

Physical and Functional Association of the Src Family Kinases Fyn and Lyn with the Collagen Receptor Glycoprotein VI-Fc Receptor γ Chain Complex on Human Platelets

By Yasuharu Ezumi, Keisuke Shindoh, Masaaki Tsuji,
and Hiroshi Takayama

*From the Department of Hematology and Oncology, Clinical Sciences for Pathological Organs,
Graduate School of Medicine, Kyoto University, Kyoto 606-8507, Japan*

Summary

We have previously shown that uncharacterized glycoprotein VI (GPVI), which is constitutively associated and coexpressed with Fc receptor γ chain (FcR γ) in human platelets, is essential for collagen-stimulated tyrosine phosphorylation of FcR γ , Syk, and phospholipase C γ 2 (PLC γ 2), leading to platelet activation. Here we investigated involvement of the Src family in the proximal signals through the GPVI-FcR γ complex, using the snake venom convulxin from *Crotalus durissus terrificus*, which specifically recognizes GPVI and activates platelets through cross-linking GPVI. Convulxin-coupled beads precipitated the GPVI-FcR γ complex from platelet lysates. Collagen and convulxin induced tyrosine phosphorylation of FcR γ , Syk, and PLC γ 2 and recruited tyrosine-phosphorylated Syk to the GPVI-FcR γ complex. Using coprecipitation methods with convulxin-coupled beads and antibodies against FcR γ and the Src family, we showed that Fyn and Lyn, but not Yes, Src, Fgr, Hck, and Lck, were physically associated with the GPVI-FcR γ complex irrespective of stimulation. Furthermore, Fyn was rapidly activated by collagen or cross-linking GPVI. The Src family-specific inhibitor PP1 dose-dependently inhibited collagen- or convulxin-induced tyrosine phosphorylation of proteins including FcR γ , Syk, and PLC γ 2, accompanied by a loss of aggregation and ATP release reaction. These results indicate that the Src family plays a critical role in platelet activation via the collagen receptor GPVI-FcR γ complex.

Key words: glycoprotein VI • Fc receptor γ chain • Src family • collagen • platelets

Platelets adhere to collagen fibers of extracellular matrix and become activated through specific membrane receptors, resulting in shape change, granule release, and aggregation. These processes are vital for the formation of a hemostatic plug under a physiologic or pathologic condition. Many platelet surface glycoproteins (GPs)¹ have been proposed as receptors for collagen, including the integrin $\alpha_2\beta_1$ (GPIIb; reference 1), GPIV (GPIIIb, CD36; reference 2), an uncharacterized protein GPVI (p62; references

3, 4), the type I collagen receptor (p65; reference 5), and 85–90-kD GPs (6). It has been well established that $\alpha_2\beta_1$ plays a major role in platelet adhesion to collagen (1), although it remains unclear whether $\alpha_2\beta_1$ directly transduces signals leading to platelet activation. Our laboratory and others have reported patients with mild bleeding disorders whose platelets lack GPVI but not $\alpha_2\beta_1$ and show selective deficiency in collagen-induced platelet aggregation and release reaction (3, 4, 7). Several lines of evidence have demonstrated that GPVI is implicated in collagen-induced tyrosine phosphorylation of proteins (8–11), including Fc receptor γ chain (FcR γ), Syk tyrosine kinase, and phospholipase C γ 2 (PLC γ 2), which are essential for platelet activation upon collagen stimulation (12–14). GPVI is constitutively associated with FcR γ (10, 11), while both of them are proportionally absent in GPVI-deficient platelets (10), suggesting their coexpression. Cross-linking GPVI by the F(ab')₂ of anti-GPVI IgG (anti-GPVI F(ab')₂) stimulates tyrosine phosphorylation of FcR γ , Syk, and PLC γ 2, and re-

¹Abbreviations used in this paper: anti-GPVI F(ab')₂, the F(ab')₂ of anti-GPVI IgG; FcR γ , Fc receptor γ chain; Fc γ RII, low affinity Fc receptor for IgG; Fc ϵ RI, high affinity Fc receptor for IgE; GP, glycoprotein; GPVI, glycoprotein VI; ITAM, immunoreceptor tyrosine-based activation motif; PLC γ 2, phospholipase C γ 2; PP1, 4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine; RGDS, Arg-Gly-Asp-Ser; RIPA, radioimmunoprecipitation assay.

cruits Syk to FcR γ , leading to Syk activation (8, 10, 11), whereas GPVI-deficient platelets specifically lack activation of Syk in response to collagen (9). These findings have revealed the GPVI-FcR γ complex as a collagen receptor responsible for platelet activation.

To further investigate the mechanisms involved in the proximal signals through the GPVI-FcR γ complex, we used the snake venom convulxin from *Crotalus durissus terrificus*, which has been known to be a powerful platelet activator (15, 16), and the Src family-specific inhibitor PP1 (4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine; reference 17). Recent reports have shown that convulxin is a heterodimeric C-type lectin and binds to platelet surface with a high affinity on a small number of binding sites (18, 19), that ¹²⁵I-convulxin specifically recognizes a glycosylated 62-kD membrane protein immunoprecipitated by the anti-GPVI IgG (18, 20), and that cross-linking GPVI with convulxin induces tyrosine phosphorylation of proteins including FcR γ , Syk, and PLC γ 2 (18). In this report, we demonstrated that the Src family tyrosine kinases Fyn and Lyn were physically associated with the GPVI-FcR γ complex and that Fyn was activated by collagen stimulation or cross-linking GPVI. PP1 dose-dependently inhibited collagen- or convulxin-induced tyrosine phosphorylation of proteins including FcR γ , Syk, and PLC γ 2, accompanied by a loss of aggregation and ATP release reaction. Analogy to the antigen and Fc receptor signals, the Src family kinases were involved in platelet activation through the collagen receptor GPVI coupled with FcR γ as a signal transducing subunit containing an immunoreceptor tyrosine-based activation motif (ITAM; references 21, 22).

