

# Role of Fc receptor $\gamma$ -chain in platelet glycoprotein Ib-mediated signaling

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**Interaction between von Willebrand factor (vWF) and glycoprotein Ib (GPIb) stimulates tyrosine kinases and subsequent tyrosine phosphorylation events in human platelets. This study found that the combination of vWF and botrocetin, by interacting with GPIb, induced tyrosine phosphorylation of Fc receptor  $\gamma$ -chain (FcR  $\gamma$ -chain), Syk, linker for activation of T cells (LAT), and phospholipase C  $\gamma$ 2 (PLC $\gamma$ 2). Pretreatment of platelets with 10  $\mu$ M PP1 completely inhibited these tyrosine phosphorylation events. On GPIb stimulation, Src and Lyn formed**

**a complex with FcR  $\gamma$ -chain and Syk, suggesting that Src and Lyn are involved in FcR  $\gamma$ -chain tyrosine phosphorylation and downstream signals. In spite of the PLC $\gamma$ 2 tyrosine phosphorylation, however, there was no intracellular calcium release and inositol 1,4,5-trisphosphate production. In Brij 35 lysates, FcR  $\gamma$ -chain was found to constitutively associate with GPIb. The number of GPIb expressed on FcR  $\gamma$ -chain-deficient platelets was comparable to that of the wild-type, as assessed by flow cytometry. However, tyrosine phosphorylation of Syk, LAT, and**

**PLC $\gamma$ 2 in response to vWF plus botrocetin was significantly suppressed, suggesting that FcR  $\gamma$ -chain mediates activation signals related to GPIb. Compared with the aggregation response of wild-type platelets, that of FcR  $\gamma$ -chain-deficient platelets in response to vWF plus botrocetin was impaired, implying that FcR  $\gamma$ -chain is required for the full activation of platelets mediated by GPIb. (Blood. 2001;97:3836-3845)**

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## Introduction

von Willebrand factor (vWF) is a multimeric protein that mediates platelet adhesion to exposed subendothelium at sites of vascular injury. The adhesive property of vWF is tightly regulated so that plasma vWF does not normally interact with circulating platelets. However, after vWF is activated by binding to damaged vessel walls, it serves to bridge the constituents of subendothelium to glycoprotein Ib (GPIb) on the membrane of circulating platelets.<sup>1,2</sup> Although much is known about the molecular basis of the interaction between vWF and GPIb, little has been clarified about the intracellular signal transduction pathway in GPIb-mediated platelet activation.

Events related to protein-tyrosine phosphorylation have emerged as important signals mediated by GPIb.<sup>3-7</sup> GPIb-mediated platelet activation in response to vWF plus botrocetin, shear stress, or vWF from patients with von Willebrand disease type IIb induces tyrosine phosphorylation of multiple proteins,<sup>3-5</sup> suggesting that the binding of vWF to GPIb causes the activation of tyrosine kinases. Studies have shown that the activation of Syk and Src and their association can be induced by GPIb stimulation.<sup>6-10</sup> To date, there is no report showing that tyrosine residues within the GPIb molecule can be phosphorylated, nor has GPIb itself intrinsic kinase activity. Thus, how clustering of GPIb induced by vWF mediates the activation of tyrosine kinases such as Src and Syk has become an important issue. Because Syk is activated by engagement of its tandem SH2 domains with phosphorylated tyrosine residues in proteins containing the immunoreceptor tyrosine-based activation motif (ITAM),<sup>11</sup> it is of interest whether ITAM-containing transmembrane molecules are also involved in GPIb signaling. Two ITAM-containing proteins have been identified in platelets, the low-affinity receptor for immunoglobulin (Ig) G, Fc $\gamma$ RIIA, and the Fc receptor  $\gamma$ -chain

(FcR  $\gamma$ -chain). Of particular note is the finding that Fc $\gamma$ RIIA is physically associated with GPIb and that it mediates some signaling events.<sup>12,13</sup> However, the level of Fc $\gamma$ RIIA tyrosine phosphorylation induced by GPIb clustering is much lower than that inducible by Fc $\gamma$ RIIA clustering. Further, murine platelets lacking Fc $\gamma$ RIIA on their membrane appear to have normal responses to GPIb-related signals. Thus, to what extent Fc $\gamma$ RIIA contributes to the GPIb signaling pathway remains to be determined.

The other ITAM-containing protein, FcR  $\gamma$ -chain, has been recognized for its role in signaling related to the high-affinity receptors for IgE (Fc $\epsilon$ RI)<sup>14</sup> and IgG (Fc $\gamma$ RI),<sup>15</sup> and the low-affinity IgG receptor (Fc $\gamma$ RIII).<sup>16</sup> In platelets, FcR  $\gamma$ -chain is colocalized with GPVI and is critical for GPVI-mediated platelet activation.<sup>17,18</sup> Clustering and activation of GPVI induces tyrosine phosphorylation of FcR  $\gamma$ -chain ITAM,<sup>19</sup> which then facilitates the recruitment of Syk, resulting in its activation.<sup>20</sup> Moreover, FcR  $\gamma$ -chain is required for tyrosine phosphorylation of phospholipase C  $\gamma$ 2 (PLC $\gamma$ 2).<sup>20</sup> Recently, Falati et al<sup>6</sup> reported that alboaggregin A, by interacting with GPIb, induces tyrosine phosphorylation of FcR  $\gamma$ -chain, PLC $\gamma$ 2 activation, and Ca<sup>++</sup> mobilization in platelets. On the basis of their studies, we sought to further define the role of FcR  $\gamma$ -chain in the GPIb-related signal transduction pathway.

## Materials and methods

### Materials

FcR  $\gamma$ -chain-deficient C57BL/6 mice and control mice were kindly provided by Dr Toshiyuki Takai (Tohoku University, Japan). The following

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materials were obtained from the indicated suppliers: monoclonal antibodies (MoAbs) against Lyn and Syk (Wako Pure Chemical Industries, Tokyo, Japan); anti-integrin  $\beta$ 1 MoAb, antiphosphotyrosine MoAb, PY 20 (Transduction Laboratories, Lexington, KY); antiphosphotyrosine MoAb, 4G10, anti-Src MoAb, anti-FcR  $\gamma$ -chain, anti-Lck, anti-LAT (linker for activation of T cells) polyclonal Abs (Upstate Biotechnology, Lake Placid, NY); anti-Fyn MoAb, polyclonal anti-PLC $\gamma$ 2, anti-Syk, peroxidase-conjugated goat antirabbit IgG Abs (Santa Cruz, CA); control fluorescence-labeled monoclonal rat antimouse antibody (Exalpha Biologicals, Boston, MA); thrombin (Green Cross, Osaka, Japan); collagen (Hormon-Chemie, Munich, Germany); PPI (Biomol, Plymouth Meeting, PA); Gly-Arg-Gly-Asp-Ser (GRGDS) peptide (Peptide Institute, Osaka, Japan); glutathione-Sepharose 4B and protein A-Sepharose 4B (Pharmacia Biotech, Uppsala, Sweden); peroxidase-conjugated goat antimouse IgG Ab (Cappel, Durham, NC); D-myo-inositol 1,4,5-trisphosphate (IP $_3$ ) assay kit (Amersham, Buckinghamshire, United Kingdom); bovine serum albumin (BSA), Tween-80, prostaglandin I $_2$ , phenylmethylsulfonyl fluoride (PMSF), Na $_3$ VO $_4$ , Triton X-100, Brij 35 (Sigma, St Louis, MO); and Fura-2 AM (Dojindo Laboratories, Kumamoto, Japan).

vWF and botrocetin were purified as described previously.<sup>7,21</sup> Nonfunctional anti-GPIIb MoAb, WGA3, was provided by Dr M. Handa (Keio University, Tokyo, Japan). Fluorescence-labeled rat antimouse GPIIb $\alpha$  (p0p4), GPIX (p0p6), and GPIIb/IIIa (JON1) MoAbs were supplied by Dr Bernhard Nieswandt (University of Witten-Herdecke, Wuppertal, Germany). Glutathione S-transferase (GST) fusion protein containing the tandem SH2 domains of Syk was obtained from Dr C.-L. Law (University of Washington, Seattle, WA). Jararaca GPIIb-binding protein was donated by Dr Y. Fujimura (Nara Medical University, Nara, Japan). Convulxin was a gift from Dr T. Morita (Meiji Pharmaceutical University, Tokyo, Japan).

### Preparation and stimulation of human and murine platelets

Murine blood was taken by cardiac puncture immediately after death. Human venous blood was obtained from healthy, drug-free volunteers on the day of the experiment, using acid citrate dextrose (120 mM sodium citrate, 110 mM glucose, 80 mM citric acid) as anticoagulant. Washed platelets were prepared as previously described<sup>7</sup> and were suspended in modified HEPES-Tyrode buffer (134 mM NaCl, 0.34 mM Na $_2$ HPO $_4$ , 2.9 mM KCl, 12 mM NaHCO $_3$ , 20 mM HEPES, 5 mM glucose, 1 mM MgCl $_2$ , pH 7.3). Unless otherwise stated, murine platelets were resuspended at a count of  $2.0 \times 10^8$  cells/mL, and human platelets were adjusted to  $1.0 \times 10^9$  cells/mL. Platelets were stimulated at 37°C under constant stirring at 1000 rpm in an AG-10 aggregation analyzer (Kowa, Tokyo, Japan). In preliminary experiments, we confirmed that human vWF alone does not induce aggregation of washed murine platelets but that aggregation is induced in the copresence of botrocetin. This finding is in agreement with previous reports that GPIIb of murine platelets can interact with human vWF.<sup>22,23</sup>

### Platelet aggregation study

Platelet aggregation was monitored by measuring light transmission with the use of an AG-10 aggregation analyzer (Kowa, Tokyo, Japan). The instrument was calibrated with either the washed platelet suspension or platelet-rich plasma (PRP) for zero light transmission and with buffer or platelet-poor plasma (PPP) for 100% transmission, respectively. Aggregation was initiated by addition of agonists under constant stirring at 1000 rpm at 37°C.

