A Novel Association of Fc Receptor γ-Chain with Glycoprotein VI and Their Co-expression as a Collagen Receptor in Human Platelets*

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The mechanism by which occupancy of collagen receptors is coupled to platelet activation has been uncertain. Our group previously demonstrated that glycoprotein (GP) VI, an uncharacterized platelet membrane protein, is specifically required for collagen-platelet interaction leading to activation of protein-tyrosine kinase Syk. Since collagen stimulation of platelets has recently been found to induce tyrosine phosphorylation of Fc receptor (FcR) γ-chain, a signal-generating subunit of FcR, we further investigated the relationships between FcR γ-chain and GPVI in human platelets. Our present study revealed the following. FcR γ-chain was physically and stably associated with GPVI in human platelets; both FcR γ-chain and GPVI were proportionally absent in GPVI-deficient platelets; GPVI cross-linking or collagen stimulation of platelets resulted in tyrosine phosphorylation of GPVI-associated FcR γ-chain accompanied by Syk association and activation. These findings strongly suggest that the associated complex of GPVI and FcR γ-chain is a collagen receptor featuring the signaling through immune receptors.

Despite the widely accepted consensus that the interactions between the extracellular matrix protein collagen and platelets are vital for the maintenance of hemostasis, the exact nature of collagen receptor on platelets has been a great enigma to date except for well characterized integrin heterodimer α b (1, 2), also called glycoprotein (GP) Ia-IIa, as a principal adhesion receptor for collagen. Among several candidates that have been proposed to be platelet collagen receptors (3–7), we have recently provided biochemical evidence that GPVI, as yet an uncharacterized member of FcR, we further investigated the relationships between FcR γ-chain and GPVI in human platelets. Our present study revealed the following. FcR γ-chain was physically and stably associated with GPVI in human platelets; both FcR γ-chain and GPVI were proportionally absent in GPVI-deficient platelets; GPVI cross-linking or collagen stimulation of platelets resulted in tyrosine phosphorylation of GPVI-associated FcR γ-chain accompanied by Syk association and activation. These findings strongly suggest that the associated complex of GPVI and FcR γ-chain is a collagen receptor featuring the signaling through immune receptors.

F(ab′) 2 fragments of anti-GPVI IgG (F(ab′) 2 ωGPVI) induces cAMP-insensitive activation of protein-tyrosine kinase Syk accompanied by tyrosine phosphorylation of phospholipase Cγ2 (PLCγ2) in a manner similar to collagen stimulation (8); GPVI-deficient platelets (6, 7, 9) expressing a normal amount of GPⅡa-Ⅱb exhibit lack of collagen-stimulated Syk activation and tyrosine phosphorylation of PLCγ2 (10). However, the question of how GPVI is involved in collagen receptor and transduces signals leading to Syk activation accompanied by tyrosine phosphorylation of PLCγ2 still remains unsolved.

One of the mechanisms by which Syk is activated is achieved via interaction between its tandem Src homology 2 (SH2) domains and a tyrosine-phosphorylated activation motif, termed the immunoreceptor tyrosine-based activation motif, found in receptors of the immune system or their associated chains (11). In platelets, this mechanism of Syk activation is a prerequisite for the activation through a low affinity Fc receptor for IgG (FcRγ) (12, 13). Among known Fc receptors belonging to the immunoglobulin superfamily (14), human platelets express only a single FcR encoded by the FcγRIIA gene (15). Recently Gibbins et al. (16) demonstrated the presence of the immunoreceptor tyrosine-based activation motif-containing Fc receptor (FcR) γ-chain, a 20-kDa disulfide-linked, homodimeric, signal-generating subunit in human platelets, and showed its tyrosine phosphorylation, Syk association, and subsequent phosphorylation of PLCγ2 upon collagen stimulation but not upon FcγRIIA cross-linking. FcR γ-chain has been shown to be associated with all three classes of FcγR, the high affinity IgE FcR (FcεR), and the IgA Fc receptor (FoR), and the T-cell receptor-CD3 complex (14, 17–21). Since platelets express FcγRIIA but lack these other known FcR γ-chain-associated membrane proteins, FcR γ-chain may be associated with a novel counterpart in platelets.