Materials and Methods

Reagents. Convulxin was purified with gel filtration chromatography as described by Polgár et al. (18) from lyophilized *Crotalus durissus terrificus* venom obtained from Sigma Chemical Co. (St. Louis, MO). CNBr-activated Sepharose 4B beads, Sepharose 4B beads, and protein A-Sepharose beads were purchased from Pharmacia Biotechnology Inc. (Uppsala, Sweden). As previously described, human anti-GPVI IgG and its F(ab')₂ fragments were prepared from serum of a patient with GPVI deficiency (3, 8), who has been followed up as an outpatient in our department. Anti-FcR γ IgG was produced by immunizing rabbits with a synthetic peptide (residues 80–86) from FcR γ as described previously (23). mAbs against Syk (clone Syk101), Lyn (clone Lyn9), and Yes (clone 3H9) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Anti-phosphotyrosine (clone 4G10) and anti-Src (clone 327) mAbs were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY) and Oncogene Science, Inc. (Uniondale, NY), respectively. Anti-low affinity Fc receptor for IgG (Fc γ RII) mAb (clone IV.3) was from Medarex Inc. (Annandale, NJ). Affinity-purified rabbit IgG specific to Fyn (FYN3), Hck (N-30), and PLC γ 2 (C19) and affinity-purified goat IgG to Lck (2102) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Affinity-purified rabbit anti-Fgr Ab was obtained from Transduction Laboratories (Lexington, KY). Mouse mAb (clone 19-1; reference 24) against the α chain of human high affinity Fc receptor for IgE (Fc ϵ R1) was a kind gift from Dr. M.-H. Jouvin and Dr. J.-P. Kinet (Beth Israel Hospital, Boston,

MA). Acid soluble fibrillar collagen prepared from equine tendon was purchased from Horm-Chemie (Munich, Germany). PP1 (17) and PGE₁ were kindly provided by Pfizer Central Research (Groton, CT) and Ono Pharmaceutical Co. (Osaka, Japan), respectively. Arg-Gly-Asp-Ser (RGDS) and enolase were obtained from Sigma Chemical Co. γ -[³²P]ATP (3,000 Ci/mmol, 10 mCi/ml) was supplied by DuPont NEN (Boston, MA). All other reagents were obtained as previously reported (25).

Preparation and Stimulation of Platelets. After informed consent was obtained, venous blood was collected from healthy adult donors. All experiments involving human subjects were conducted according to the principles expressed in the Declaration of Helsinki. Anticoagulation of blood and preparation of platelet-rich plasma were performed as described previously (26). Platelet-rich plasma was incubated with 1 mM aspirin for 30 min at 37°C. Gel-filtered platelets were then prepared as described previously (27) at a final concentration of 1 \times 10⁹ cells/ml in Hepes buffer (137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 5.6 mM glucose, 1 mg/ml BSA, 3.3 mM NaH₂PO₄, and 20 mM Hepes, pH 7.4), containing 1 U/ml apyrase and 500 μ M RGDS to avoid secondary effects via released ADP and aggregation. Platelets were stimulated by 50 μ g/ml of collagen, 50 ng/ml of convulxin, or 150 μ g/ml of anti-GPVI F(ab')₂ with gentle agitation for appropriate periods at 37°C. In some experiments, gel-filtered platelets were incubated for 3 min at 37°C with indicated concentrations of PP1 or DMSO (0.25%) as a control before stimulation. Resting or stimulated platelets were lysed with an equal volume of ice-cold 2 \times Triton X-100 lysis buffer (150 mM NaCl, 10 mM EGTA, 2% Triton X-100, 2 mM PMSF, 2 mM Na₃VO₄, 40 μ g/ml leupeptin, 40 μ g/ml aprotinin, and 20 mM Hepes, pH 7.4) or 2 \times radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 10 mM EGTA, 2% Triton X-100, 2% sodium deoxycholate, 0.2% SDS, 2 mM PMSF, 2 mM Na₃VO₄, 40 μ g/ml leupeptin, 40 μ g/ml aprotinin, and 20 mM Hepes, pH 7.4). For preparation of whole platelet lysates, gel-filtered platelets were suspended in Hepes buffer without BSA and directly lysed in an equal volume of 2 \times SDS sample buffer (\times 1; 2% SDS, 5% glycerol, 5% β -ME, and 62.5 mM Tris-HCl, pH 6.8), and boiled for 5 min.

Affinity Precipitation of Convulxin-binding Proteins. 100 μ g of purified convulxin was covalently coupled to 1 ml of CNBr-activated Sepharose 4B beads according to the manufacturer's instructions. 1.2 ml of platelet lysates (from 6 \times 10⁸ cells/sample) was clarified by centrifugation at 16,000 *g* for 30 min, incubated for 1 h with 40 μ l of Sepharose 4B (50% slurry), and then pre-cleared by sedimentation at 16,000 *g* for 30 min. These steps and all subsequent steps were carried out at 4°C. The supernatants were incubated for 1 h with 40 μ l of convulxin-coupled Sepharose 4B (50% slurry) or Sepharose 4B as a control. The beads were sedimented by brief centrifugation and washed four times in lysis buffer containing 0.5 M NaCl. Proteins were eluted from the beads in 90 μ l of SDS sample buffer and boiled for 5 min.

Immunoprecipitation. Immunoprecipitation of each protein and its associated proteins was performed as described previously (25), followed by two-step elution with different buffers. 1.2 ml of lysates prepared from 6 \times 10⁸ platelets in Triton X-100 lysis buffer was clarified by centrifugation at 16,000 *g* for 30 min, pre-cleared with protein A-Sepharose beads, and incubated for 2 h with 5 μ g of anti-FcR γ IgG, preimmune rabbit IgG, anti-Fc γ RII mAb, anti-Fyn Ab, anti-Lyn mAb, anti-Yes mAb, anti-Src mAb, and anti-PLC γ 2 Ab. This step and all subsequent steps were performed at 4°C. For immunoprecipitation of Yes and Src, lysates were further incubated for 1 h with 25 μ g of rabbit anti-mouse

IgG. Immune complexes were then precipitated with protein A-Sepharose beads for 1 h, sedimented by brief centrifugation, and washed four times in Triton X-100 lysis buffer. Coprecipitated proteins were first eluted in 60 μ l of RIPA buffer. Eluates were diluted with 30 μ l of 3 \times SDS sample buffer and boiled for 5 min. The left beads were further washed four times in RIPA buffer. Immunoprecipitates were then eluted in 60 μ l of SDS sample buffer and boiled for 5 min. These samples eluted by SDS sample buffer were checked with immunoblotting whether each Ab precipitated its recognizing protein.

