Platelet glycoprotein (GP) VI is a so-far uncharacterized 62-kDa membrane protein, whose deficiency results in selective impairment in collagen-induced platelet aggregation. Our group previously reported a human polyclonal antibody (anti-p62 IgG) that induces activation of normal, but not of GPVI-deficient, platelets in an FC-independent manner. The F(ab')_2 fragments of this antibody (F(ab')_2-anti-p62) stimulated tyrosine phosphorylation of numerous proteins, which was not prevented even in the presence of cAMP-increasing agents such as prostacyclin. Pretreatment of platelets with the protein-tyrosine kinase (PTK) inhibitor tyrphostin A47 completely abolished F(ab')_2-anti-p62-induced platelet aggregation in parallel with dose-dependent inhibition of protein-tyrosine phosphorylation, indicating an essential requirement of PTK activity for generating GPVI-mediated signaling. We found that two cytosolic PTKs, c-Src and Syk, became rapidly activated in response to F(ab')_2-anti-p62 in a way insensitive to elevation of cAMP. In contrast, in the presence of prostacyclin, F(ab')_2-anti-p62 did not stimulate tyrosine phosphorylation of the focal adhesion kinase. cAMP-insensitive activation of c-Src and Syk was also observed in collagen-but not thrombin-stimulated platelets. Moreover, either F(ab')_2-anti-p62 or collagen stimulated cAMP-insensitive tyrosine phosphorylation of phospholipase C-γ2. These results indicate that the receptor-mediated activation of several PTKs in platelets is regulated through a cAMP-sensitive or -insensitive mechanism depending on the nature of each stimulus, and also suggest that GPVI engagement is coupled to cAMP-insensitive activation of c-Src and Syk accompanied by tyrosine phosphorylation of numerous substrates including phospholipase C-γ2 in a manner similar to collagen stimulation.

Investigations of platelet membrane glycoproteins have unveiled a great deal about how the interaction between cell surface protein molecules and their specific ligands harmoniously regulates diverse platelet functions, such as adhesion and aggregation. Although the structures and functions of several major glycoproteins on platelets have been steadily clarified, the molecular characterization of platelet glycoprotein (GP) VI, a 62-kDa membrane protein that was originally described in the pioneer studies by Phillips and Agin (1, 2), remains a great enigma. A breakthrough in the elucidation of this molecule was a clinical report on a platelet activating antibody (anti-p62 IgG) found in a patient with autoimmune thrombocytopenia whose platelets showed selective deficiency in collagen-induced platelet aggregation (3). This antibody immunoprecipitated a 62-kDa/57-kDa (reducing/nonreducing) membrane protein from normal platelets but did not significantly react with membrane proteins of the patient’s platelets. The 62-kDa protein recognized by this antibody was later identified as GPVI through the analysis of platelets from a patient with familial GPVI-deficiency (4), and there have since been two other reports on patients with GPVI-deficiency (5, 6). Platelets from these patients were not reactive with anti-p62 IgG and also showed defective responses only to collagen despite the normal expression of GPIa-IIa (integrin α₂β₃), a primary platelet adhesion receptor for collagen (7, 8). Although these clinical findings have suggested a potential role of GPVI in collagen-induced platelet activation (9, 10), very little is known about the mechanism for signaling through GPVI.

Like other platelet agonists, collagen stimulates an increase in intracellular Ca²⁺, phosphoinositide metabolism, activation of protein kinase C, and accumulation of phosphatidic acid (11–13). However, recent studies pointed out that collagen-induced signal transduction is unique compared to other stimuli; while elevation of cyclic AMP is thought to prevent many aspects of platelet activating signal transduction, it does not inhibit several features of collagen-induced signaling events. This cAMP-insensitive signaling stimulated by collagen includes intracellular Ca²⁺ mobilization, phosphatidic acid formation, and protein-tyrosine phosphorylation (14, 15). Among these, a key signaling event insensitive to cAMP appears to be protein-tyrosine phosphorylation (16), because the recent reports revealed that collagen stimulates tyrosine phosphorylation of phospholipase C (PLC)-γ2 which could lead to cytosolic Ca²⁺ mobilization and phosphatidic acid formation (17, 18).