Therefore, we hypothesized that FcR γ-chain might associate with GPVI composing a collagen receptor and play a pivotal role in collagen-induced Syk activation through GPVI in human platelets. In testing this hypothesis, we studied the following points: whether a complex of FcR γ-chain and GPVI can be demonstrated in human platelets; how FcR γ-chain is present in GPVI-deficient platelets; whether GPVI cross-linking of platelets with F(ab′) 2 ωGPVI induces tyrosine phosphorylation of FcR γ-chain and its association with Syk; and how GPVI is involved in the binding of the tandem SH2 domains of Syk to FcR γ-chain in collagen-stimulated platelets. Here we report that FcR γ-chain is physically and stably associated with platelet membrane GPVI but not expressed in GPVI-deficient platelets and also that GPVI cross-linking or collagen stimulation of platelets induces tyrosine phosphorylation of GPVI-associated FcR γ-chain accompanied by Syk association and activation.

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1 The abbreviations used are: GP, glycoprotein; F(ab′) 2 ωGPVI, F(ab′) 2 fragments of anti-GPVI IgG; PLCγ2, phospholipase Cγ2; SH2, Src homology 2; FcγR, Fc receptor for IgG; FcR, Fc receptor; FcεRI, high affinity IgE Fc receptor; FcαRI, IgA Fc receptor; GST, glutathione S-transferase; GST-Syk-SH2, GST fusion protein containing the tandem SH2 domains of Syk; ECL, enhanced chemiluminescence; PAGE, polyacrylamide gel electrophoresis.
A Collagen Receptor Complex of Fc Receptor γ-Chain and Glycoprotein VI

EXPERIMENTAL PROCEDURES

Materials—Glutathione S-transferase (GST) fusion protein containing the tandem SH2 domains of Syk (GST-Syk-SH2) was a gift from Dr. T. Kurosaki (Kansai Medical School, Osaka, Japan), Dr. S. Yanagi (Kobe University School of Medicine, Kobe, Japan), and Dr. H. Yamamura (Kobe University School of Medicine, Kobe, Japan). Anti-serum against FcR γ-chain (22) was kindly provided by Dr. M.-H. Jouvin and Dr. J.-P. Kinet (Beth Israel Hospital, Boston, MA). Anti-GPVII (CD36) monoclonal antibody (clone 73) (23) was obtained from Transduction Laboratories (Lexington, Kentucky). Anti-α2β1 integrin (G19) (24) and anti-FcγRII (IV.3) (25) antibodies were from Immunotech S.A. (Marseille, France) and from Medarex Inc. (Annandale, NJ), respectively. Other reagents were from previously described sources (8, 10).

Preparation of anti-GPVII IgG and F(ab’2)GPVII was performed as described previously (8), using the serum of a patient with GPVII deficiency (6) who was followed up as an outpatient in our department. For immunoblotting, anti-GPVII IgG was labeled with biotin using a standard method as described previously (26).

Preparation and Stimulation of Platelets—Blood samplings were performed by venipuncture from healthy donors or from patients with GPVII-deficient platelets with informed consent. Anticoagulation of blood and preparation of washed platelets were performed as described previously (8). Washed platelets (1.0 × 10^9 cells/ml) were stimulated with 150 μg/ml F(ab’2)GPVII or 100 μg/ml collagen for the indicated times at 37 °C in an aggregometer with continuous stirring at 800 rpm.

Platelet Lysis and Immunoprecipitation—Unstimulated or stimulated platelets were lysed in an ice-cold lysis buffer (16), and immunoprecipitation of each specified protein was performed as described previously (8, 10).

Fusion Protein Precipitation Studies—Precipitation of platelet protein with GST-Syk-SH2 was performed as described previously (16).

Immunoblotting Studies—Immunoblotting analysis of whole platelets lysates, immunoprecipitated proteins, or precipitated proteins with GST-Syk-SH2 followed by enhanced chemiluminescence (ECL) detection was performed as described previously (8, 10). When immunoblotting was performed with biotinylated anti-GPVII IgG, the blots were then incubated with avidin-horseradish peroxidase and developed with ECL. In some experiments, immunoprecipitated protein bands were measured by densitometry (Densitorol DMU-33C, Toyo Kagaku Sangyo Ltd., Osaka, Japan). Absorption of proteins in normal control samples was set to 100%.

Preparation of Erythrocytes, Granulocytes, and Lymphocytes—Washed erythrocytes were prepared and erythrocyte membranes were obtained by hemolysing cells as described previously (27). Erythrocyte hemoglobin-free membrane was lysed in the lysis buffer.