Immunoblotting. Immunoblot analysis for each protein was done as described previously (25). In brief, samples (the whole platelet lysates from 5×10^6 cells/lane or affinity precipitates and immunoprecipitates from 1×10^8 cells/lane) were subjected to 10% SDS-PAGE and transferred electrophoretically to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA) with a semidry blotter. For detection of FcR γ , samples were resolved on 12.5% SDS-PAGE with Tris-Tricine buffer system. The membranes were treated with Ab against phosphotyrosine (1 μ g/ml), GPVI (2.5 μ g/ml), FcR γ (1 μ g/ml), α chain of Fc ϵ RI (0.5 μ g/ml), Syk (2 μ g/ml), Fyn (1 μ g/ml), Lyn (5 μ g/ml), Yes (1 μ g/ml), Src (0.2 μ g/ml), Fgr (1 μ g/ml), Hck (2 μ g/ml), Lck (1 μ g/ml), or PLC γ 2 (1 μ g/ml), followed by the ECL chemiluminescence reaction (Amersham International plc., Little Chalfont, UK). For detection of GPVI, anti-GPVI IgG was biotinylated to enhance sensitivity (10). The densities of some protein bands on scanned images were analyzed using the public domain NIH Image program (written by Wayne Rasband at the National Institutes of Health).

Immune Complex Kinase Assay. To examine in vitro kinase activity of Fyn and Lyn, the kinases were isolated by immunoprecipitation with 1 μ g of specific Abs as described above from platelet lysates (from 3×10^8 cells/sample) prepared in RIPA buffer. Immunoprecipitates were washed four times with RIPA buffer and twice with kinase buffer (20 mM Hepes, pH 7.5, and 10 mM MnCl $_2$), and then incubated for 5 min at room temperature with 10 μ g of acid-treated enolase in 30 μ l of kinase buffer including 5 μ Ci of γ -[32 P]ATP and 1 μ M of ATP. Reactions were stopped by addition of 30 μ l of 2 \times SDS sample buffer. The samples were boiled for 3 min and subjected to 10% SDS-PAGE. Radioactivity of each protein was visualized and quantified with the Bio-Imaging Analyzer BAS-2000II (Fuji Photo Film Co., Tokyo, Japan).

Platelet Aggregation and ATP Release Reaction. Gel-filtered platelets were prepared from platelet-rich plasma as described above but without aspirin pretreatment, suspended at a final concentration of 3×10^8 cells/ml in Hepes buffer without RGDS, and incubated with PP1 or DMSO for 3 min at 37°C before stimulation by collagen, convulxin, or thrombin. Standard aggregometry of platelets was performed according to the Born method (28) in an aggregometer (HEMA TRACER 1; Nikko Bioscience, Tokyo, Japan). Release reaction from dense granules of platelets was monitored as ATP release using luciferin-luciferase reaction in a lumi-aggregometer (Chrono-Log, Harverton, PA) as described previously (8).

Results

Precipitation of the GPVI-FcR γ Complex with Convulxin-coupled Beads from Platelet Lysates. Convulxin specifically and strongly binds GPVI expressed on the platelet surface (19). 125 I-convulxin also specifically recognizes GPVI blot-

ted on polyvinylidene difluoride or nitrocellulose membranes (18, 20). Here we used Sepharose 4B beads that were covalently coupled with convulxin to isolate GPVI and associated proteins from platelet lysates. Immunoblot analysis showed that convulxin-coupled Sepharose 4B but not Sepharose 4B alone effectively precipitated both GPVI and FcR γ , which are constitutively associated with each other (10, 11), when we used Triton X-100 lysis buffer (Fig. 1, A and B). If we used more stringent RIPA buffer to solubilize platelets, only GPVI but not FcR γ was precipitated with convulxin-coupled Sepharose 4B from platelet lysates (data not shown). These findings indicate that GPVI and FcR γ were dissociated in RIPA buffer and that convulxin directly and strongly binds to GPVI. Thus, we used Triton X-100 lysis buffer to analyze molecules associated with the GPVI-FcR γ complex in the rest of studies.

Collagen- or Convulxin-induced Tyrosine Phosphorylation of Proteins Associated with the GPVI-FcR γ Complex. Collagen, convulxin, and anti-GPVI F(ab') $_2$ stimulate tyrosine phosphorylation of signaling molecules including FcR γ , Syk, and PLC γ 2, which are essential for platelet activation by these agonists (8–14, 18). Recent studies have shown that convulxin activates platelets via cross-linking GPVI (18, 20). We examined tyrosine phosphorylation of proteins coprecipitated with the GPVI-FcR γ complex purified by convulxin-coupled Sepharose 4B from intact platelets and platelets stimulated by collagen or convulxin. Upon collagen and convulxin stimulation, we observed an increase in tyrosine phosphorylation of proteins with molecular masses of 140, 95, 85, 72, 53/60, and 10/12 kD (Fig. 2 A). We confirmed that the 72-kD band corresponded to accu-

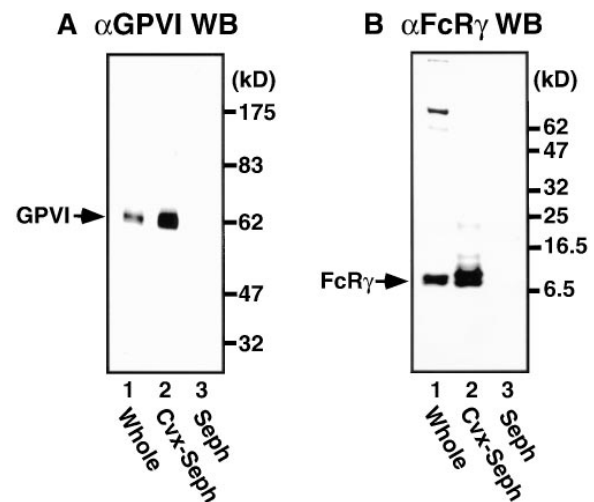


Figure 1. Affinity precipitation of the GPVI-FcR γ complex with convulxin-coupled Sepharose 4B from platelet lysates. Unstimulated gel-filtered platelets were lysed directly in SDS sample buffer or in Triton X-100 lysis buffer. Whole lysates (lane 1) and proteins precipitated by affinity with convulxin-coupled Sepharose 4B (lane 2) or Sepharose 4B alone (lane 3) were resolved by 10% (A) or 12.5% SDS-PAGE (B), transferred to nitrocellulose membranes, and immunoblotted with anti-GPVI IgG (A) or anti-FcR γ IgG (B). Molecular mass markers are indicated in kD on the right of panels.

mulation of tyrosine-phosphorylated Syk (Fig. 2 *B*) and that the 10/12-kD doublets were FcR γ , which showed mobility retardation on SDS-PAGE due to phosphorylation (Fig. 2 *D*). GPVI, whose molecular mass is 62 kD under the reduced condition (3, 4, 8), was equally precipitated (Fig. 2 *C*) but not tyrosine phosphorylated irrespective of stimulation as previously reported (8, 10). Whereas collagen and convulxin stimulated tyrosine phosphorylation of PLC γ 2 as previously reported (8, 14, 18, 29, 30), we could not detect PLC γ 2 in the precipitated proteins with convulxin-coupled Sepharose 4B regardless of stimulation (data not shown).