Platelet aggregation of mice was measured not only by changes in light transmission but also by a particle-counting technique using light scatter.<sup>24</sup> Briefly, a diode laser light beam (width: 40  $\mu$ m; wavelength: 675 nm) was passed through mouse PRP in a cylindrical glass cuvette maintained at 37°C with constant stirring. The light scatter by particles in a limited volume (33  $\times$  65  $\times$  65  $\mu$ m) was measured with an optical system that minimized multiple light scatter. The signals were digitized to quantitate the number and size of platelet aggregates. Light-scattering method provides a sensitive, in situ continuous measurement of platelet aggregation by counting the number and size of aggregates. On the basis of the level of scattered light intensity, platelet aggregates can be divided into small aggregates (0.2 to 2.0

V/min, consisting of less than 500 platelets) and large aggregates (2.0 to 10 V/min).<sup>24</sup>

### Immunoprecipitation and GST-fusion protein precipitation

After the indicated time intervals of activation, platelets were solubilized with an equal volume of  $2 \times$  ice-cold lysis buffer (100 mM Tris/HCl, pH 7.4, 5.0 mM EGTA, 2.0 mM PMSF, 2.0 mM Na $_3$ VO $_4$ , 100  $\mu$ g/mL leupeptin, and 2% Triton X-100) and kept on ice for 30 minutes. All subsequent steps were performed at 4°C. The lysates were centrifuged at 15 000g for 5 minutes, and the resulting supernatants were precleared by incubation for 30 minutes with protein A-Sepharose for immunoprecipitation experiments or with glutathione-Sepharose for GST-fusion protein precipitation. For immunoprecipitation, the supernatants were then incubated with the appropriate antibodies, followed by the addition of protein A-Sepharose. For GST-fusion protein precipitation, the supernatants were incubated with GST-Syk-SH2 (20  $\mu$ g/sample), followed by the addition of glutathione-Sepharose beads. The precipitates obtained after centrifugation were washed 3 times in  $1 \times$  lysis buffer before the addition of Laemmli sample buffer.

As indicated elsewhere, the precipitates with GST-Syk-SH2 were subjected to re-immunoprecipitation in some experiments. Briefly, the precipitated proteins with GST fusion protein bound to glutathione-Sepharose beads were eluted with Glutathione Elution Buffer (10 mM reduced glutathione in 50 mM Tris/HCl, pH 8.0). The eluates were collected, dialyzed against 50 mM Tris/HCl (pH 8.0) containing 1 mM PMSF, 50  $\mu$ g/mL leupeptin, and 1 mM Na $_3$ VO $_4$ , and subjected to immunoprecipitation with the indicated antibody.

### Immunoblotting

Precipitated proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes. The membranes were blocked with 1% BSA in phosphate-buffered saline (PBS). After extensive washing with PBS containing 0.4% Tween-80, the immunoblots were incubated with the appropriate antibodies for 2 hours at room temperature, or overnight at 4°C. Antibody binding was detected by using peroxidase-conjugated secondary antibodies diluted at 1:7500 and was visualized with the enhanced chemiluminescence reaction reagent. For reprobing with other antibodies, the antibody bound to PVDF membranes was removed with a stripping buffer (2% SDS, 62.5 mM Tris/HCl, pH 6.8, 100  $\mu$ M 2-mercaptoethanol) at 50°C for 20 minutes. After washing, the membranes were blocked with 1% BSA and reprobed with the indicated antibodies. When indicated, the level of proteins as detected by immunoblotting was quantified by using a PDI4200e scanner (PDI, New York, NY).

### Flow cytometry

Washed murine platelets ( $8 \times 10^7$  cells/mL) in a volume of 25  $\mu$ L were incubated with fluorescence-labeled rat antimouse GPIIb $\alpha$  (p0p4), antimouse GPIX (p0p6), or antimouse GPIIb/IIIa (JON1) MoAbs at the final concentration of 10  $\mu$ g/mL for 15 minutes at 37°C, respectively. After dilution with 400  $\mu$ L PBS, the samples were analyzed immediately on a FACScan (Becton Dickinson, San Jose, CA).

### Measurement of IP $_3$ production

Platelets were suspended in HEPES-Tyrode buffer at a count of  $3 \times 10^9$ /mL. After stimulation, an equal volume of ice-cold 15% trichloroacetic acid (TCA) was added to the platelet suspension to terminate reactions, and the mixtures were kept on ice for 30 minutes. The mixtures were centrifuged at 4000g for 15 minutes at 4°C, and the resultant supernatant was treated 5 times with 5 mL water-saturated diethyl ether to extract trichloroacetic acid. Residual ether was further removed under vacuum for 1 hour. The samples were neutralized by titration with 0.2 N NaOH, and the production of IP $_3$  was measured with an IP $_3$  assay kit (Amersham) according to the manufacturer's instructions.

### Measurement of the intracellular $Ca^{++}$ concentration

Measurement of intracellular  $Ca^{++}$  concentration ( $[Ca^{++}]_i$ ) was performed with the use of  $Ca^{++}$ -sensitive fluorophore, fura2, as described previously.<sup>25</sup> The fura2-loaded platelets were adjusted to a count of  $2 \times 10^8$ /mL, and 200  $\mu$ M EGTA was added to the platelet suspension to inhibit  $Ca^{++}$  influx, as GPIb-mediated platelet activation appears to induce  $Ca^{++}$  influx.<sup>5,26</sup> After stimulation, changes in  $[Ca^{++}]_i$  were measured with a Hitachi 2000 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). The  $[Ca^{++}]_i$  values were determined from the ratio of the fura2 fluorescence intensity at 340 nm excitation wavelength to that at 380 nm.

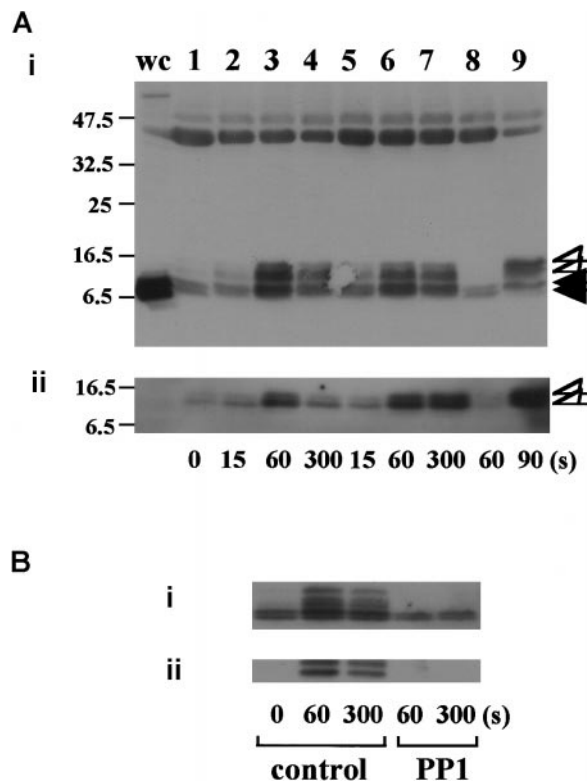
### Statistical analysis

The data are expressed as the mean  $\pm$  SEM. With the use of Statworks statistical software (Cricket Software, Philadelphia, PA), statistical analysis was done by Student *t* test with *P* < .05 taken to indicate significance.

## Results

### vWF plus botrocetin stimulates tyrosine phosphorylation of FcR $\gamma$ -chain

In preliminary experiments, we used 2 commercially available anti-FcR  $\gamma$ -chain polyclonal antibodies to detect tyrosine phosphorylation of FcR  $\gamma$ -chain in GPIb-mediated or collagen-induced platelet activation. However, there was little detectable change, if any. These antibodies appeared to be unsuitable for immunoprecipitation. Thus, we sought another way to address this issue. Human platelets have only 2 ITAM-containing proteins, Fc $\gamma$ RIIA and FcR  $\gamma$ -chain. Because the tandem SH2 domains of Syk bind specifically to the ITAM-containing proteins, they are often employed to detect the tyrosine-phosphorylated ITAMs.<sup>19,20</sup> Thus, we used a GST-fusion protein expressing the tandem SH2 domains of Syk, GST-Syk-SH2. Human platelets were stimulated with 10  $\mu$ g/mL vWF plus 6  $\mu$ g/mL botrocetin, or 50  $\mu$ g/mL collagen, the subsequent platelet lysates were incubated with GST-Syk-SH2, and proteins precipitated by GST-Syk-SH2 were checked for the presence of FcR  $\gamma$ -chain by using anti-FcR  $\gamma$ -chain polyclonal antibodies (Figure 1A). Approximately 4 proteins of 13, 11, 8.5, and 7 kd were detected as FcR  $\gamma$ -chain in platelets stimulated with vWF plus botrocetin [Figure 1Ai, lanes 1-4]. The anti-FcR  $\gamma$ -chain polyclonal antibodies were thus suitable for Western blotting but not for immunoprecipitation. Immunoblotting with antiphosphotyrosine antibodies revealed that the upper 2 bands (13 and 11 kd) were heavily tyrosine-phosphorylated, indicating that tyrosine phosphorylation of FcR  $\gamma$ -chain leads to a mobility shift on SDS-PAGE [Figure 1Aii, lanes 1-4]. Because only polyclonal antibodies against FcR  $\gamma$ -chain are available, whether the lower 2, nonphosphorylated bands correspond to the nonphosphorylated FcR  $\gamma$ -chain awaits elucidation. The pattern of FcR  $\gamma$ -chain tyrosine phosphorylation, including those of nonphosphorylated bands, is similar to that in GPVI-stimulated platelets, observed by Tsuji et al.<sup>18</sup> Tyrosine phosphorylation of FcR  $\gamma$ -chain reached its peak 60 seconds after stimulation, and partial dephosphorylation occurred at 300 seconds [Figure 1Aii, lanes 1-4]. Precipitates with GST-Syk-SH2 from collagen-activated platelets showed patterns similar to vWF plus botrocetin [Figure 1Aii, lane 9]. It is established that vWF contains not only a binding site for GPIb $\alpha$ , the A1 domain, but also a C1 domain peptide sequence, Arg-Gly-Asp (RGD), which mediates vWF binding to integrin  $\alpha$ IIB $\beta$ 3.<sup>27</sup> To rule out the possibility that integrin  $\alpha$ IIB $\beta$ 3 signaling is involved in tyrosine phosphorylation of FcR  $\gamma$ -chain, the platelets were pretreated with RGDS and EGTA and then stimulated with vWF