Washed granulocytes and lymphocytes were prepared with a modification of a previously described method (28). Briefly, after removing of platelet-rich plasma, erythrocytes were eliminated from the remaining blood by dextran sedimentation. The upper leukocyte layer was centrifuged over Ficoll-Paque (Pharmacia Biotech Inc.) at 400 g for 30 min. Each granulocyte- or lymphocyte-containing fraction was taken and washed once with phosphate-buffered saline. Contaminating erythrocytes in the granulocyte pellets were lysed in hypotonic saline. The granulocytes or lymphocytes were further washed and lysed in the lysis buffer.

Protein concentration in cell lysates was measured with BCA protein assay reagent (Pierce). After addition of SDS sample buffer and boiling, the samples containing an equal amount of protein (20 μg/lane) were subjected to SDS-PAGE and subsequent immunoblotting as described above.

RESULTS AND DISCUSSION

Fcγ γ-Chain Is Physically Associated with Platelet Membrane GPVII—We first examined whether there is physical association between Fcγ γ-chain and GPVII. Cell lysates of unstimulated washed platelets were prepared and tested for the interaction between Fcγ γ-chain and GPVII by immunoprecipitation, followed by immunoblotting with anti-Fcγ γ-chain serum and anti-GPVII IgG. When Fcγ γ-chain and associated proteins were immunoprecipitated from platelet cell lysates with anti-Fcγ γ-chain serum, GPVII was co-precipitated with Fcγ γ-chain (Fig. 1A). Conversely, when GPVII and associated proteins were immunoprecipitated from platelet cell lysates with anti-GPVII IgG, Fcγ γ-chain was co-precipitated with GPVII (Fig. 1B). Immunoblotting with anti-Fcγ γ-chain serum revealed the presence of an apparent doublet of Fcγ γ-chain, which has been observed previously (16, 29). We further studied the possible association of Fcγ γ-chain with integrin α2β1, FcγRII, or GPIV (CD36), which is another candidate for collagen receptor (3). Unstimulated washed human platelets were lysed and subjected to immunoprecipitation with antibodies specific to integrin α2β1, FcγRII, or GPIV. Immunoblotting with anti-Fcγ γ-chain serum revealed the absence of Fcγ γ-chain in those immunoprecipitates (Fig. 1B). These findings demonstrate that Fcγ γ-chain is physically associated with GPVII but not with integrin α2β1, FcγRII, or GPIV in human platelets.

Fcγ γ-Chain Is Absent Specifically in GPVII-deficient Platelets—We then questioned how Fcγ γ-chain is present in GPVII-deficient platelets. Whole cell lysates of platelets obtained from a patient with complete GPVII deficiency (6) were tested for the presence of Fcγ γ-chain by immunoblotting with anti-Fcγ γ-chain serum. Surprisingly, Fcγ γ-chain was not immunodetected with anti-Fcγ γ-chain serum in the GPVII-deficient platelets, indicating the complete deficiency of Fcγ γ-chain in these platelets (Fig. 2A). We next examined the presence of Fcγ γ-chain in the other peripheral blood cells such as granulocytes, lymphocytes, and erythrocytes obtained from the same patient (Fig. 2B). The results showed that Fcγ γ-chain was normally present in those blood cells compared with normal control.
FcR requires the expression of FcRg. Although it has been reported that the expression of some of the levels of FcRg, suggests another possibility that the expression of FcRg also requires its counterpart.

If so, the amount of FcRg-chain should be proportional to that of GPVI in human platelets. The same results were also confirmed by the other patient (data not shown).

Therefore, these data indicate that the expression of FcRg-chain is altered specifically in GPVI-deficient platelets. A, washed platelets were obtained from one patient (Pt1) with complete deficiency of platelet GPVI, another patient (Pt2) with incomplete deficiency of platelet GPVI, and a normal subject (N) and lysed in the lysis buffer. Whole cell lysates were resolved on SDS-PAGE and immunoblotted (WB) with anti-FcRg-chain serum (αFcRg-chain) or biotinylated anti-GPVI IgG (αGPVI IgG) as described in Fig. 1. B, washed granulocytes (Gr), lymphocytes (Lym), and erythrocytes (Ery) were obtained from normal control (N) and the patient (Pt1). Immunoblotting (WB) with anti-FcRg-chain serum or biotinylated anti-GPVI IgG was performed as described in Fig. 1. The data of platelets (Pt) obtained from normal control are shown for comparison. The same results were also confirmed by the other patient (data not shown).

Therefore, we concluded that both GPVI and FcRg-chain were co-expressed in human platelets and that the lack of GPVI might cause the expression deficiency of FcRg-chain. Although it has been reported that the expression of some of FcR requires the expression of FcRg-chain (30, 31), this may suggest another possibility that the expression of FcRg-chain also requires its counterpart.