Physical Association of Fyn and Lyn with the GPVI-FcR γ Complex. We next focused on the heavily tyrosine-phosphorylated proteins with molecular masses of 53–60 kD. The Src family kinases are cytosolic protein tyrosine kinases with molecular masses \sim 60 kD and usually tyrosine phosphorylated themselves. Previous reports have shown that platelets abundantly express the Src family kinases including Src, Fyn, Yes, Lyn, Hck, Fgr, and Lck (31–36). We then examined whether the Src family kinases existed in the precipitated proteins with convulxin-coupled Sepharose 4B by immunoblotting with Ab specific to each kinase. Fig. 3 shows that convulxin-coupled Sepharose 4B precipitated Fyn (59 kD) and Lyn (53–56 kD), but not Yes (62 kD), Src (60 kD), Fgr (59 kD), and Lck (56 kD, data not shown), from lysates of resting platelets and collagen- or convulxin-

stimulated platelets. Upon stimulation, protein levels of precipitated Fyn and Lyn did not change (Fig. 3, *A* and *B*). Sepharose 4B alone as a control precipitated none of these kinases (Fig. 3). We could not detect Hck with immunoblotting in whole platelet lysates or in the precipitated proteins with convulxin-coupled Sepharose 4B (data not shown). To confirm whether Fyn and Lyn precipitated with convulxin-coupled Sepharose 4B were associated with the GPVI-FcR γ complex, we next analyzed proteins coimmunoprecipitated with anti-FcR γ IgG and Abs against the Src family kinases. There was a problem that H chain of IgG used for immunoprecipitation overlapped the Src family kinases or GPVI on immunoblots due to their similar molecular masses. Hence, RIPA buffer was used to elute only coprecipitated proteins from immunoprecipitates as described in Materials and Methods, since RIPA buffer could not elute IgG and its recognizing proteins from immune complexes bound to protein A-Sepharose beads (data not shown). Fyn and Lyn but not Src were coimmunoprecipitated with anti-FcR γ IgG from platelet lysates (Fig. 4, *A–C*). Yes, Fgr, Hck, and Lck were not detected in coimmunoprecipitates with anti-FcR γ IgG (data not shown). Conversely, anti-Fyn or anti-Lyn Ab, but not anti-Yes or anti-Src mAb coimmunoprecipitated both GPVI and FcR γ (Fig. 4, *D* and *E*). These data clearly demonstrate that Fyn and Lyn were constitutively and specifically associated with the GPVI-FcR γ complex. Because very recent studies indicated that platelets heterogeneously express Fc ϵ RI (37, 38), which is associated with FcR γ and Lyn in other cells (39, 40), we examined whether Fc ϵ RI existed in the precipitated proteins with convulxin-coupled Sepharose 4B. We did not detect the α chain of Fc ϵ RI among them with immunoblotting (data not shown). Although collagen stimulates tyrosine phosphorylation of Fc γ RIIA in platelets (14), convulxin did not induce it (data

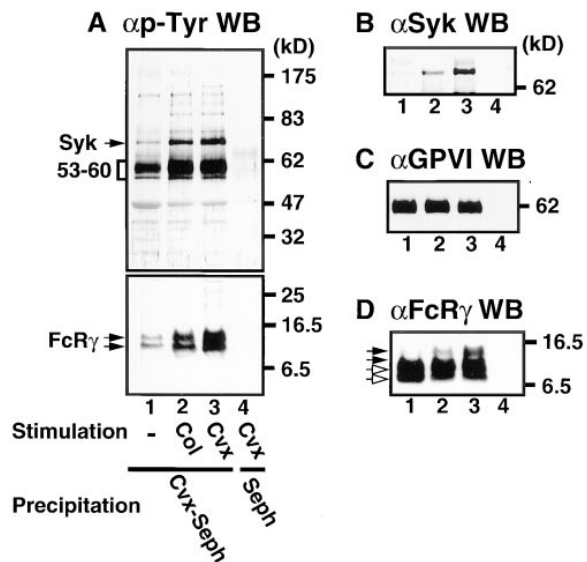


Figure 2. Tyrosine phosphorylation of proteins coprecipitated with the GPVI-FcR γ complex in collagen- and convulxin-stimulated platelets. Gel-filtered platelets were unstimulated (lane 1) or stimulated for 1 min at 37°C with 50 μ g/ml of collagen (lane 2) or 50 ng/ml of convulxin (lanes 3 and 4) and lysed in Triton X-100 lysis buffer. Precipitated proteins with convulxin-coupled Sepharose 4B (lane 1–3) or Sepharose 4B (lane 4) were resolved by 10% (*A*, *top*, *B*, and *C*) or 12.5% SDS-PAGE (*A*, *bottom* and *D*), transferred to nitrocellulose membranes, and immunoblotted with Abs against phosphotyrosine (*A*), Syk (*B*), GPVI (*C*), and FcR γ (*D*). Molecular mass markers are indicated in kD on the right of panels. In *A* (*bottom*) and *D* open and closed arrows mark unphosphorylated and phosphorylated FcR γ , respectively.

