platelets from mice rendered deficient for Fcγ were not to respond to collagen or collagen-like peptides (25). Aggretin was therefore tested on normal and Fcγ-deficient mouse platelets. A flow cytometric method was used to measure activation of GPⅠb-Ⅲa (fibrinogen-binding sites) as a parameter of platelet stimulation using FITC-labeled fibrinogen. As expected the Fcγ-deficient mouse platelets did not respond to convulxin, the GPVI-specific C-type lectin, as agonist, whereas the control platelets were activated (Fig. 5A). The Fcγ-deficient mouse platelets gave a stronger response to aggretin than the control platelets (Fig. 5B) and also aggregated, whereas although both types of platelets gave a similar FITC-fibrinogen binding response with collagen (Fig. 5C), the Fcγ-deficient platelets did not give detectable aggregates. The data shown are the means of results from three different experiments.

**Tyrosine Phosphorylation in Platelets Induced by Aggretin Compared with Collagen and Convulxin**—Fig. 6 shows a tyrosine phosphorylation time range for platelets activated by 100 ng/ml aggretin compared with 1.5 μg/ml collagen and 30 ng/ml convulxin. Comparison of the proteins phosphorylated on tyrosine showed several differences between platelets activated by aggretin and the others. A band at 90 kDa is persistently phosphorylated in aggretin, rapidly transiently phosphorylated in convulxin-activated, but not phosphorylated in collagen platelets. The bands at 36–38 kDa, which are closely associated with signaling via GPVI/Fcγ are very strongly phosphorylated in platelets activated by convulxin and much weaker in those activated by collagen as already described (15). In aggretin-activated platelets either the tyrosine-phosphorylated bands in the 36–38-kDa range were very weakly phosphorylated or a different, slightly lower band was phosphorylated in response to this agonist. Unlike platelet responses to convulxin, neither the aggregation nor the tyrosine phosphorylation response to aggretin is virtually instantaneous. There is a clear dose-dependent lag phase during which the phosphorylation of p72SYK increases slowly (Fig. 7).

**Signal Transduction by Aggretin involves p72SYK and PLCγ2 but Not Fcγ**—Platelets activated by either aggretin, convulxin, or collagen as above were solubilized in Triton X-100 and centrifuged, and the supernatant was used for immunoprecipitation with antibodies to p72SYK and PLCγ2 (Fig. 8, A and B). Both p72SYK and PLCγ2 were activated and tyrosine-phosphorylated by all three agonists. However, tyrosine-phosphorylated Fcγ was directly co-immunoprecipitated with p72SYK platelets activated by collagen and convulxin but not in those activated by aggretin.

**Aggretin Causes Strong Activation of p125FAK**—Even When GPIb-Ⅲa Is Blocked—Platelets activated by aggretin, convulxin, or collagen in the presence or the absence of GPIb-Ⅲa inhibitor (Ro44–9883, 1 μM) were solubilized in Triton X-100 and centrifuged, and the supernatant was used for immunoprecipitation with antibodies to p125FAK (Fig. 8C). Convulxin with GPIb-Ⅲa inhibitor gave little phosphorylation of p125FAK but collagen and aggretin induced phosphorylation of p125FAK in the presence of the inhibitor, with aggretin having the strongest effect.

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**Aggretin Involves α2β1 as Receptor**—The monoclonal antibody to α2β1, 6P1, blocked platelet aggregation to aggretin in a dose-dependent way with the addition of 2.5, 12.5, or 50 μg/ml, giving 58, 36, or 11%, respectively, of the initial aggregation (Fig. 3A). The monoclonal antibody BHA2.1 against α2β1 also inhibited aggretin-induced platelet aggregation. The protein-tyrosine phosphorylation occurring in platelets after activation with aggretin was completely blocked by antibodies against α2β1 (Fig. 3B).

**Aggretin Binds Both α2β1 and GPIb from Platelet Lysates**—

α-subunit is defined as the larger. N-terminal sequencing gave the 30-amino acid sequences GLEDCDPGWSPPYQH-CYQAFNQKTWDAEA for α-subunit and DCPGWSSYEGHCYKPFNNEPKWNWADERFC for the β-subunit. The sequences of both subunits shows full identity to the sequence of the cloned aggretin established by Chung et al. (18). Aggretin is a heterodimeric C-type lectin with strong sequence similarity to other venom C-type lectins interacting with platelet receptors (Fig. 2).