**Figure 1. Stimulation of platelets with vWF plus botrocetin induces tyrosine phosphorylation of FcR  $\gamma$ -chain.** (A) Human platelets were preincubated for 10 minutes at 37°C with a vehicle solution (lanes 1-4, 9), 1 mM RGDS plus 1 mM EGTA (lanes 5-7), or 20  $\mu$ g/mL jararaca GPIb-BP (lane 8). After stimulation with 6  $\mu$ g/mL botrocetin plus 10  $\mu$ g/mL vWF (lanes 1-8) or 50  $\mu$ g/mL collagen (lane 9) for the indicated time periods, the reactions were terminated by the addition of lysis buffer. The platelet lysates were then precipitated with GST-Syk-SH2. (B) After preincubation with 0.25% dimethyl sulfoxide (DMSO) as control or 10  $\mu$ M PP1 for 5 minutes at 37°C, the human platelets were activated by the addition of 6  $\mu$ g/mL botrocetin plus 10  $\mu$ g/mL vWF for the indicated time frames. The cells were processed for precipitation with GST-Syk-SH2 as described in the legend for A. For A and B, precipitated proteins were then separated by SDS-PAGE (15% polyacrylamide) and immunoblotted with anti-FcR  $\gamma$ -chain (i) and 4G10 plus PY20 (ii). The numbers below the panels show time in seconds. The data are representative of 3 experiments. The open arrowheads indicate the proteins of 13, 11 kd. The closed arrowheads indicate the proteins of 8.5, 7 kd. wc indicates whole cell lysates.

plus botrocetin. As shown in Figure 1Aii, lanes 5-7, blockage of integrin  $\alpha$ IIB $\beta$ 3 by RGDS and EGTA did not inhibit tyrosine phosphorylation of FcR  $\gamma$ -chain. We next used jararaca GPIb-binding protein (GPIb-BP) to confirm that FcR  $\gamma$ -chain tyrosine phosphorylation is mediated by GPIb-vWF interaction. Jararaca GPIb-BP itself induced neither platelet aggregation nor serotonin release from platelets, and it completely inhibited vWF binding to GPIb in the presence of botrocetin or ristocetin.<sup>28</sup> In jararaca GPIb-BP-pretreated platelets, tyrosine phosphorylation of FcR  $\gamma$ -chain was abolished [Figure 1Aii, lane 8]. These findings indicate that GPIb stimulation specifically induces tyrosine phosphorylation of FcR  $\gamma$ -chain. Falati et al<sup>6</sup> also found that both ristocetin plus normal vWF and ristocetin plus RGGs-vWF, a mutant vWF able to bind to GPIb but not to integrin  $\alpha$ IIB $\beta$ 3, induced both tyrosine phosphorylation of FcR  $\gamma$ -chain and its association with Syk. Our findings are in good accord with their report and confirm that tyrosine phosphorylation of FcR  $\gamma$ -chain indeed takes place in the GPIb-mediated signal transduction pathway. Recently, Fc $\gamma$ RIIA, which also contains one ITAM, has been shown to be physically proximal to the GPIb-IX-V complex and functionally related to it.<sup>13</sup> In our study, however, tyrosine

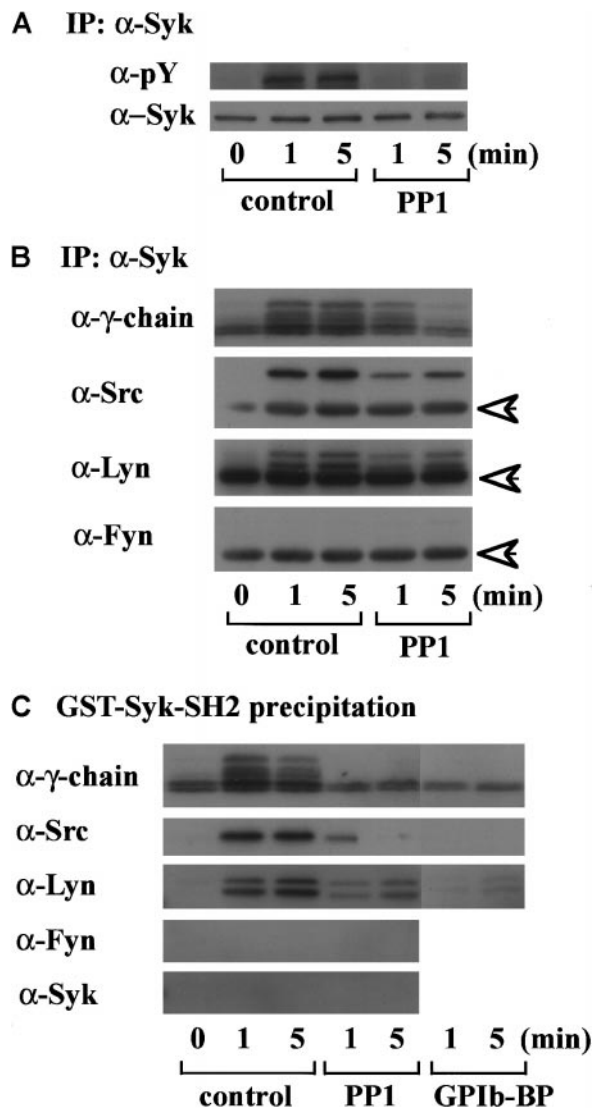
phosphorylation of Fc $\gamma$ R1IA was not detectable in the GST-Syk SH2 precipitates (data not shown).

Tyrosine phosphorylation of FcR  $\gamma$ -chain, in response to the activation of immune receptors in lymphocytes or of GPVI in platelets, is catalyzed by Src family tyrosine kinases.<sup>29,30</sup> Because previous reports suggest the involvement of Src in GPIIb-mediated platelet activation,<sup>7,8</sup> the platelets were pretreated with a specific Src family kinase inhibitor, PP1, to examine whether FcR  $\gamma$ -chain is also tyrosine-phosphorylated by Src family kinases. PP1 is selective to Src family kinase over Syk by 10 000-fold in both *in vitro* and functional studies.<sup>31,32</sup> As shown in Figure 1B, PP1 at 10  $\mu$ M completely inhibited tyrosine phosphorylation of FcR  $\gamma$ -chain, suggesting that Src kinase(s) is responsible for FcR  $\gamma$ -chain tyrosine phosphorylation, a finding in agreement with that of Falati et al.<sup>6</sup>

#### FcR $\gamma$ -chain tyrosine-phosphorylated by Src kinases recruits Syk

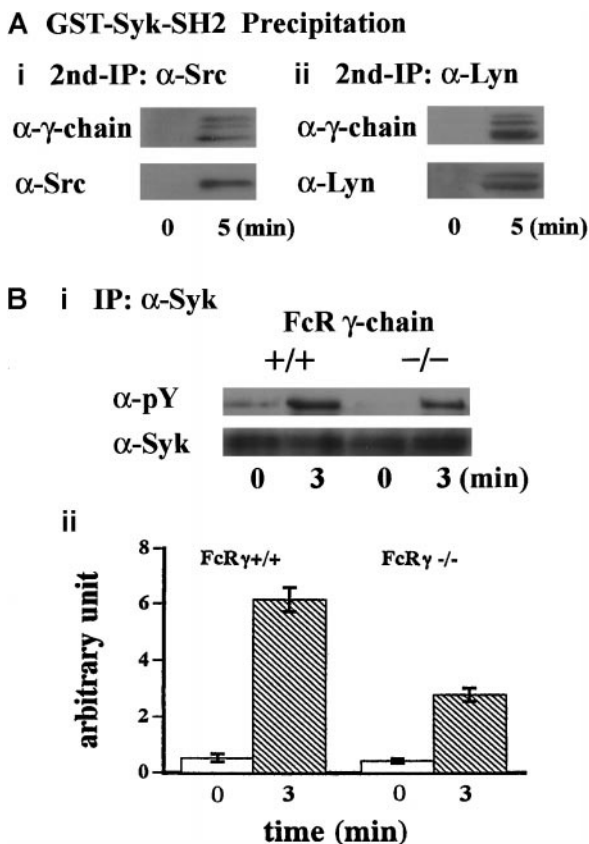
An accumulating body of evidence suggests that tyrosine phosphorylation of FcR  $\gamma$ -chain catalyzed by Src kinases is required for the membrane recruitment and activation of Syk in signal transduction related to immune receptors and GPVI.<sup>11,20</sup> Because we found in a previous report that GPIIb mediates tyrosine phosphorylation of Syk and its association with Src,<sup>7</sup> we then asked if Syk locates downstream of Src kinase activation and FcR  $\gamma$ -chain tyrosine phosphorylation in GPIIb signaling. As shown in Figure 2A, PP1 pretreatment completely suppressed tyrosine phosphorylation of Syk, suggesting that Src family kinase activation lies upstream of Syk tyrosine phosphorylation. To determine which member(s) of the Src family tyrosine kinases is responsible for Syk activation, the proteins precipitated with anti-Syk MoAb were probed with the antibodies against various Src kinases. As shown in Figure 2B, in platelets stimulated with vWF plus botrocetin, not only Src but also Lyn were found to associate *in vivo* with Syk, which is also complexed with tyrosine-phosphorylated FcR  $\gamma$ -chain. Fyn and other kinases of the Src family were not detected (Figure 2B and data not shown). To investigate whether Src and Lyn associate with Syk via the SH2 domain of Syk, the platelet lysates were precipitated with GST-Syk-SH2, instead of anti-Syk MoAb. As shown in Figure 2C, the pattern of association of Src/Lyn, FcR  $\gamma$ -chain with GST-Syk-SH2 was consistent with their association with Syk (Figure 2B), and the precipitates were also negative for Fyn. Pretreatment of platelets with jararaca GPIIb-BP inhibited association of Src, Lyn, FcR  $\gamma$ -chain with GST-Syk-SH2 (Figure 2C), implying that their association is specifically mediated by GPIIb stimulation. Additionally, PP1 pretreatment also inhibited the association of Src/Lyn, FcR  $\gamma$ -chain with Syk or GST-Syk-SH2. This inhibition was not complete at an early time point; the reason remains elusive. In platelets, Src kinases and FcR  $\gamma$ -chain are membrane-associated proteins, and this complex formation may serve to recruit Syk to the membrane, thereby resulting in its tyrosine phosphorylation.