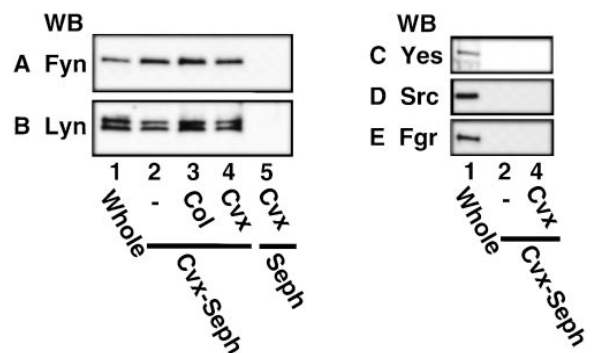


Figure 3. Coprecipitation of Fyn and Lyn, but not Yes, Src, and Fgr, with the GPVI-FcR γ complex from platelet lysates. Gel-filtered platelets were unstimulated (lanes 1 and 2) or stimulated for 1 min at 37°C with 50 μ g/ml of collagen (lane 3) or 50 ng/ml of convulxin (lanes 4 and 5) and lysed in Triton X-100 lysis buffer (lanes 2–5) or directly in SDS sample buffer (lane 1). Whole lysates (lane 1) and precipitated proteins with convulxin-coupled Sepharose 4B (lanes 2–4) or Sepharose 4B (lane 5) were resolved by 10% SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with Abs against Fyn (*A*), Lyn (*B*), Yes (*C*), Src (*D*), and Fgr (*E*).

not shown). Convulxin-coupled Sepharose 4B did not coprecipitate Fc γ R1IA (data not shown), confirming our previous findings that it is not associated with the GPVI-FcR γ complex (10).

Rapid Activation of Fyn upon Collagen Stimulation or GPVI Cross-linking. Next we examined whether collagen stimulation or cross-linking GPVI activated Fyn and Lyn. Fyn and Lyn were immunoprecipitated and subjected to an *in vitro* kinase assay with enolase as an exogenous substrate. We confirmed with immunoblotting that an equal amount of each kinase was immunoprecipitated (data not shown). Judging from both autophosphorylating activity and kinase activity toward enolase, Fyn was very rapidly activated by collagen stimulation or by cross-linking GPVI with convulxin or anti-GPVI F(ab')₂ (Fig. 5, A–C). Activation of Fyn reached the maximum level at 10 s and returned to the basal level within 60 s upon stimulation of convulxin or anti-GPVI F(ab')₂, whereas collagen-stimulated activation of Fyn was observed through 2 min of stimulation. We did not detect an increase in kinase activity of Lyn by collagen or cross-linking GPVI (data not shown). In some but not all experiments, we observed a slight decrease in kinase activity of Lyn upon convulxin (Fig. 5 D) or collagen stimulation (data not shown).

Inhibition of the Collagen- or Convulxin-induced Protein Tyrosine Phosphorylation by the Src Family-specific Inhibitor PP1. We used the Src family-specific inhibitor PP1 (17) to examine whether activation of Src family kinases was involved in the collagen- or convulxin-induced tyrosine phosphorylation of molecules including FcR γ , Syk, and PLC γ 2. Gel-filtered platelets were preincubated with or without various concentrations of PP1 for 3 min and then stimulated with 50 μ g/ml of collagen or 50 ng/ml of convulxin for appropriate times. Whole lysates were prepared from those platelets and subjected to the analysis of immu-

noblotting with anti-phosphotyrosine mAb (Fig. 6). Collagen stimulation induced tyrosine phosphorylation of proteins with molecular masses of 140, 120, 100, 85, 72/75, 65, 38/40, and 28/30 kD. Preincubation of platelets with PP1 inhibited the collagen-induced tyrosine phosphorylation of these proteins in a dose-dependent manner (Fig. 6 A). Convulxin stimulation induced tyrosine phosphorylation of proteins with the same molecular masses as described above, but the intensity of those protein bands was much stronger than upon collagen stimulation (Fig. 6 B). Preincubation of platelets with PP1 also inhibited the convulxin-induced tyrosine phosphorylation in a dose-dependent manner as was observed with collagen stimulation (Fig. 6 B). The heavily tyrosine-phosphorylated 60-kD protein band in both resting and activated platelets corresponded to Src with immunoblotting analysis (data not shown). Next we examined the effects of PP1 on the collagen- or convulxin-induced tyrosine phosphorylation of FcR γ , Syk, and PLC γ 2 in platelets. PP1 (1–20 μ M) dose-dependently inhibited the collagen-stimulated tyrosine phos-

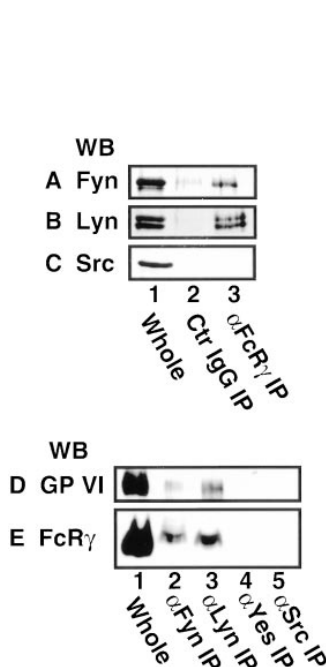


Figure 4. Coimmunoprecipitation of Fyn and Lyn with anti-FcR γ IgG and coimmunoprecipitation of GPVI and FcR γ with anti-Fyn and anti-Lyn Abs. Unstimulated gel-filtered platelets were lysed directly in SDS sample buffer or in Triton X-100 lysis buffer. (A–C) Whole lysates (lane 1) and immunoprecipitates with preimmune rabbit IgG (lane 2) and anti-FcR γ IgG (lane 3) were resolved by 10% SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with Abs against Fyn (A), Lyn (B), and Src (C). (D and E) Whole lysates (lane 1) and immunoprecipitates with Abs against Fyn (lane 2), Lyn (lane 3), Yes (lane 4), and Src (lane 5) were resolved by 10% (D) or 12.5% (E) SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with anti-GPVI IgG (D) and anti-FcR γ IgG (E).