**Aggretin Acts via GPIb**—Aggretin is a powerful platelet agonist and induced maximal platelet aggregation at concentrations in the 40–60 ng/ml range. Apart from convulxin, most agonist and induced maximal platelet aggregation at concentrations in the 40–60 ng/ml range. Apart from convulxin, most agonist and induced maximal platelet aggregation at concentrations in the 40–60 ng/ml range. Apart from convulxin, most agonist and induced maximal platelet aggregation at concentrations in the 40–60 ng/ml range. Apart from convulxin, most agonist and induced maximal platelet aggregation at concentrations in the 40–60 ng/ml range.
DISCUSSION

Several earlier studies reported proteins from the venom of C. rhodostoma that activate platelets, and it was suggested that one of these, called aggregoserpentin or aggretin, acts via $\alpha_2\beta_1$ (17). Aggretin was recently cloned, and the full amino acid sequence was established (18). We isolated a protein from C. rhodostoma venom that has N-terminal sequences of $\alpha$- and $\beta$-subunits identical to aggretin. This protein has a molecular mass of 28 kDa with subunits of 12 and 14 kDa on SDS-PAGE. Under nondenaturing conditions by gel filtration this protein has a molecular mass around 60 kDa. This implies that two subunits of the protein are linked together by disulfide bridges and that two heterodimers interact noncovalently to form tetramers. Aggretin aggregated platelets in a dose-dependent manner with a long lag phase as previously described (17).

In all previous reports (17, 18) the authors showed that aggretin acts via the $\alpha_2\beta_1$ receptor. We confirmed that aggretin binds to $\alpha_2\beta_1$ but showed that it binds to GPIb as well. GPIb involvement in activation of platelets via aggretin was shown by affinity chromatography and inhibition of aggretin-induced platelet aggregation using an anti-GPIb monoclonal antibody. The monoclonal antibody VM16d completely inhibited aggretin-induced platelet aggregation as well as changes in protein-tyrosine phosphorylation (27). Both GPIb and $\alpha_2\beta_1$ were detected in eluted material specifically bound to biotinylated aggretin-avidin-Sepharose. These receptors bound to aggretin only when the platelet lysate was prepared in the absence of EDTA or EGTA. When the platelet lysate was prepared with EDTA, proteins were not specifically bound to aggretin, showing that divalent cations are important for aggretin interactions with platelet receptors. It is not yet clear whether divalent cations are necessary for the active structure of aggretin or whether they are necessary for receptors to maintain a structure that is recognized by aggretin.

**FIG. 2.** A comparison of the N-terminal sequences of the subunits of aggretin with members of the snake venom C-type lectin family. The sequences are highly similar, and aggretin clearly belongs to the same protein family. Assignment to $\alpha$- and $\beta$-subunits was on the basis of the subunit size. The positions with amino acids conserved in at least four of the six subunit sequences, respectively, are shown with bold letters.

**FIG. 3.** Antibodies against $\alpha_2\beta_1$ and GPIb inhibit aggretin-induced platelet aggregation and protein-tyrosine phosphorylation. A, platelet aggregation was induced by 40 ng/ml of aggretin. Anti-$\alpha_2\beta_1$ antibody (6F1, left panel) or anti-GPIb antibody (VM16d, right panel) at various concentrations were added to platelets 1 min before aggretin. The amounts of antibodies added are indicated. B, washed human platelets (700 μl, $5 \times 10^8$ platelets/ml) were stirred at 1000 rpm at 37°C. Aggretin at 40 ng/ml was added, and aliquots were withdrawn at the times indicated and dissolved in SDS buffer containing inhibitors. After separation by SDS-PAGE (7–17% acrylamide gradient) and transfer to PVDF membranes, the proteins were incubated with anti-phosphotyrosine antibody 4G10 before detection by peroxidase-linked second antibody and chemiluminescence. Anti-$\alpha_2\beta_1$ antibody (6F1, 50 μg/ml) or anti-GPIb antibody (VM16d, 20 μg/ml) were added to the platelets 1 min before aggretin. The left panel shows changes in protein-tyrosine phosphorylation in platelets activated by aggretin without any antibodies; the middle panel shows changes in platelets pretreated with 6F1; and the right panel shows changes in platelets pretreated with VM16d.
Shin and Morita (19) isolated a C-type lectin that activates platelets from venom of *C. rhodostoma* that they called rhodocytin. They did not establish an activation mechanism but showed that it was not via GPIb. The N-terminal sequences that they determined are identical with the first 22 and 19 amino acids, respectively, of aggretin. Recently, the same group reported that the \( \alpha_2 \) subunit of \( \alpha_2 \beta_1 \) is involved in the platelet interaction with rhodocytin and investigated some aspects of signal transduction occurring in platelets after activation with rhodocytin (20, 21). The situation has been complicated by a recent report that rhodocytin does not bind to a recombinant \( \alpha_2 \beta_1 \) complex, whereas another *C. rhodostoma* venom C-type lectin, rhodocetin, composed of noncovalently associated \( \alpha \)- and \( \beta \)-subunits with different sequences than those of aggretin or rhodocytin, bound strongly to the recombinant complex and blocked its binding to collagen (22).