To further clarify the mechanism of this complex formation, the lysates were first precipitated with GST-Syk-SH2. The precipitates with GST fusion proteins were eluted with glutathione, then the resultant eluates were subjected to immunoprecipitation with anti-Src or anti-Lyn MoAb. As shown in Figure 3A, FcR  $\gamma$ -chain was recovered with anti-Src and anti-Lyn immunoprecipitates, suggesting that Src and Lyn bind to FcR  $\gamma$ -chain. Additionally, Syk was not present in GST-Syk-SH2 precipitates (Figure 2C), suggesting that association of Src/Lyn with FcR  $\gamma$ -chain is not mediated by Syk associated with FcR  $\gamma$ -chain. To verify that FcR  $\gamma$ -chain is



**Figure 2. Tyrosine phosphorylation of Syk and its association with FcR  $\gamma$ -chain, Src, and Lyn are dependent on Src kinase activity.** (A) After incubation with 0.25% DMSO as the vehicle control or 10  $\mu$ M PP1 for 5 minutes at 37°C, human platelets were stimulated with 6  $\mu$ g/mL botrocetin plus 10  $\mu$ g/mL vWF for the indicated time periods. The lysates were immunoprecipitated with anti-Syk MoAb. Precipitated proteins were then separated by SDS-PAGE and immunoblotted with 4G10 plus PY20 and anti-Syk MoAb. (B) Precipitated proteins with anti-Syk MoAb obtained in A were separated by SDS-PAGE and analyzed by immunoblotting with anti-FcR  $\gamma$ -chain, anti-Src, anti-Lyn, and anti-Fyn Abs as indicated in the left of each panel. (C) After pretreatment with 0.25% DMSO (control), 10  $\mu$ M PP1 or 20  $\mu$ g/mL jararaca-GPIIb-BP, human platelets were stimulated with vWF plus botrocetin. The lysates were precipitated with GST-Syk-SH2, and the precipitated proteins were separated by SDS-PAGE and analyzed by immunoblotting with anti-FcR  $\gamma$ -chain, anti-Src, anti-Lyn, anti-Fyn, and anti-Syk Abs. The data are representative of 3 experiments. The arrows represent the band derived from the heavy chains of IgG. pY indicates phosphorytyrosine.

required for tyrosine phosphorylation of Syk, we performed similar experiments with FcR  $\gamma$ -chain knockout mice. As shown in Figure 3B (i and ii), tyrosine phosphorylation of Syk was reduced by approximately 60% in the FcR  $\gamma$ -chain-deficient platelets. Although FcR  $\gamma$ -chain plays an important role in Syk activation, there may be an additional signal transduction pathway(s) for Syk activation other than FcR  $\gamma$ -chain in GPIIb-mediated platelet activation. Collectively, these findings suggest that Src and Lyn first bind to FcR  $\gamma$ -chain or an FcR  $\gamma$ -chain-associated protein and tyrosine-phosphorylate FcR  $\gamma$ -chain and that Syk subsequently joins in this complex.



**Figure 3. FcR  $\gamma$ -chain associated with Src and Lyn is required for tyrosine phosphorylation of Syk.** (A) Samples were prepared as described in the legend for Figure 2C. The precipitates with GST-Syk-SH2 from human platelet lysates were eluted with Glutathione Elution Buffer (10 mM glutathione in 50 mM Tris/HCl, pH 8.0), and the resultant eluates were subjected to re-immunoprecipitation with anti-Src and anti-Lyn MoAbs, respectively. Precipitated proteins were separated by SDS-PAGE and were analyzed by immunoblotting with anti-FcR  $\gamma$ -chain (i and ii), anti-Src (i), and anti-Lyn (ii) Abs. (B) Washed platelets prepared from wild-type C57BL/6 and FcR  $\gamma$ -chain-deficient mice were stimulated with 6  $\mu$ g/mL botrocetin plus 10  $\mu$ g/mL vWF for 3 minutes. Syk was immunoprecipitated from the lysates, and precipitates were separated by SDS-PAGE and analyzed with 4G10 plus PY20 and anti-Syk immunoblotting. pY indicates phosphorylated tyrosine. (Bii) Level of Syk tyrosine phosphorylation shown in Bi was quantified by densitometry. Columns and error bars represent means  $\pm$  SEM (n = 3).

#### Association of FcR $\gamma$ -chain with GPIb in platelets

FcR  $\gamma$ -chain, which is tyrosine-phosphorylated on GPVI stimulation, binds constitutively to GPVI as a coreceptor.<sup>18</sup> Because tyrosine phosphorylation of FcR  $\gamma$ -chain occurs early in GPIb-mediated platelet activation, it is possible that FcR  $\gamma$ -chain also locates proximal to GPIb. To determine whether there was a constitutive association between FcR  $\gamma$ -chain and GPIb, the GPIb immunoprecipitates obtained from Triton X-100 lysates were immunoblotted with anti-FcR  $\gamma$ -chain antibody. As shown in Figure 4, there was little, if any, FcR  $\gamma$ -chain associated with GPIb in Triton X-100 lysates. When a weaker detergent, Brij 35, was used instead of Triton X-100, FcR  $\gamma$ -chain association with GPIb was clearly detected, suggesting that FcR  $\gamma$ -chain is loosely linked with GPIb. In contrast, integrin  $\beta$ 1, another integrate transmembrane protein, could not be recovered in any lysates (Figure 4). There was no significant change in the level of FcR  $\gamma$ -chain associated with GPIb, irrespective of stimulation. As described above, tyrosine phosphorylated FcR  $\gamma$ -chain is distinguished from the nonphosphorylated form by its mobility shift. However, no band shift was detectable in GPIb-associated FcR  $\gamma$ -chain. Although our findings hitherto suggest that Src and/or Lyn tyrosine-

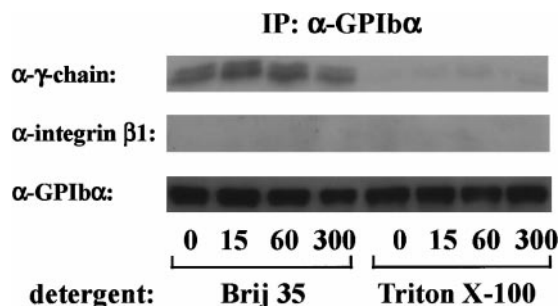
phosphorylates FcR  $\gamma$ -chain with subsequent binding of Syk, we were unable to detect Src/Lyn or Syk in GPIb immunoprecipitates. This discrepancy needs to be addressed in the future.

#### Tyrosine phosphorylation of PLC $\gamma$ 2 by botrocetin plus vWF is sensitive to PP1 pretreatment and independent of integrin $\alpha$ IIB $\beta$ 3 signaling

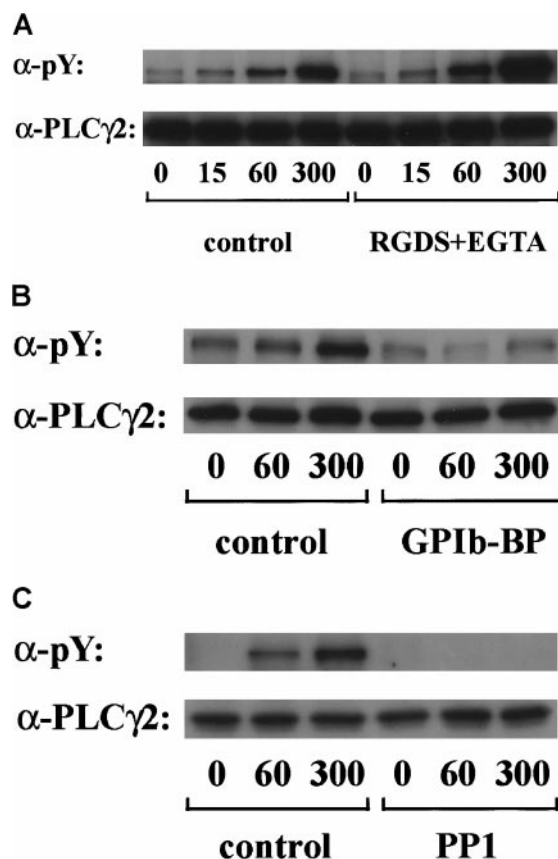
PLC $\gamma$ 2 is the major PLC $\gamma$  isoform expressed in platelets<sup>33</sup> and is the only type reported to undergo tyrosine phosphorylation on stimulation.<sup>34</sup> Tyrosine phosphorylation of PLC $\gamma$ 2 has been recently reported to occur in response to alboaggregin A, which presumably interacts with GPIb.<sup>6</sup> We confirmed in this study that stimulation of platelets by vWF plus botrocetin caused marked tyrosine phosphorylation of PLC $\gamma$ 2 in a time-dependent manner. Densitometric analysis revealed that the level of PLC $\gamma$ 2 tyrosine phosphorylation increased 2-fold at 15 seconds and reached a maximum level at 300 seconds (Figure 5A and data not shown). As shown in Figure 5B, tyrosine phosphorylation of PLC $\gamma$ 2 was markedly inhibited by jararaca GPIb-BP pretreatment. Moreover, the pretreatment of platelets with RGDS and EGTA did not suppress the level of PLC $\gamma$ 2 tyrosine phosphorylation (Figure 5A). These findings suggest that tyrosine phosphorylation of PLC $\gamma$ 2 induced by vWF plus botrocetin is specifically mediated by GPIb and is not dependent on integrin  $\alpha$ IIB $\beta$ 3 signaling. Tyrosine phosphorylation of PLC $\gamma$ 2 induced by vWF plus botrocetin was completely inhibited by PP1 (Figure 5C), suggesting the contribution of Src family kinases.