Platelets have been known to possess a number of nonreceptor protein-tyrosine kinases (PTKs) including five Src family kinases (c-Src, Fyn, Yes, two variants of Lyn, and Hck) (19, 20), focal adhesion kinase (FAK) (21), and Syk (22, 23). Thrombin

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The abbreviations used are: GP, glycoprotein; PLC, phospholipase C; PTK, protein-tyrosine kinase; FAK, focal adhesion kinase; SH2, src homology 2; F(ab')_2-anti-p62, F(ab')_2 fragments of anti-p62 IgG; RGDS, Arg-Gly-Asp-Ser tetrapeptide; PGI₂, prostacyclin; mAb, monoclonal antibody; RIPA, radiolmunoimmuno precipitation assay; PBS, phosphate-buffered saline; G-protein, guanine nucleotide-binding protein; Me₂SO, dimethyl sulfoxide.
stimulation affects four of five Src family kinases. c-Src is enzymatically activated (24–26); c-Src and Fyn form a signaling complex with phosphatidylinositol 3-kinase (27; Fyn, Lyn, and Yes become associated with the GTPase-activating protein of p21ras (28). Although all of these events occur independently of platelet aggregation mediated by fibrinogen binding to GPIIb-IIIa (integrin αIIbβ3), redistribution of c-Src, Fyn, Lyn, and Yes into the Triton-insoluble “cytoskeleton” fraction takes place in an aggregation-dependent manner (26, 29). In addition, activation of FAK which does not belong to the Src family is believed to be brought about by fibrinogen binding or adherence to GPIIb-IIIa and to be involved in the relatively late phase of protein-tyrosine phosphorylation in activation of platelets (21, 30).

Another cytosolic PTK that is also implicated in platelet activation by a variety of platelet agonists is Syk, a member of the Syk/ZAP-70 family (23, 31). Unlike the Src family PTKs, Syk possesses two tandemly arranged SH2 (src homology 2) domains, which are considered essential for the association of Syk with its target molecules that contain a cytoplasmic consensus motif called a tyrosine-based activation motif (32). It has been reported that cross-linking of multiple glycoproteins by wheat germ agglutinin (21), ligation of GPIIb-IIIa with disintegrin or fibrinogen (33), and clustering of the low affinity Fc receptors (FcγRIIA) (34) lead to tyrosine phosphorylation and activation of Syk. In addition, antibody-mediated CD9 clustering has been shown to induce activation of Syk which becomes associated with c-Src (35).

With these backgrounds in mind, it was of great interest to us to clarify the nature of GPVI-mediated signaling with special reference to protein-tyrosine phosphorylation and activation of PTKs, since specific stimulation of GPVI can be achieved by use of the F(ab′)2 fragments of anti-p62 IgG (F(ab′)2-anti-p62). Surprisingly, stimulation of GPVI with F(ab′)2-anti-p62 was found to induce AMP-insensitive tyrosine phosphorylation of numerous substrates including PLC-γ2, as is the case with collagen stimulation. In this paper, we report the unique nature of F(ab′)2-anti-p62-induced activation of PTKs through GPVI and its comparison with collagen stimulation.

**EXPERIMENTAL PROCEDURES**

Reagents—Tyrosmin, A47 (3,4-dihydroxy-α-cyanothiannimide) and cheletrymine chloride (36) were purchased from LC Laboratories (Woburn, MA). Acetylsalicylic acid was from Nakarai Tesque Inc. (Kyoto, J. apan). Lyophilized pepsin, phosphocholine, creatine phosphokinase, sodium orthovanadate, Arg-Gly-Asp-Ser (RGDS) tetrapeptide, and histone (subgroup f2b, from calf thymus) were from Sigma. PGI2 (sodium salt) and a stable mimetic of thromboxane A2, STA2 (9,11-epithio-11,12-methano-thromboxaneA2), were kindly provided by Ono Pharmaceutical Co. (Osaka, Japan). Acid-soluble collagen was obtained from Horm-Chemie (Munich, Germany). All other reagents were obtained as previously reported (3, 37).