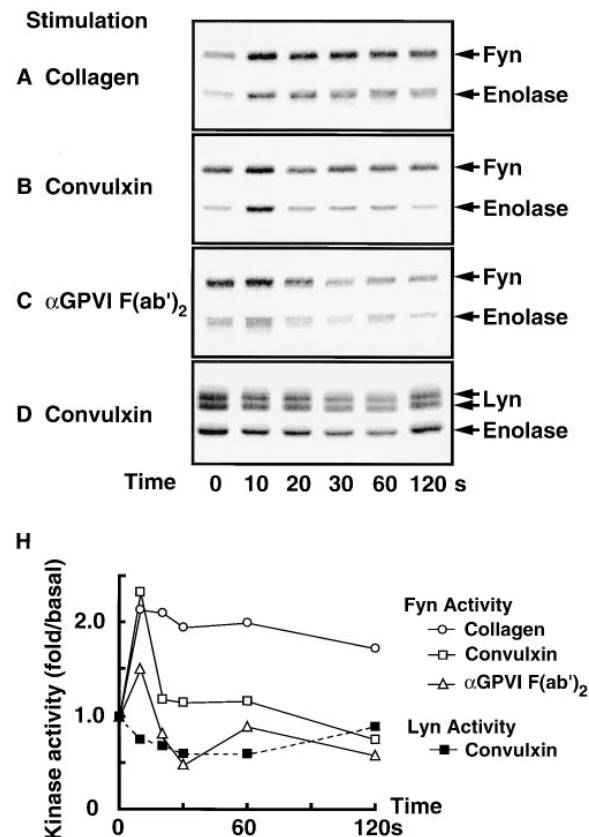


Figure 5. Kinase activity of Fyn and Lyn in platelets stimulated by collagen, convulxin, or anti-GPVI F(ab')₂. Gel-filtered platelets were stimulated for the indicated periods at 37°C with collagen (A, 50 μ g/ml), convulxin (B and D, 50 ng/ml), or anti-GPVI F(ab')₂ (C, 150 μ g/ml) and lysed in RIPA buffer. Fyn (A–C) and Lyn (D) were immunoprecipitated from lysates and subjected to *in vitro* kinase assay with enolase as an exogenous substrate, followed by 10% SDS-PAGE. Radioactivity of each protein was visualized (A–D) and quantified with the Bio-Imaging Analyzer BAS-2000II. Kinase activity toward enolase was calculated (H). Positions of Fyn, Lyn, and enolase are indicated on the right.

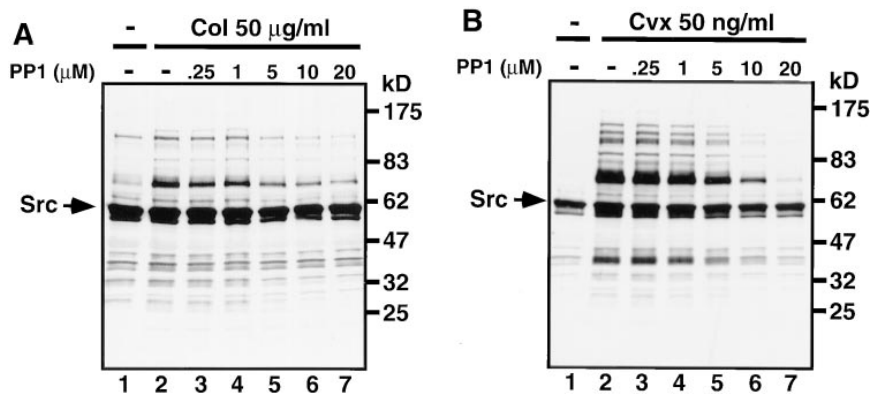


Figure 6. The Src family-specific inhibitor PP1 dose-dependently inhibits collagen- or convulxin-induced tyrosine phosphorylation of whole lysates. Gel-filtered platelets were preincubated for 3 min at 37°C with 0.25% DMSO (lanes 1 and 2) or the indicated concentrations of PP1 (lanes 3–7). Platelets were unstimulated (lane 1) or stimulated with 50 μg/ml of collagen for 30 s (A, lanes 2–7) or 50 ng/ml of convulxin for 15 s (B, lanes 2–7), directly lysed in SDS sample buffer, resolved by 10% SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with anti-phosphotyrosine mAb. Molecular mass markers are indicated in kD on the right of panels. Arrows mark the position of Src.

phorylation of GPVI-associated FcR γ (Fig. 7 C) as well as the recruitment of tyrosine-phosphorylated Syk to the GPVI–FcR γ complex (Fig. 7, A and B). We confirmed that convulxin-coupled Sepharose 4B equally precipitated both GPVI and FcR γ , irrespective of PP1 pretreatment (Fig. 7, D and E). PP1 also reduced collagen-stimulated tyrosine phosphorylation of PLC γ 2 in a dose-dependent fashion (Fig. 7 F). Essentially similar results on the PP1 effects were obtained with convulxin-stimulated platelets (data not shown).

Inhibition of the Collagen- or Convulxin-induced Aggregation and ATP Release Reaction by PP1. To investigate functional involvement of the Src family kinases in platelet activation through the GPVI–FcR γ complex, we examined the effects of PP1 on aggregation and ATP release reaction upon collagen or convulxin stimulation. PP1 alone did not cause any shape change, aggregation, or ATP release up to 20 μM of concentrations (data not shown). PP1 ranging in concentrations from 1 to 10 μM dose-dependently inhibited aggregation and ATP release caused by 2 μg/ml of collagen (Fig. 8, A and a), whereas PP1 little affected the extent of shape change but markedly delayed it (Fig. 8 A). Similar findings were also obtained when platelets were pretreated with 1–10 μM of PP1 and stimulated with 10 ng/ml of convulxin (Fig. 8, B and b). In contrast, 20 μM of PP1 did not affect aggregation and ATP release induced by 0.1 U/ml of thrombin (Fig. 8, C and c).

Discussion

Because the snake venom convulxin specifically binds to GPVI (18, 20), we used convulxin-coupled Sepharose 4B beads for affinity precipitation of the GPVI–FcR γ complex and associated proteins from platelet lysates. Convulxin-coupled Sepharose 4B efficiently precipitated GPVI, FcR γ , and their associated proteins such as Syk. We also found that collagen or convulxin stimulation induced tyrosine phosphorylation of FcR γ associated with GPVI and recruitment of Syk to the GPVI–FcR γ complex, confirming the complex as a collagen receptor. These experiments showed that convulxin-coupled Sepharose 4B was a powerful tool to isolate and study the GPVI–FcR γ complex. We noticed that convulxin-coupled Sepharose 4B copre-