#### vWF plus botrocetin stimulates tyrosine phosphorylation of LAT

LAT is a 36- to 38-kd transmembrane protein that is found in glycolipid-enriched membrane domains.<sup>35</sup> Recent studies have shown that LAT plays an essential role in regulation of PLC $\gamma$ 2 following stimulation of GPVI in platelets.<sup>36</sup> Hence, we asked whether LAT is also involved in the GPIb-mediated signal transduction pathway. As shown in Figure 6A, stimulation with vWF plus botrocetin induced tyrosine phosphorylation of LAT in a time-dependent manner. Densitometric analysis revealed that tyrosine phosphorylation of LAT increased rapidly by 7-fold as early as 15 seconds after stimulation. Because pretreatment with RGDS and EGTA failed to inhibit phosphorylation of LAT (Figure 6A), it is suggested that integrin  $\alpha$ IIB $\beta$ 3 signaling is not involved. The potent



**Figure 4. FcR  $\gamma$ -chain is associated with GPIb.** After stimulation with 6  $\mu$ g/mL botrocetin plus 10  $\mu$ g/mL vWF for the indicated time periods, human platelets were solubilized with an equal volume of lysis buffer containing 2% Brij 35 (vol/vol) or 2% Triton X-100 (vol/vol). GPIb was immunoprecipitated with WGA3, an anti-GPIb MoAb. Precipitated proteins were separated by SDS-PAGE and were analyzed by anti-FcR  $\gamma$ -chain, anti-integrin  $\beta$ 1, and anti-GPIb immunoblotting, respectively. The data are representative of 4 experiments. The numbers below the panels show time in seconds.

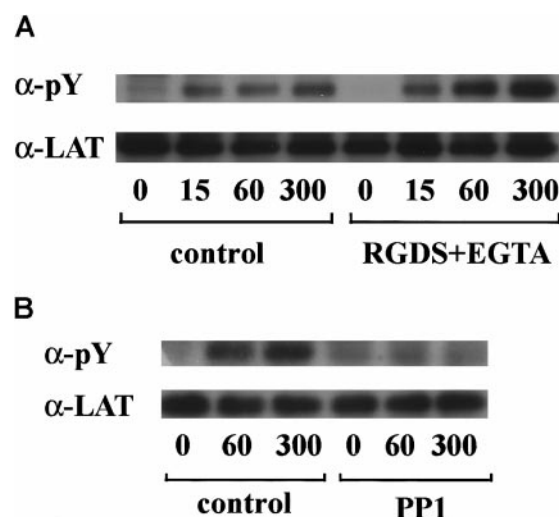


**Figure 5. GPIIb-vWF interaction induces tyrosine phosphorylation of PLC $\gamma$ 2, independently of integrin  $\alpha$ IIb $\beta$ 3 signaling.** Human platelets were preincubated with a vehicle solution as the control (A-C, left panels), 1 mM RGDS peptide plus 1 mM EGTA (A, right panel), 20  $\mu$ g/mL jararaca GPIIb-BP (B, right panel), or 10  $\mu$ M PP1 (C, right panel) for 5 minutes and then stimulated with 6  $\mu$ g/mL botrocetin plus 10  $\mu$ g/mL vWF for the indicated time periods. After cells were solubilized, PLC $\gamma$ 2 was isolated by immunoprecipitation with the corresponding Ab. Precipitated proteins were then separated by SDS-PAGE and analyzed by immunoblotting with 4G10 plus PY20. The membranes were reprobed by immunoblotting with anti-PLC $\gamma$ 2. The data are representative of 3 experiments. The numbers below the panels show time in seconds. pY indicates phosphotyrosine.

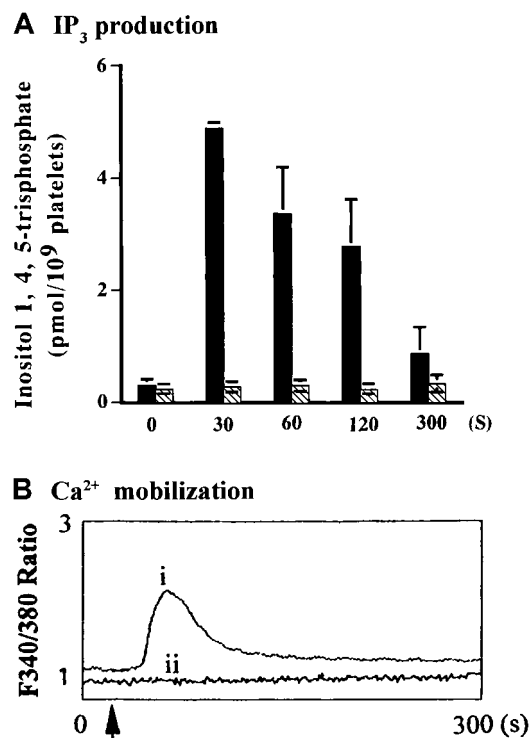
inhibition of LAT tyrosine phosphorylation by PP1 suggests that this is also downstream of Src family kinase activation (Figure 6B).

#### Stimulation with vWF plus botrocetin fails to induce IP<sub>3</sub> production and calcium release

In platelets, stimulation with collagen or Fc $\gamma$ RIIA cross-linking induces activation of PLC $\gamma$ 2, which leads to IP<sub>3</sub> production and intracellular calcium mobilization.<sup>37,38</sup> Because vWF plus botrocetin also stimulates tyrosine phosphorylation of PLC $\gamma$ 2, we then asked whether PLC $\gamma$ 2 is activated in GPIIb-mediated platelet activation. Activation of PLC $\gamma$ 2 can be monitored by measurement of IP<sub>3</sub> production and intracellular Ca<sup>++</sup> release. As shown in Figure 7A, although collagen induced a rapid and prominent increase in IP<sub>3</sub>, platelet stimulation with vWF plus botrocetin failed to induce IP<sub>3</sub> production. In addition, vWF plus botrocetin did not induce intracellular calcium release, whereas collagen was able to induce calcium release as early as 20 seconds after stimulation (Figure 7B), which is in good correlation with the time course of IP<sub>3</sub> production. These findings suggest that PLC $\gamma$ 2 is not activated in vWF/botrocetin GPIIb-mediated platelet activation *in vitro* despite its tyrosine phosphorylation.



**Figure 6. LAT is tyrosine-phosphorylated in GPIIb-mediated platelet activation.** Human platelets were preincubated with a vehicle solution as the control (A and B, left panels), 1 mM RGDS peptide plus 1 mM EGTA (A, right panel), or 10  $\mu$ M PP1 (B, right panel) for 5 minutes and then activated with 6  $\mu$ g/mL botrocetin plus 10  $\mu$ g/mL vWF for the indicated time periods. After cell lysis, LAT was isolated by immunoprecipitation with the corresponding Ab. Precipitated proteins were then separated by SDS-PAGE and analyzed by immunoblotting with 4G10 plus PY20. The membranes were reprobed for anti-LAT by immunoblotting. The data are representative of 3 experiments. The numbers below the panels show time in seconds. pY indicates phosphotyrosine.



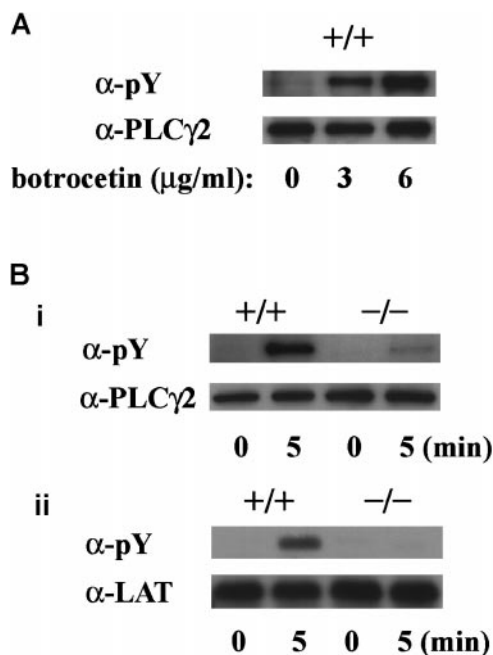
**Figure 7. IP<sub>3</sub> production and calcium release are not observed in platelets stimulated with vWF plus botrocetin.** (A) IP<sub>3</sub>: after human platelets were stimulated with 75  $\mu$ g/mL collagen (■) or 6  $\mu$ g/mL botrocetin plus 10  $\mu$ g/mL vWF (▨) for the indicated time periods, the production of IP<sub>3</sub> was measured as described in "Materials and Methods." The data are expressed as means  $\pm$  SEM of 3 separate experiments. (B) Ca<sup>++</sup>: stimulation of human platelets with 75  $\mu$ g/mL collagen (i) or 6  $\mu$ g/mL botrocetin plus 10  $\mu$ g/mL vWF (ii) was initiated at the time indicated by the arrow. The [Ca<sup>++</sup>]<sub>i</sub> elevation in the absence of extracellular calcium was monitored as changes in fura2 fluorescence for 300 seconds. The ordinate represents the ratio of fura2 fluorescence. The results are representative of 3 experiments.