Preparation of anti-p62 IgG and F(ab′)2-anti-p62—Purification of anti-p62 IgG from serum of a patient with GPVI-deficiency was performed essentially as described previously (3). To prepare F(ab′)2-anti-p62, purified anti-p62 IgG was incubated with peptic (10 units of peptic/1 mg of IgG) in 50 mM citrate buffer, pH 3.5, for 20 at 37 °C. Fc fragments and undigested IgG were removed on protein A-Sepharose beads, and incubated for 2 h at 4 °C. The precipitated proteins were eluted in 50 μl of 1% SDS sample buffer and boiled for 5 min. For immunoblotting analysis, the precipitated proteins were eluted in 50 μl of 1% SDS sample buffer and boiled for 5 min. For the immunoprecipitation of the Src family kinases, the same procedures as described above were also performed by replacing the RIPA buffer with the 1% Triton X-100-containing buffer which was prepared by Clark and Brugge (26).

Fractionation of Platelets—Subcellular fractionation of platelets into the 10,000 × g pellet and into the 1,000 × g supernatant was performed as described previously (38). The Triton X-100-insoluble cytoskeleton fraction was resuspended in the lysis buffer containing 0.5 mM NaCl, 0.3 M sucrose, and 0.2% TX-100. The anti-Syk and anti-Src family immunoprecipitates were divided into two aliquots; one was subjected to in vitro kinase assays and the other to immunoblotting analysis. For immunoblotting analysis, the precipitated proteins were eluted in 50 μl of 1% SDS sample buffer and boiled for 5 min. For the immunoprecipitation of the Src family kinases, the same procedures as described above were also performed by replacing the RIPA buffer with the 1% Triton X-100-containing buffer which was prepared by Clark and Brugge (26).

**Immunoblotting—Whole platelet lysates, subcellular fractions, and immunoprecipitates from the same cell equivalents were electrophoresed on 8% SDS-polyacrylamide gels and transferred onto a nitrocellulose membrane (Trans-blot, Bio-Rad). The membranes were blocked overnight in 5% non-fat dry milk (Difco) or 5% bovine serum albumin (Intergen) in TBPS (PBS supplemented with 1% Tween 20), washed four times in TBPS, and incubated for 2 h in the antibody solution of interest in TBPS with 1% bovine serum albumin. The antibodies used for anti-phosphotyrosine, anti-Syk, anti-Src, anti-PLC-γ1, anti-PLC-γ2, and anti-FAK immunoblotting were 4G10 (1 μg/ml), 101 (3 μg/ml), and 327 (1 μg/ml), rabbit anti-PLC-γ1 (1 μg/ml), rabbit anti-PLC-γ2 (1 μg/ml), and 2A7 (5 μg/ml), respectively. After the membranes were washed,
enhanced chemiluminescence detection (Amersham) of antibody binding and the reprobing of some blots with another antibody was performed as described previously (38).

Immune Complex Kinase Assay—To examine in vitro kinase activity of Syk or Src family kinases, the anti-Syk or anti-Src family immunoprecipitates prepared as above were washed once with low-salt buffer (25 mM Hepes, pH 7.5, 50 mM NaCl, and 10 mM MnCl2) for 10 min. The kinase reaction was started by the addition of 25 μl of kinase buffer (25 mM Hepes, pH 7.5, 10 mM MnCl2, and 1 μM ATP including 5 μCi of [γ-32P]ATP) with 0.2 mg/ml of histone as an exogenous substrate for 5 min at 25 °C, and terminated with 25 μl of 2 × SDS sample buffer. On the other hand, the reprobing of some blots with another antibody was performed as described previously (38).