In earlier studies, lack of involvement of GPIb as a receptor for aggretin or rhodocytin was based on the failure of monoclonal antibodies or echicetin to affect platelet activation. In fact, Shin and Morita (19) do show an increase in the lag phase response to rhodocytin in the presence of echicetin, and the slope of aggregation was also slightly inhibited. We also found that echicetin had a minor inhibitory effect on platelet responses to low concentrations of aggretin (data not shown). The monoclonal antibodies that had no effect on aggretin were AP-1 and 6D1 (17). Thus, the binding site for aggretin on GPIb must partly overlap with the echicetin-binding site but not with the epitopes of AP-1, 6D1, SZ-2, or Ib-23. On the other hand, VM16d shares the same binding site as aggretin. VM16d is a well characterized monoclonal antibody to GPIb with its epitope in the double-loop region (27) that inhibits thrombin-binding but not von Willebrand factor binding (28) and must therefore bind to one face of the GPIb\(\alpha\) molecule. These results suggest that the previous studies did not detect aggretin (rhodocytin) binding to GPIb because the anti-GPIb reagents used did not bind to the same region (or face) of GPIb.

![Platelet surface glycoproteins binding to biotinylated aggretin-avidin Sepharose 4B](image_url)

FIG. 4. Platelet surface glycoproteins binding to biotinylated aggretin-avidin Sepharose 4B. Platelets were lysed with 1.2% Triton X-100 in buffer containing inhibitors (without EDTA), and glycoproteins were isolated by wheat germ agglutinin-affinity chromatography. Biotinylated aggretin was added to aliquots and incubated together for 2 h. Then avidin-Sepharose 4B was added, and the mixture was incubated for a further 4 h. As a negative control, avidin-Sepharose was incubated with an aliquot of platelet glycoproteins without adding biotinylated aggretin. After intensive washing both avidin-Sepharose samples were boiled in 1% SDS. Eluted proteins were separated by SDS-PAGE, transferred to a PVDF membrane, and treated with anti-\( \alpha_2 \) (lanes 1–3) or anti-GPIb (lanes 4–6) monoclonal antibodies before detection by peroxidase-linked secondary antibody and chemiluminescence. Lanes 1 and 4, platelet glycoproteins; lanes 2 and 5, eluate from avidin-Sepharose without biotinylated aggretin; lanes 3 and 6, eluate from biotinylated aggretin-avidin-Sepharose.

![Flow cytometry analysis of fibrinogen-binding sites exposed on Fcy-deficient and control platelets activated by aggretin, convulxin, and collagen](image_url)

FIG. 5. Flow cytometry analysis of fibrinogen-binding sites exposed on Fcy-deficient and control platelets activated by aggretin, convulxin, and collagen. Platelets from control (C) or Fcy-deficient mice (○) were activated with convulxin (A), aggretin (B), and collagen (C) in the presence of fibrinogen-FITC. After 120 s the platelets were fixed, washed, and analyzed by flow cytometry. The data shown are the means of the results of three different experiments.
FIG. 6. Time dependence of tyrosine phosphorylation in proteins from platelets activated by aggretin, collagen, or convulxin. Washed platelets (700 μl, 5 × 10^8 platelets/ml) were stirred at 1,000 rpm at 37 °C. After separation by SDS-PAGE (7–17% acrylamide gradient) and transfer to PVDF membranes, the proteins were incubated with anti-phosphotyrosine antibody 4G10 before detection by peroxidase-linked secondary antibody and chemiluminescence.

Considerable evidence has accumulated that among the potential candidates for collagen receptors, at least α2β1 and GPVI/Fcγ are critical. It was suggested earlier that α2β1 is essentially an adhesion receptor, whereas GPVI/Fcγ is the main activation receptor for collagen, although this is controversial (11, 29–31). Collagen-related peptides based on a repetitive motif (line C) (an arrow marks the point of addition). Lower panel, aliquots of aggretin-activated platelets were lysed with SDS at the times shown, and proteins were separated by SDS-PAGE, transferred to a PVDF membrane, and detected with anti-phosphotyrosine antibody (4G10), peroxidase-linked secondary antibody, and chemiluminescence.