### GPIIb-mediated tyrosine phosphorylation of PLC $\gamma$ 2 and LAT is absent in murine platelets lacking FcR $\gamma$ -chain

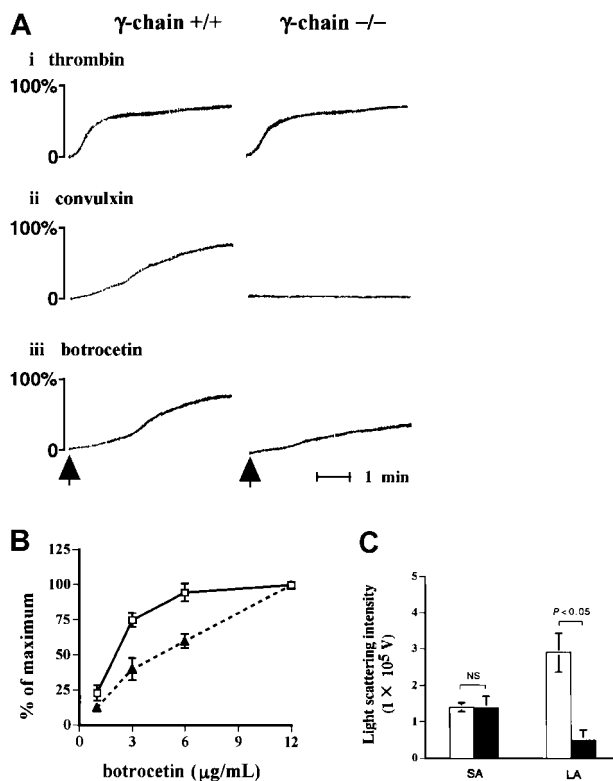
To determine whether tyrosine phosphorylation of PLC $\gamma$ 2 and LAT is dependent on FcR  $\gamma$ -chain, we performed experiments with platelets obtained from FcR  $\gamma$ -chain knockout mice. The level of tyrosine phosphorylation of PLC $\gamma$ 2 by vWF plus botrocetin stimulation was dependent on the botrocetin dose in platelets of wild-type C57BL/6 mice (Figure 8A). In platelets lacking FcR  $\gamma$ -chain, tyrosine phosphorylation of PLC $\gamma$ 2 on botrocetin plus vWF stimulation was reduced by approximately 80%, and tyrosine phosphorylation of LAT was virtually absent (Figure 8B<sub>i,ii</sub>).

### Impaired large aggregate formation of FcR $\gamma$ -chain-deficient platelets

Because the findings hitherto point to an important role of FcR  $\gamma$ -chain in the signal transduction pathway in GPIIb-mediated platelet activation, we evaluated the functional role of FcR  $\gamma$ -chain in GPIIb signaling. As detected by the light-transmission method (Figure 9A), platelets lacking FcR  $\gamma$ -chain failed to respond to convulxin, but they aggregated normally on thrombin stimulation as was the case with the platelets from wild-type C57BL/6 mice. Platelet aggregation in response to vWF plus botrocetin with FcR  $\gamma$ -chain-deficient mice was partially impaired compared with that of wild-type mice (Figure 9A,B). Platelet aggregation was further evaluated with a particle-counting method using light scattering. This method is unique in that it distinguishes the size of aggregates, based on the intensity of light scattering in PRP. Although the formation of small aggregates with FcR  $\gamma$ -chain-deficient mice



**Figure 8.** GPIIb-stimulated tyrosine phosphorylation of LAT and PLC $\gamma$ 2 are absent in platelets lacking FcR  $\gamma$ -chain. (A) Washed platelets from wild-type C57BL/6 mice were stimulated with 10  $\mu$ g/mL vWF plus 0, 3, or 6  $\mu$ g/mL botrocetin for 5 minutes. After lysis, platelets were immunoprecipitated with anti-PLC $\gamma$ 2 Ab. (B) Washed platelets from wild-type C57BL/6 and FcR  $\gamma$ -chain-deficient mice were stimulated with 6  $\mu$ g/mL botrocetin plus 10  $\mu$ g/mL vWF for the indicated time periods. After lysis, PLC $\gamma$ 2 (i) and LAT (ii) were immunoprecipitated with the corresponding Ab, respectively. The precipitated proteins in A and B were separated by SDS-PAGE and were analyzed by 4G10 plus PY20 immunoblotting. The loaded proteins were confirmed to be at comparable levels by immunoblotting with the corresponding Ab. The data are representative of 3 experiments. pY indicates phosphoryrosine.



**Figure 9.** Formation of large aggregates is suppressed in platelets from FcR  $\gamma$ -chain-deficient mice. (A) Platelets from wild-type or FcR  $\gamma$ -chain-deficient mice were challenged with 1 U/mL thrombin (i), 10 ng/mL convulxin (ii), or 6  $\mu$ g/mL botrocetin (iii), respectively. In the case of thrombin, washed platelets were used instead of platelet-rich plasma (PRP). The aggregation was evaluated for 5 minutes by the light transmission method. The arrows show the time points for the addition of the reagents. (B) PRPs from wild-type (□) or FcR  $\gamma$ -chain-deficient mice (▲) were stimulated with increasing amounts of botrocetin for 5 minutes. The maximal light transmission of platelet aggregation induced by 12  $\mu$ g/mL was set as 100%. The ordinate represents the percentage maximal magnitude of platelet aggregation induced by various concentrations of botrocetin. The data are expressed as means  $\pm$  SEM (n = 3). (C) The formation of small and large aggregates was assessed by a light-scattering method, as described in "Materials and methods." □, platelet aggregate formation from wild-type mice (n = 7); ■, FcR  $\gamma$ -chain-deficient mice (n = 5). The data are expressed as means  $\pm$  SEM. NS indicates not significant; SA, small aggregates; LA, large aggregates.

was detected at a level comparable to that of the control, the formation of large aggregates was markedly reduced in FcR  $\gamma$ -chain-deficient platelets (Figure 9C).

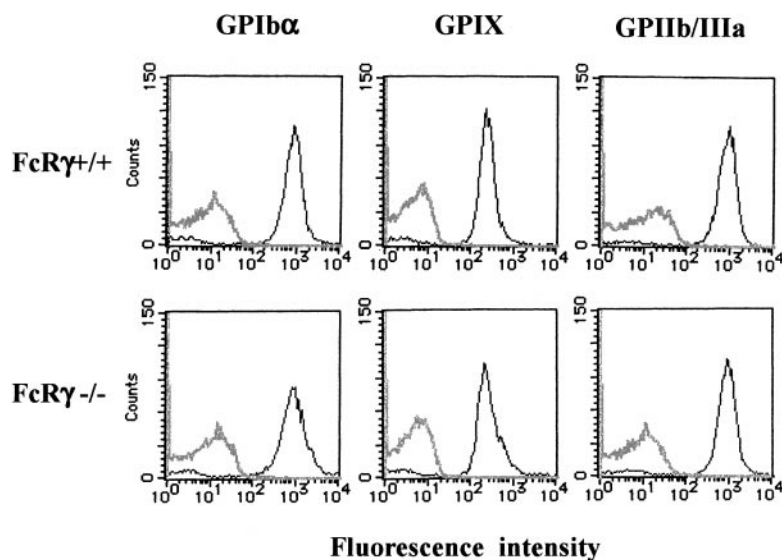
### GPIIb expression in wild-type and FcR $\gamma$ -chain-deficient platelets

Because FcR  $\gamma$ -chain is coexpressed with many receptors, we then asked whether it is also required for the expression of GPIIb-IX complex. As shown in Figure 10, flow cytometric studies indicated that the expression of GPIIb, IX, or GPIIb/IIIa in FcR  $\gamma$ -chain-deficient platelets was comparable to those of the wild-type.

## Discussion

The FcR  $\gamma$ -chain constitutes an integral part of several Fc receptors and is coexpressed with GPVI, forming a platelet collagen receptor.<sup>18</sup> On stimulation of this complex, Lyn and Fyn, Src family kinases, are activated and phosphorylate the ITAM of FcR  $\gamma$ -chain.<sup>29,30</sup> Subsequently, Syk binds to the tyrosine-phosphorylated FcR  $\gamma$ -chain and sends downstream signals, leading to PLC $\gamma$ 2

**Figure 10. Comparable platelet GPIb-IX expression between FcR  $\gamma$ -chain-deficient and wild-type mice.** Platelets from wild-type (upper panels) or FcR  $\gamma$ -chain-deficient (lower panels) mice were stained with phycoerythrin-labeled p0p4 ( $\alpha$ -mouse GPIb $\alpha$ ), fluorescein isothiocyanate-labeled p0p6 ( $\alpha$ -mouse GPIX), or phycoerythrin-labeled JON1 ( $\alpha$ -mouse GPIIb/IIIa), respectively. The fluorescence was analyzed by flow cytometry (solid lines). Gray lines represent the control, the fluorescence of rat  $\alpha$ -mouse Ab. The results shown are representative of 4 experiments.



activation.<sup>20</sup> Recently, Falati et al<sup>6</sup> reported that the GPIIb-mediated activation signal, elicited by alboaggregin A, induces tyrosine phosphorylation of FcR  $\gamma$ -chain, a complex formation between Lyn, Fyn, FcR  $\gamma$ -chain, and Syk, as well as activation of PLC $\gamma$ 2 with resultant Ca<sup>++</sup> release. Their findings imply that the GPIIb-mediated signal transduction pathway has several features in common with that of the GPVI collagen receptor. In this report, we sought to define changes in FcR  $\gamma$ -chain-related signaling molecules in response to GPIIb-vWF interaction.

By using GST-Syk-SH2, we confirmed that vWF plus botrocetin specifically stimulates tyrosine phosphorylation of FcR  $\gamma$ -chain, an event completely blocked by jararaca GPIIb-BP, a specific GPIIb inhibitor. Tyrosine phosphorylation of FcR  $\gamma$ -chain was also independent of integrin  $\alpha$ IIB $\beta$ 3. Our findings confirm those of Falati et al<sup>6</sup> and suggest that the activation signal mediated by GPIIb induces FcR  $\gamma$ -chain tyrosine phosphorylation. Furthermore, we showed that Src/Lyn and FcR  $\gamma$ -chain associate with Syk by its tandem SH2 domains, suggesting that a complex of Src/Lyn, FcR  $\gamma$ -chain, and Syk is formed *in vivo* on GPIIb stimulation. This complex formation is specifically mediated by GPIIb, as jararaca GPIIb-BP significantly abrogated its formation. In the present study, we also found that PP1 significantly inhibits the association of Src/Lyn, FcR  $\gamma$ -chain with Syk, as well as Syk phosphorylation. This finding suggests that the complex formation is dependent of Src kinase activity and that Syk is downstream of activated Src kinase(s). However, the incomplete inhibition by PP1 implies that there may be an additional mechanism of the complex formation, distinct for Src kinase activation. By re-immunoprecipitation experiments, we demonstrated that Src and Lyn either bind directly to FcR  $\gamma$ -chain or to an FcR  $\gamma$ -chain-associated protein. On the basis of these findings, it is most likely that Src/Lyn first phosphorylates the tyrosine residues of FcR  $\gamma$ -chain, which then results in Syk association via its tandem SH2 domains, thereby inducing autophosphorylation of Syk. However, our findings are not in accord with the previous report of Falati et al<sup>6</sup> who showed that Lyn, Fyn, FcR  $\gamma$ -chain, and Syk form a complex in alboaggregin-A-stimulated platelets. We could not detect the presence of Fyn in the complex, whereas they apparently could not detect Src. Although an anti-GPIIb MoAb blocked alboaggregin A-induced platelet aggregation, those researchers did not show any effects of specific GPIIb blockade on complex formation.<sup>6</sup> It, therefore, remains possible that alboaggregin A interacts with membrane

molecules other than GPIIb, inducing the association between FcR  $\gamma$ -chain and Lyn/Fyn. Indeed, the mode of complex formation reported by them is quite reminiscent of that mediated by GPVI, the collagen receptor.<sup>11</sup>