cipitated tyrosine-phosphorylated proteins with molecular masses of 53–60 kD with the GPVI–FcR γ complex. It has been established that the Src family tyrosine kinases are essential for generating initial signals through the immune receptors, which consist of ITAM-containing subunits, leading to tyrosine phosphorylation of ITAMs and Syk activation (22, 39, 40). Platelets abundantly express the Src family kinases, whose molecular masses are 53–62 kD and usually tyrosine phosphorylated (31–36). Using coprecipitation methods with convulxin-coupled Sepharose 4B, anti-FcR γ IgG, and Abs to the Src family kinases, we clearly showed that Fyn and Lyn, but not Yes, Src, Fgr, Hck, and Lck, were constitutively associated with the GPVI–FcR γ complex, irrespective of stimulation. Although GPIV (CD36) is also stably associated with Fyn, Lyn, and Yes in platelets (36), GPIV is not associated with either GPVI or FcR γ (10). Recent reports showed that platelets heterogeneously express Fc ϵ RI (37, 38), which consists of the α , β , and γ chains, and is associated with Lyn in other cells such as mast cells (39, 40). However, we denied association of the α chain of Fc ϵ RI with the GPVI–FcR γ complex. Fc γ RIIA, which is another ITAM-containing receptor in platelets, is not associated with the GPVI–FcR γ complex (10). These data indicate that the GPVI–FcR γ complex is a newly identified receptor that is physically associated with the Src family kinases in platelets.

Rapid activation of Src (41) and Lyn (42) but not of Fyn has been previously reported in thrombin-stimulated platelets under specific experimental conditions, such as weak stimulation or specified lysis buffers. We have reported that Src is activated upon collagen stimulation or cross-linking GPVI (8, 9). In addition, here we showed for the first time that Fyn became rapidly activated by collagen or cross-linking GPVI. We could detect the Src activation only if we used 1% Triton X-100 containing lysis buffer to lyse platelets as previously reported (8, 9). In contrast to Src, we could observe the Fyn activation only if we used RIPA buffer as described in Materials and Methods. However, we could not detect Lyn activation by collagen stimulation or cross-linking GPVI with either RIPA buffer or Triton X-100 lysis buffer. It has been well documented that members of the Src family kinases are differently distributed in resting platelets (35) and relocate to the membrane skeleton or

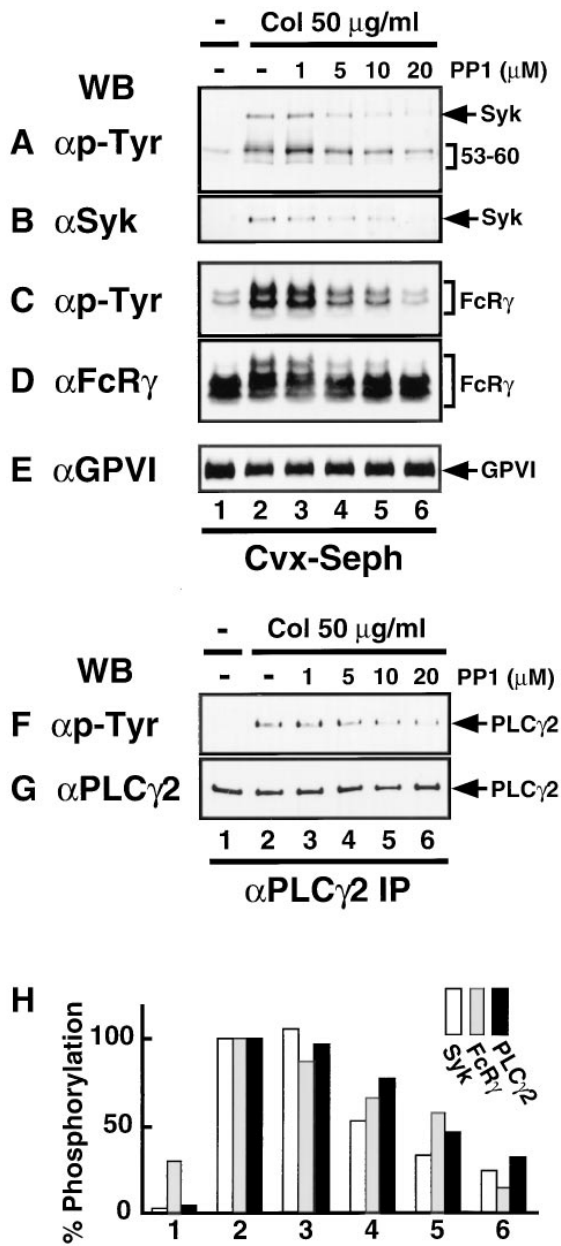


Figure 7. PP1 dose-dependently inhibits collagen-induced Syk recruitment and tyrosine phosphorylation of FcR γ and PLC γ 2. Gel-filtered platelets were preincubated for 3 min at 37°C with 0.25% DMSO (lanes 1 and 2) or the indicated concentrations of PP1 (lanes 3–6). Platelets were unstimulated (lane 1) or stimulated with 50 μ g/ml of collagen for 30 s (lanes 2–6) and lysed in Triton X-100 lysis buffer. Precipitated proteins with convulxin-coupled Sepharose 4B (A–E) or immunoprecipitates with anti-PLC γ 2 Ab (F and G) were resolved by 10% (A, B, and E–G) or 12.5% (C and D) SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with Abs against phosphotyrosine (A, C, and F), Syk (B), FcR γ (D), GPVI (E), and PLC γ 2 (G). In H the band densities from tyrosine-phosphorylated Syk (A), FcR γ (C), and PLC γ 2 (F) were quantified and graphed as a percentage of the band density in lane 2 of each panel. The position of each protein is indicated on the right of panels.

the cytoskeleton (41–44), which is insoluble in 1% Triton X-100 containing lysis buffer, upon stimulation. These different natures of the kinases may cause the different requirements for lysis buffers to detect their activation. We