Surprisingly, although the normal mouse platelets reacted strongly, the Fcγ-deficient platelets responded even more powerfully (Fig. 5B), suggesting either that more GPIb-α2β1 is expressed or that signaling through these receptors is up-regulated by the loss of the Fcγ pathway. Both types of platelet also aggregated in response to aggretin. The GPIb-IIIa responses of both the Fcγ-deficient platelets and the normal platelets to collagen were almost identical (Fig. 5C) (note that total GPIIb-IIIa activation after 2 min was measured here; therefore differences in rates were not studied). However, neither Fcγ-deficient mouse nor GPVI-deficient human platelets (31) aggregate to collagen. Therefore, a signal from GPVI/Fcγ is essential for the aggregation response to collagen. The clear results obtained with convulxin as well as Western blot studies with anti-Fcγ antibodies (data not shown) confirmed the Fcγ-deficient status of these platelets. Keely and Parise (35) reported that FcγRIIA played an essential role in activation of platelets via cross-linking with antibodies to α2β1. Because mouse platelets do not express FcγRIIA (36), it is unlikely that this receptor is involved in platelet activation by aggretin.

Earlier studies on Fcγ-deficient mouse platelets reported that they show only low responses to collagen (37) including a remnant low activation of p72SYK and PLCγ2. Although the exposure of the fibrinogen-binding sites is not identical to aggregation, a close correlation might be expected between these two parameters. However, Poole et al. (37) did not study aggregation to collagen with the Fcγ-deficient mouse platelets. In the case of human platelets from a patient with GPVI deficiency, also lacking Fcγ (38), the platelets also still responded to collagen but much more slowly than normal human platelets (31). It was suggested that the remnant response of GPVI-deficient human platelets to collagen could be due to its interactions with α2β1 alone.

Suzuki-Inoue et al. (21) showed that rhodocytin induced aggregation of Fcγ-deficient mouse platelets and that α2 bound to a rhodocytin affinity column. Rhodocytin binding to liposomes containing recombinant α2β1 was not inhibited by EDTA (21). Eble et al. (22), however, found no rhodocytin binding to recombinant α2β1 whether in an enzyme-linked immunosorbent assay or in competition with α2β1 binding to collagen. The question of whether aggretin and rhodocytin are identical or variants with the same N-terminal sequences is not yet clear.

In platelets stimulated by aggretin, p125FAK was strongly activated. In contrast to platelets treated with convulxin,
Aggretin Activation of Platelets via $\alpha_2\beta_1$ and GPIb, Not GPVI

where the activation of p125$^{FAK}$ is a consequence of the secondary involvement of GPIb-IIIa (15) in aggretin-treated platelets, the activation of p125$^{FAK}$ was only slightly blocked by M Ro-44–9883, a GPIIb-IIIa inhibitor. Aliquots were immunoprecipitated with anti-p72$^{SYK}$ antibodies. After SDS-PAGE separation and transfer to PVDF membrane, the immunoprecipitates were incubated with 4G10 anti-phosphotyrosine antibody. The membrane was stripped and reprobed for p72$^{SYK}$. The absence of a direct, early, agonist with more than one binding site for platelet receptors, such as collagen is still open. Because aggretin is again an agonist with more than one binding site for platelet receptors, this suggests that binding and clustering of $\alpha_2\beta_1$ may not be enough to activate platelets, and therefore GPIb or GPVI (in the case of collagen) is necessary to give adequate stimulation of signaling pathways. Platelet activation with aggretin-induced activation of p72$^{SYK}$ and of PLC$\gamma 2$ but not FC$\gamma$ co-immunoprecipitated with p72$^{SYK}$. The absence of a direct, early, signaling role for FC$\gamma$ in the platelet response to aggretin supports the results obtained using platelets from FC$\gamma$-deficient mice where the GPIIb-IIIa activation response to aggretin was stronger than that obtained with platelets from normal, control mice. Aggretin, by activating and clustering $\alpha_2\beta_1$ and GPIb, gives a powerful signaling response in platelets. This strongly supports a major signaling role for $\alpha_2\beta_1$ integrin in collagen-induced platelet activation as well. Results measuring “soluble collagen” binding to resting and activated platelets (41) suggest that, although there is a residual binding to resting platelets, this is considerably enhanced in activated platelets. Because the structure of soluble collagen is poorly defined, a role for GPVI/FC$\gamma$ in resting platelets in binding this collagen can still not be excluded. In the platelet response to collagen, $\alpha_2\beta_1$ acts synergistically with GPVI/FC$\gamma$ and modulates and controls the response to this receptor. This is clearly seen in the strength of the platelet response to convulxin compared with collagen. In this, $\alpha_2\beta_1$ shows a number of parallels to GPIIb-IIIa. Normally, GPIIb-IIIa needs to be activated by signaling from other receptors to change conformation and allow fibrinogen binding. However, platelets can adhere to and be activated by a fibrinogen-coated surface (42), so there must be a recognition potential even in the “nonactivated” conformation. Clustering of GPIIb-IIIa leads to activation of p72$^{SYK}$ without obvious FC$\gamma$ involvement; however, the mechanism for this is not yet known (43). Aggretin clustering of $\alpha_2\beta_1$ and GPIb appears to induce a similar signaling pathway that may be common to several integrins. The availability of aggretin as a specific reagent, activating platelets via $\alpha_2\beta_1$ and GPIb independently of GPVI/FC$\gamma$, should allow further analysis of the signaling pathways from these receptors. Together with reagents specific for GPVI/FC$\gamma$, such as convulxin (15) or collagen-related peptides (32), it should provide the tools to analyze how these major receptors work together in collagen-induced platelet activation.