In the present study, we show that phosphorylation of Syk is significantly suppressed in platelets of FcR  $\gamma$ -chain-deficient mice, suggesting that FcR  $\gamma$ -chain is located upstream of Syk and is required for phosphorylation of Syk in GPIIb-mediated platelet activation, similar to that of GPVI. However, the incomplete suppression of Syk tyrosine phosphorylation (approximately 60%) in the absence of FcR  $\gamma$ -chain suggests the existence of an FcR  $\gamma$ -chain-independent pathway for Syk activation. This situation may be compatible with the fact that mice lacking Syk suffer severe hemorrhage during fetal development,<sup>39,40</sup> whereas FcR  $\gamma$ -chain-deficient mice present no hemorrhagic diathesis. In contrast with incomplete suppression of Syk phosphorylation in FcR  $\gamma$ -chain-deficient platelets, tyrosine phosphorylation of LAT, known as a substrate of Syk, is completely abrogated, suggesting that LAT phosphorylation is more strictly dependent on FcR  $\gamma$ -chain. It is, therefore, possible that FcR  $\gamma$ -chain-bound Syk is responsible for tyrosine phosphorylation of LAT, whereas FcR  $\gamma$ -chain-independent Syk activation exists in GPIIb-mediated platelet activation.

The precise mechanism by which the GPIIb-vWF interaction activates Src/Lyn with resultant FcR  $\gamma$ -chain tyrosine phosphorylation is still elusive. In a previous report, we found that GPIIb-vWF interaction induces an increase in GPIIb-associated tyrosine kinase activity.<sup>7</sup> However, we were unable to identify the kinase responsible for this increase at that time. In this study, we found that FcR  $\gamma$ -chain coprecipitated with GPIIb in Brij 35 lysates but not in Triton X-100 lysates. Thus, FcR  $\gamma$ -chain appears to be constitutively but loosely associated with GPIIb. However, we could neither detect tyrosine-phosphorylated FcR  $\gamma$ -chain associated with GPIIb nor Src family kinases associated with GPIIb. It is possible that a portion of FcR  $\gamma$ -chain undergoes tyrosine phosphorylation and that the phosphorylated form rapidly dissociates from GPIIb. In collagen-stimulated platelets, tyrosine phosphorylated FcR  $\gamma$ -chain does not detectably coprecipitate with GPVI, suggesting that FcR  $\gamma$ -chain after being phosphorylated dissociates from GPVI.<sup>18</sup> Further investigation is needed to address this issue.

Falati et al<sup>6</sup> reported that alboaggregin A, a putative GPIIb activator, induces PLC $\gamma$ 2 tyrosine phosphorylation in platelets. In the present study, we have confirmed that GPIIb stimulation by



vWF plus botrocetin is able to induce tyrosine phosphorylation of PLC $\gamma$ 2. In hematopoietic cells, including platelets, LAT, a transmembrane protein, and PLC $\gamma$ 2 have been shown to be downstream of Syk and FcR  $\gamma$ -chain.<sup>11,35,36</sup> Stimulation of platelets with collagen induces LAT tyrosine phosphorylation,<sup>36</sup> and tyrosine-phosphorylated LAT serves as a docking site to recruit PI 3-K to the membrane.<sup>41</sup> These events are required for tyrosine phosphorylation and activation of PLC $\gamma$ 2.<sup>36</sup> In the present study, we found that LAT is also tyrosine-phosphorylated by vWF plus botrocetin. Because PP1 completely blocks tyrosine phosphorylation of LAT and PLC $\gamma$ 2, it is suggested that these events are also catalyzed by a Src family kinase, as with FcR  $\gamma$ -chain and Syk. In platelets lacking FcR  $\gamma$ -chain, LAT and PLC $\gamma$ 2 tyrosine phosphorylation are significantly suppressed, indicating that FcR  $\gamma$ -chain plays a critical role in these signaling events mediated by GPIb-vWF interaction. Whereas tyrosine phosphorylation of LAT was entirely absent in FcR  $\gamma$ -chain-deficient platelets, PLC $\gamma$ 2 tyrosine phosphorylation still partially occurred on GPIb stimulation, suggesting the existence of an alternative pathway for PLC $\gamma$ 2 tyrosine phosphorylation. A previous report suggested that PLC $\gamma$ 2 becomes activated and induces calcium release in platelets activated by alboaggregin A and that these phenomena are mediated by GPIb.<sup>6</sup> However, we found in this study that tyrosine phosphorylation of PLC $\gamma$ 2 induced by the GPIb-vWF interaction is not sufficient for its activation, because there was no IP<sub>3</sub> production and subsequent calcium release. Although platelet aggregation induced by alboaggregin A was blocked by an anti-GPIb MoAb, there was no corresponding blocking experiment on Ca<sup>++</sup> release.<sup>6</sup> Thus, it cannot be entirely ruled out that alboaggregin A induces Ca<sup>++</sup> release by binding to some membrane molecule(s) other than GPIb. There has been an accumulating body of evidence to suggest that, in addition to its tyrosine phosphorylation, activation of PLC $\gamma$ 2 requires additional factors, such as PLC $\gamma$ 2 translocation to the membrane,<sup>42</sup> the production of PI(3,4,5)P<sub>3</sub> and phosphatidic acid, or the presence of AHNAK (a Hebrew term for a giant, presumably required for PLC $\gamma$  activation in presence of arachidonic acid).<sup>43-46</sup> Further studies are necessary to understand what is specifically lacking to allow PLC $\gamma$ 2 activation in GPIb stimulation.

It is, therefore, reasonable to ask whether the FcR  $\gamma$ -chain-related signaling molecules, Syk, LAT, and PLC $\gamma$ 2, play any role at all in GPIb-mediated platelet activation. There is an increasing body of evidence indicating that tyrosine-phosphorylated FcR

$\gamma$ -chain and its downstream signal, LAT, play an important role in cell activation.<sup>36,47</sup> They facilitate the membrane recruitment of multiple signaling molecules, including Grb2, Grb2-related adaptor downstream of Shc, the p85 subunit of PI 3-K, Vav, SLP-76, and others.<sup>48-50</sup> Of particular interest is that LAT can also serve as a docking site to recruit PI 3-K to the membrane,<sup>41</sup> the function of which appears to be important in GPIb-mediated platelet activation.<sup>8,51</sup> This issue needs to be addressed in the future. We then sought to determine whether there is any functional impairment in platelets deficient in FcR  $\gamma$ -chain. In response to a maximal amount of botrocetin, 12  $\mu$ g/mL, there was no difference in the magnitude of aggregation between control and FcR  $\gamma$ -chain-deficient platelets. However, at lower doses of botrocetin, there was a slight, but distinct, difference in the magnitude of aggregation. By using a light-scattering method, we found that the formation of large aggregates is selectively impaired in FcR  $\gamma$ -chain-deficient mice. The factors that determine the size of platelet aggregates remain elusive. However, it is conceivable that FcR  $\gamma$ -chain-related signals consolidate GPIb-mediated aggregate formation. Because flow cytometric analysis demonstrated that the expression of GPIb-IX complex is not distinguishable between the wild-type and FcR  $\gamma$ -chain-deficient platelets, it is most likely that there is certain functional impairment with FcR  $\gamma$ -chain-deficient platelets.

In conclusion, the present study has demonstrated that FcR  $\gamma$ -chain, which is constitutively associated with GPIb, is critical for the signal transduction of activated Src kinases and downstream tyrosine phosphorylation of Syk, LAT, and PLC $\gamma$ 2 in GPIb-stimulated platelets. In platelets lacking FcR  $\gamma$ -chain, large aggregate formation is significantly impaired, suggesting that FcR  $\gamma$ -chain is required for the full activation of platelets mediated by GPIb.