\( F(ab')_2\alpha GPVI \) Induces Tyrosine Phosphorylation of GPVI-associated FcRg-chain Followed by Syk Association—We reported previously that the cross-linking of platelet GPVI with \( F(ab')_2\alpha GPVI \)-induced activation of Syk in a similar manner to collagen stimulation (8). Therefore we questioned how GPVI-associated FcRg-chain is involved in GPVI cross-linking or collagen-induced activation of Syk. Washed human platelets were stimulated with \( F(ab')_2\alpha GPVI \) (150 μg/ml) for various times up to 10 min and lysed in the lysis buffer. Whole cell lysates were immunoprecipitated with anti-FcRg-chain serum (αFcRg-chain). Anti-FcRg-chain immunoprecipitates (IP) were resolved on SDS-PAGE and immunoblotted (WB) with αFcRg-chain, anti-Syk antibody (αSyk), or biotinylated anti-GPVI IgG (αGPVI IgG), followed by reprobing of stripped immunoblots with anti-phosphotyrosine antibody (αPY). Densitometric analysis revealed that band intensities of GPVI-associated FcRg-chain were unchanged before and after GPVI cross-linking with \( F(ab')_2\alpha GPVI \).
anti-GPVI IgG, or anti-phosphotyrosine antibody. As shown in Fig. 3, FeR γ-chain was tyrosine-phosphorylated in a time-dependent manner. Immunoblot analysis with anti-FeR γ-chain serum confirmed similar and specific efficiencies of immunoprecipitation of FeR γ-chain. Protein-tyrosine kinase Syk became associated with FeR γ-chain in a time-dependent manner upon GPVI cross-linking, and the associated Syk was tyrosine-phosphorylated. During these events, FeR γ-chain was stably associated with GPVI. Similar findings were also obtained with collagen-stimulated platelets: collagen induced time-dependent tyrosine phosphorylation of FeR γ-chain, which was stably associated with GPVI and recruited Syk (data not shown). Since parallel increases in the activity of Syk with its tyrosine-phosphorylated state are well known and also confirmed in our previous reports (8, 10), these data strongly suggest that GPVI engagement including collagen stimulation causes tyrosine phosphorylation of FeR γ-chain, which is involved in recruitment and activation of Syk.

Binding of the Tandem SH2 Domains of Syk to Tyrosine-phosphorylated FeR γ-Chain Associated with GPVI in Collagen-stimulated Platelets—It has been demonstrated that Syk binds tyrosine-phosphorylated FeR γ-chain through its tandem SH2 domains (11). To show the relationships between GPVI, FeR γ-chain, and the tandem SH2 domains of Syk in collagen-stimulated platelets, we used GST-Syk-SH2. Following collagen stimulation, platelets were lysed and the lysates were incubated with GST-Syk-SH2. Precipitated proteins with GST-Syk-SH2 were resolved on SDS-PAGE and subjected to immunoblotting with anti-FeR γ-chain serum, anti-GPVI IgG, or anti-phosphotyrosine antibody. As shown in Fig. 4, the co-precipitation of FeR γ-chain with the fusion protein reached a maximum after 60 s of collagen stimulation and declined thereafter. Both GPVI and FeR γ-chain showed similar time-dependent appearances in the fusion protein precipitates, where FeR γ-chain was tyrosine-phosphorylated but GPVI was not (data not shown). Therefore, taken together with our previous findings that GPVI is specifically required for collagen-platelet interaction leading to Syk activation (8, 10), these data indicate that collagen signals through GPVI, and induces tyrosine phosphorylation of its associated FeR γ-chain, followed by the recruitment of Syk through the tandem SH2 domains.

FeR γ-chain has been widely accepted to be involved in immune responses in association with Fe receptors and T-cell receptor. Our present work demonstrates that FeR γ-chain is associated and co-expressed with a so far uncharacterized membrane GPVI in human platelets and that this novel association plays essential roles in tyrosine phosphorylation of FeR γ-chain followed by the association of Syk, which is triggered by signaling through GPVI upon collagen stimulation. This could emphasize that FeR γ-chain is involved not only in immune responses but also in a nonimmune response with a novel counterpart GPVI as shown here.

Although we do not deny that GPVI may associate with more than one protein, our findings are relevant to the concept that the associated complex between GPVI and FeR γ-chain is a collagen receptor featuring the signaling through immune receptors.

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