The main platelet activation pathway by both collagen and convulxin was shown to involve phosphorylation of FC$\gamma$, leading to activation of p72$^{SYK}$ and hence of PLC$\gamma 2$. A role for $\alpha_2\beta_1$ in activation of p72$^{SYK}$ and of PLC$\gamma 2$ by pathways requiring FC$\gamma$RIIA was also suggested by studies using specific antibodies and inhibitors (35), and Polanowska-Grabowska et al. (40) showed that $\alpha_2\beta_1$ signals via dephosphorylation of a heat shock protein complex. However, these investigations are limited by the use of collagen as agonist where interactions with more than one receptor cannot be excluded or by the use of activating antibodies where a role of FC$\gamma$RIIA was critical. It has been suggested that aggretin or rhodocytin may provide a good tool for investigating signaling via $\alpha_2\beta_1$. However, because aggretin binds to both $\alpha_2\beta_1$ and GPIb the question about the role of other receptors in preactivation of $\alpha_2\beta_1$ before it can bind agonists such as collagen is still open. Because aggretin is again an agonist with more than one binding site for platelet receptors, this suggests that binding and clustering of $\alpha_2\beta_1$ may not be enough to activate platelets, and therefore GPIb or GPVI (in the case of collagen) is necessary to give adequate stimulation of signaling pathways. Platelet activation with aggretin-induced activation of p72$^{SYK}$ and of PLC$\gamma 2$ but not FC$\gamma$ co-immunoprecipitated with p72$^{SYK}$. The absence of a direct, early, signaling role for FC$\gamma$ in the platelet response to aggretin supports the results obtained using platelets from FC$\gamma$-deficient mice where the GPIIb-IIIa activation response to aggretin was stronger than that obtained with platelets from normal, control mice. Aggretin, by activating and clustering $\alpha_2\beta_1$ and GPIb, gives a powerful signaling response in platelets. This strongly supports a major signaling role for $\alpha_2\beta_1$ integrin in collagen-induced platelet activation as well. Results measuring “soluble collagen” binding to resting and activated platelets (41) suggest that, although there is a residual binding to resting platelets, this is considerably enhanced in activated platelets. Because the structure of soluble collagen is poorly defined, a role for GPVI/FC$\gamma$ in resting platelets in binding this collagen can still not be excluded. In the platelet response to collagen, $\alpha_2\beta_1$ acts synergistically with GPVI/FC$\gamma$ and modulates and controls the response to this receptor. This is clearly seen in the strength of the platelet response to convulxin compared with collagen. In this, $\alpha_2\beta_1$ shows a number of parallels to GPIIb-IIIa. Normally, GPIIb-IIIa needs to be activated by signaling from other receptors to change conformation and allow fibrinogen binding. However, platelets can adhere to and be activated by a fibrinogen-coated surface (42), so there must be a recognition potential even in the “nonactivated” conformation. Clustering of GPIIb-IIIa leads to activation of p72$^{SYK}$ without obvious FC$\gamma$ involvement; however, the mechanism for this is not yet known (43). Aggretin clustering of $\alpha_2\beta_1$ and GPIb appears to induce a similar signaling pathway that may be common to several integrins. The availability of aggretin as a specific reagent, activating platelets via $\alpha_2\beta_1$ and GPIb independently of GPVI/FC$\gamma$, should allow further analysis of the signaling pathways from these receptors. Together with reagents specific for GPVI/FC$\gamma$, such as convulxin (15) or collagen-related peptides (32), it should provide the tools to analyze how these major receptors work together in collagen-induced platelet activation.
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