## Acknowledgments

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## References

- Ruggeri ZM. von Willebrand factor. *J Clin Invest*. 1997;99:559-564.
- Clemetson KJ. Platelet glycoprotein Ib-IX-V complex. *Thromb Haemost*. 1997;78:266-270.
- Francesconi M, Casonato A, Pagan S, et al. Inhibitory effect of prostacyclin and nitroprusside on type IIB von Willebrand factor-promoted platelet activation. *Thromb Haemost*. 1996;76:469-474.
- Razdan K, Hellums JD, Kroll MH. Shear-stress-induced von Willebrand factor binding to platelets causes the activation of tyrosine kinase(s). *Biochem J*. 1994;302:681-686.
- Ozaki Y, Satoh K, Yatomi Y, Miura S, Fujimura Y, Kume S. Protein tyrosine phosphorylation in human platelets induced by interaction between glycoprotein Ib and von Willebrand factor. *Biochim Biophys Acta*. 1995;1243:482-488.
- Falati S, Edmead CE, Poole AW. Glycoprotein Ib-V-IX, a receptor for von Willebrand factor, couples physically and functionally to the Fc Receptor  $\gamma$ -chain, Fyn, and Lyn to activate human platelets. *Blood*. 1999;94:1648-1656.
- Asazuma N, Ozaki Y, Satoh K, et al. Glycoprotein Ib-von Willebrand factor interactions activate tyrosine kinases in human platelets. *Blood*. 1997;90:4789-4798.
- Jackson SP, Schoenwaelder SM, Yuan Y, Rabinowitz I, Salem HH, Mitchell CA. Adhesion receptor activation of phosphatidylinositol 3-kinase: von Willebrand factor stimulates the cytoskeletal association and activation of phosphatidylinositol 3-kinase and pp60c-src in human platelets. *J Biol Chem*. 1994;269:27093-27099.
- Yanabu M, Ozaki Y, Nomura S, et al. Tyrosine phosphorylation and p72<sup>syk</sup> activation by an anti-GPIb monoclonal antibody. *Blood*. 1997;89:1590-1598.
- Satoh K, Asazuma N, Yatomi Y, et al. Activation of protein-tyrosine kinase pathways in human platelets stimulated with the A1 domain of von Willebrand factor. *Platelets*. 2000;11:171-176.
- Watson SP. Collagen receptor signaling in platelets and megakaryocytes. *Thromb Haemost*. 1999;82:365-376.
- Sullam PM, Hyun WC, Szollosi J, Dong JF, Foss WM, Lopez JA. Physical proximity and functional interplay of the glycoprotein Ib-IX-V complex and the Fc receptor Fc $\gamma$ RIIA on the platelet plasma membrane. *J Biol Chem*. 1998;273:5331-5336.
- Torti M, Bertoni A, Canobbio I, et al. Rap1B and Rap2B translocation to the cytoskeleton by von Willebrand factor involves FcII receptor-mediated protein tyrosine phosphorylation. *J Biol Chem*. 1999;274:13690-13697.
- Paolini R, Renard V, Vivier E, et al. Different roles for the Fc epsilon RI gamma chain as a function of the receptor context. *J Exp Med*. 1995;181:247-255.
- Duchemin AM, Ernst LK, Anderson CL. Clustering of the high affinity Fc receptor for immunoglobulin G (Fc gamma RI) results in phosphorylation of its associated gamma-chain. *J Biol Chem*. 1994;269:12111-12117.
- Greenberg S, Chang P, Silverstein SC. Tyrosine phosphorylation of the gamma subunit of Fc gamma receptors, p72<sup>syk</sup>, and paxillin during Fc receptor-mediated phagocytosis in macrophages. *J Biol Chem*. 1994;269:3897-3902.
- Gibbins JM, Okuma M, Farndale R, Barnes M,

- Watson SP. Glycoprotein VI is the collagen receptor in platelets which underlies tyrosine phosphorylation of the Fc receptor gamma-chain. *FEBS Lett.* 1997;413:255-259.
18. Tsuji M, Ezumi Y, Arai M, Takayama H. A novel association of Fc receptor  $\gamma$ -chain with glycoprotein VI and their co-expression as a collagen receptor in human platelets. *J Biol Chem.* 1997; 272:23528-23531.
  19. Gibbins JM, Asselin J, Farndale R, Barnes M, Law CL, Watson SP. Tyrosine phosphorylation of the Fc receptor  $\gamma$ -chain in collagen-stimulated platelets. *J Biol Chem.* 1996;271:18095-18099.
  20. Poole JM, Gibbins M, Turner MJ, et al. The Fc receptor  $\gamma$ -chain and the tyrosine kinase Syk are essential for activation of mouse platelets by collagen. *EMBO J.* 1997;16:2333-2341.
  21. Andrews RK, Booth WJ, Gorman JJ, Castaldi PA, Berndt MC. Purification of botrocetin from *Bothrops jararaca* venom: analysis of the botrocetin-mediated interaction between von Willebrand factor and human platelet membrane glycoprotein Ib-IX complex. *Biochemistry.* 1989;28:8317-8326.
  22. Kawasaki T, Kaida T, Arnout J, Vermeylen J, Hoylaerts MF. A new animal model of thrombophilia confirms that high plasma factor VIII levels are thrombogenic. *Thromb Haemost.* 1999;81:306-311.
  23. Ware J, Russell S, Ruggeri ZM. Cloning of the murine platelet glycoprotein Ibalpha gene highlighting species-specific platelet adhesion. *Blood Cells Mol Dis.* 1997;23:292-301.
  24. Ozaki Y, Satoh K, Yatomi Y, Yamamoto T, Shirasawa Y, Kume S. Detection of platelet aggregates with a particle counting method using light scattering. *Anal Biochem.* 1994;218:284-294.
  25. Asazuma N, Yatomi Y, Ozaki Y, et al. Protein tyrosine phosphorylation and p72<sup>Syk</sup> activation in human platelets stimulated with collagen is dependent upon glycoprotein Ia/IIa and actin polymerization. *Thromb Haemost.* 1996;75:648-654.
  26. Kuwahara M, Sugimoto M, Tsuji S, Miyata S, Yoshioka A. Cytosolic calcium changes in a process of platelet adhesion and cohesion on a von Willebrand factor-coated surface under flow conditions. *Blood.* 1999;94:1149-1155.
  27. Titani K, Kumar S, Takio K, et al. Amino acid sequence of human von Willebrand factor. *Biochemistry.* 1986;25:3171-3184.
  28. Fujimura Y, Ikeda Y, Miura S, et al. Isolation and characterization of *jararaca* GPIb-BP, a snake venom antagonist specific to platelet glycoprotein Ib. *Thromb Haemost.* 1995;74:743-750.
  29. Briddon SJ, Watson SP. Evidence for the involvement of p59fyn and p53/56lyn in collagen receptor signaling in human platelets. *Biochem J.* 1999;338:203-209.
  30. Ezumi Y, Shindoh K, Tsuji M, Takayama H. Physical and functional association of the Src family kinases Fyn and Lyn with the collagen receptor glycoprotein VI-Fc receptor  $\gamma$ -chain complex on human platelets. *J Exp Med.* 1998;188:267-276.
  31. Hanke JH, Gardner JP, Dow RL, et al. Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. *J Biol Chem.* 1996;271: 695-701.
  32. Amoui M, Draber P, Draberova L. Src family selective tyrosine kinase inhibitor, PP1, inhibits both Fc epsilon RI- and Thy-1-mediated activation of rat basophilic leukemia cells. *Eur J Immunol.* 1997;27:1881-1886.
  33. Lee SB, Rao AK, Lee KH, Yang X, Bae YS, Rhee SG. Decreased expression of phospholipase C-beta 2 isozyme in human platelets with impaired function. *Blood.* 1996;88:1684-1691.
  34. Blake RA, Schieven GL, Watson SP. Collagen stimulates tyrosine phosphorylation of phospholipase C- $\gamma$ 2 but not phospholipase C- $\gamma$ 1 in human platelets. *FEBS Lett.* 1994;353:212-216.
  35. Zhang W, Triple RP, Samelson LE. LAT palmitoylation: its essential role in membrane microdomain targeting and tyrosine phosphorylation during T cell activation. *Immunity.* 1998;9:239-246.
  36. Pasquet JM, Gross B, Quek L, et al. LAT is required for tyrosine phosphorylation of PLC $\gamma$ 2 and platelet activation by the collagen receptor GPVI. *Mol Cell Biol.* 1999;19:8326-8334.
  37. Daniel JL, Dangelmaier C, Smith JB. Evidence for a role for tyrosine phosphorylation of phospholipase C gamma2 in collagen-induced platelet cytosolic calcium mobilization. *Biochem J.* 1994; 302:617-622.
  38. Gratacap MP, Payrastra B, Viala C, Mauco G, Plantavid M, Chap H. Phosphatidylinositol 3,4,5-trisphosphate-dependent stimulation of phospholipase C- $\gamma$ 2 is an early key event in Fc $\gamma$ RIIA-mediated activation of human platelets. *J Bio Chem.* 1998;273:24314-24321.
  39. Turner M, Mee PJ, Costello PS, et al. Perinatal lethality and blocked B-cell development in mice lacking the tyrosine kinase Syk. *Nature.* 1995; 378:298-302.
  40. Cheng AM, Rowley B, Pao W, Hayday A, Bolen JB, Pawson T. Syk tyrosine kinase required for mouse viability and B-cell development. *Nature.* 1995;378:303-306.
  41. Gibbins JM, Briddon S, Shutes A, et al. The p85 subunit phosphatidylinositol 3-kinase associates with the Fc receptor  $\gamma$ -chain and linker for activator of T cells in platelets stimulated by collagen and convulxin. *J Biol Chem.* 1998;273:34437-34443.
  42. Rittenhouse SE. Phosphoinositide 3-kinase activation and platelet function. *Blood.* 1996;88: 4401-4414.
  43. Sekiya F, Bae YS, Rhee SG. Regulation of phospholipase C isozymes: activation of phospholipase C- $\gamma$  in the absence of tyrosine phosphorylation. *Chem Phys Lipids.* 1999;98:3-11.
  44. Rhee SG, Bae YS. Regulation of phosphoinositide-specific phospholipase C isozymes. *J Biol Chem.* 1997;272:15045-15048.
  45. Sekiya F, Bae YS, Jhon DY, Hwang SC, Rhee SG. AHNAK, a protein that binds and activates phospholipase C- $\gamma$ 1 in the presence of arachidonic acid. *J Biol Chem.* 1999;274:13900-13907.
  46. Falasca M, Logan SK, Lehto VP, Baccante G, Lemmon MA, Schlessinger J. Activation of phospholipase C $\gamma$  by PI 3-kinase-induced PH domain-mediated membrane targeting. *EMBO J.* 1998; 17:414-422.
  47. Zhang W, Sommers CL, Burshtyn DN, et al. Essential role of LAT in T cell development. *Immunity.* 1999;10:323-332.
  48. Asazuma N, Wilde JI, Berlanga O, et al. Interaction of linker for activation of T cells with multiple adapter proteins in platelets activated by the glycoprotein VI-selective ligand, convulxin. *J Biol Chem.* 2000;275:33427-33434.
  49. Zhang W, Sloan-Lancaster J, Kitchen J, et al. LAT: the ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation. *Cell.* 1998;92:83-92.
  50. Lin J, Weiss A, Finco TS. Localization of LAT in glycolipid-enriched microdomains is required for T cell activation. *J Biol Chem.* 1999;274:28861-28864.
  51. Munday AD, Berndt MC, Mitchell CA. Phosphoinositide 3-kinase forms a complex with platelet membrane glycoprotein Ib-IX-V complex and 14-3-3 $\zeta$ . *Blood.* 2000;96:577-584.