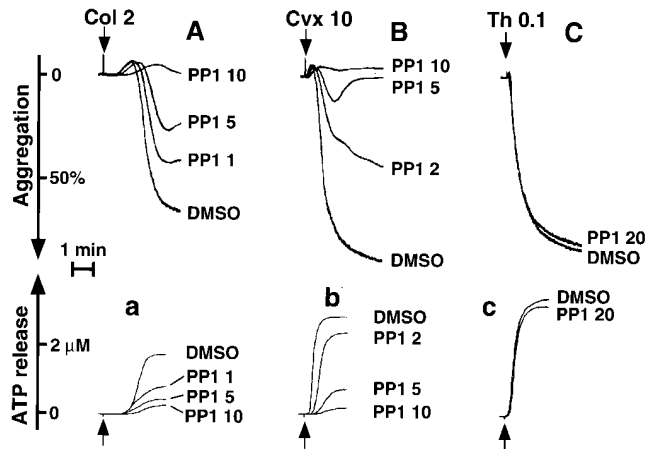


Figure 8. PP1 dose-dependently inhibits collagen- or convulxin-induced aggregation and ATP release reaction. Gel-filtered platelets were preincubated for 3 min at 37°C with 0.25% DMSO or various concentrations of PP1 (μ M) as indicated. Platelets were stimulated with 2 μ g/ml of collagen (A and a), 10 ng/ml of convulxin (B and b), or 0.1 U/ml of thrombin (C and c) under constant stirring conditions. Aggregation curves (A–C) and ATP release (a–d) were monitored. This experiment is representative of four similar experiments from different donors.

observed a slight decrease in kinase activity of Lyn upon convulxin or collagen stimulation in some but not all experiments. Such a decrease in Lyn activity seems to be similar to Src activity as reported previously (9, 41). Although Src is activated by either collagen or cross-linking GPVI in normal platelets, Src is still activated by collagen in GPVI-deficient platelets lacking collagen-induced Syk activation and PLC γ 2 phosphorylation (9). Taken together with our new findings that Src was not physically associated with the GPVI–FcR γ complex, Src does not seem to be directly involved at least in Syk activation and subsequent tyrosine phosphorylation of PLC γ 2 upon collagen stimulation.

It has been demonstrated that PP1 selectively inhibits the Src family kinases, including Lck, Fyn, Src, Hck, and Lyn, but not the Syk family kinases, including ZAP-70 and Syk, even at concentrations over 100 μ M (17, 45). We observed that PP1 (1–20 μ M) dose-dependently inhibited collagen- or convulxin-induced tyrosine phosphorylation of whole lysates. PP1 also abolished tyrosine phosphorylation of GPVI-associated FcR γ , Syk, and PLC γ 2 as well as recruitment of Syk to the GPVI–FcR γ complex. The concentrations of PP1 used here were similar ones that have been reported to inhibit induction of tyrosine phosphorylation or cell functions upon stimulation in T cells, RBL-2H3 cells, fibroblasts, or Mo7e cells (17, 45–47). As for immune receptors, it has been reported that FcR γ is phosphorylated on tyrosine by the Src family kinases that are associated with the receptors, such as Lyn for Fc ϵ R1 (22, 39, 40). Therefore, Fyn and Lyn that were associated with the GPVI–FcR γ complex would be most probable candidate kinases responsible for phosphorylating FcR γ and subsequent signals in GPVI-mediated platelet activation by collagen. Finally, we showed that PP1 inhibited collagen- or convulxin-stimulated aggregation and ATP release reaction

but not extent of shape change in a dose-dependent manner. The inhibition of aggregation and release reaction by PP1 was observed parallel to that of the tyrosine phosphorylation discussed above, suggesting that PP1 blocks platelet functions through inhibiting activation of the Src family and its downstream signals. However, when we used higher concentrations of collagen (20–50 $\mu\text{g}/\text{ml}$), aggregation and ATP release reactions became somewhat insensitive to even 20 μM of PP1, although PP1 still notably delayed beginning of aggregation and ATP release by these concentrations of the agonists (data not shown). It is probably because PP1 could not completely inhibit activation of the Src family upon strong stimulation of collagen. If it is not, there may be another possibility that PP1-insensitive bypass signals might function in platelets stimulated with higher concentrations of collagen. Although previous studies have shown that platelets are enriched in the Src family kinases (31–36), this is the first report demonstrating that the Src family is functionally related to platelet activation.

The collagen-stimulated tyrosine phosphorylation including Syk and PLC γ 2 is compromised by blocking of $\alpha_2\beta_1$ with anti- $\alpha_2\beta_1$ mAbs (14, 48) or proteolytic cleavage of the β_1 subunit with the snake venom metalloproteinase Jararhagin (49), suggesting that $\alpha_2\beta_1$ is also essential for the collagen signals. However, direct cross-linking of $\alpha_2\beta_1$ alone with mAbs does not cause platelet activation and protein tyrosine phosphorylation (14). In contrast to our

present findings on the GPVI–FcR γ complex, $\alpha_2\beta_1$ has not been shown to be physically associated with protein tyrosine kinases in platelets (48, 49). On the other hand, cross-linking the GPVI–FcR γ complex with anti-GPVI F(ab') $_2$, convulxin, or collagen-related peptides, which also stimulate platelets via GPVI (50), causes platelet activation and tyrosine phosphorylation of proteins including FcR γ , Syk, and PLC γ 2 independently of $\alpha_2\beta_1$ (8, 10–12, 18, 51, 52). In GPVI-deficient platelets, collagen stimulates $\alpha_2\beta_1$ -dependent activation of Src and tyrosine phosphorylation of multiple proteins but not that of Syk, PLC γ 2, and Vav (9). These data seem to support the two-site, two-step model of platelet activation by collagen (53, 54). At the first step of the platelet–collagen interaction, $\alpha_2\beta_1$ plays a critical role in adhesion and might modulate the GPVI–FcR γ complex-mediated responses including protein tyrosine phosphorylation as a coreceptor, leading to aggregation and release reaction. Roles of the other receptors (2, 5, 6) remain to be less clear than $\alpha_2\beta_1$ and the GPVI–FcR γ complex in the collagen signals. Platelets from patients lacking GPIV (the Nak^a-negative phenotype) aggregate (55) and show tyrosine phosphorylation (56) normally to collagen. However, GPIV may play some roles in the collagen-induced tyrosine phosphorylation, since GPIV is physically associated with Fyn, Yes, and Lyn (36). Further studies are necessary to elucidate the complete nature of collagen signals through the GPVI–FcR γ complex, $\alpha_2\beta_1$, and possibly others.

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Address correspondence to Hiroshi Takayama, The Department of Hematology and Oncology, Clinical Sciences for Pathological Organs, Graduate School of Medicine, Kyoto University, 54 Shogoin-Kawaracho, Sakyo-ku, Kyoto 606-8507, Japan. Phone: 81-75-751-3151; Fax: 81-75-751-3201; E-mail: hiro@kuhp.kyoto-u.ac.jp

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