RESULTS

F(\text{ab}′)_2-anti-p62 Induces Tyrosine Phosphorylation of Multiple Proteins in Platelets—As previously reported (3–5), anti-p62 IgG and its F(\text{ab}′)_2 fragments recognize platelet GPVI and are capable of inducing full activation of normal but not of GPVI-deficient platelets, including release reaction and aggregation. These observations were confirmed by radioligand precipitation analysis of platelet membrane proteins with anti-p62 IgG that yielded a single 62-kDa/57-kDa (reducing/nonreducing) protein which was absent in the GPVI-deficient platelets (Fig. 1A) and also by the platelet aggregation studies showing that F(\text{ab}′)_2-anti-p62 induced irreversible aggregation of normal but not of GPVI-deficient platelets (Fig. 1B, upper panels). In addition, preincubation of normal platelets with the monoclonal Fc-blocking antibody IV.3 did not affect platelet aggregation induced by anti-p62 IgG (data not shown). To eliminate any contributions from Fc receptors, we exclusively used F(\text{ab}′)_2-anti-p62 as an agonist for GPVI in the following experiments.

We first evaluated the profile of time-dependent protein-tyrosine phosphorylation in F(\text{ab}′)_2-anti-p62-stimulated platelets. Whole lysates from resting and F(\text{ab}′)_2-anti-p62-stimulated platelets (5.0 × 10⁶ platelets/each lane) were subjected to analysis on anti-phosphotyrosine immunoblots (Fig. 1B, lower panels). In normal platelets, F(\text{ab}′)_2-anti-p62 induced tyrosine phosphorylation of multiple proteins, which were subdivided into the following three groups. The first group with the earliest appearance peaked at 1 min and gradually diminished (bands at 150, 130, 72-, and 42-kDa). The second group also appeared early but declined more slowly (102- and 68-kDa). Coincident with platelet aggregation, the last group with the specific platelet inhibitors on F(\text{ab}′)_2-anti-p62-stimulated protein-tyrosine phosphorylation. These inhibitors included (a) acetyl salicylic acid, which irreversibly inhibits platelet cyclooxygenase to prevent the generation of prostaglandin endoperoxides and thromboxane A₂; (b) creatine diphosphate plus creatine phosphokinase that removes ADP secreted from the platelet dense granules; and (c) PGJ₂, which elevates cAMP and negatively affects most agonist-stimulated platelet responses.

Platelets were preincubated with these inhibitors and stimulated with F(\text{ab}′)_2-anti-p62. Either acetyl salicylic acid or creatine diphosphate plus creatine phosphokinase did not affect the profile of F(\text{ab}′)_2-anti-p62-induced protein-tyrosine phosphorylation and platelet aggregation (data not shown). In contrast, PGJ₂ completely inhibited platelet aggregation and ATP secretion induced by F(\text{ab}′)_2-anti-p62 (Fig. 2B). However, even under this condition, F(\text{ab}′)_2-anti-p62 did stimulate protein-tyrosine phosphorylation of a number of proteins (Fig. 2D), which were not dephosphorylated and persistently observed in a profile similar to that observed in RGDS-treated platelets. Similar results were obtained when we used dibutyryl cAMP instead of PGJ₂ (data not shown). On the other hand, in the presence of PGJ₂ or dibutyryl cAMP, either thrombin or a stable TXA₂ mimetic STA₂ failed to induce detectable increase in tyrosine-phosphorylated proteins in platelets (data not shown). These findings...
examined whether the inhibition of platelet PTKs would affect F(ab)\(^{2}\)-anti-p62-stimulated platelet aggregation and protein-tyrosine phosphorylation induced by F(ab)\(^{2}\)-anti-p62. Among several PTKs so far identified in platelets, it is only Syk that has been reported to become directly activated by clustering of platelet surface glycoproteins (31, 34, 35). Therefore we first analyzed tyrosine phosphorylation and the activation state of Syk in normal, PGI\(_2\)-treated, and Glanzmann's thrombasthenic platelets. Panel A, washed platelets from a normal donor were stirred and stimulated with 150 \(\mu\)g/ml of F(ab)\(^{2}\)-anti-p62 for the times indicated below each lane. Anti-Syk immunoprecipitates were prepared and analyzed as described above. In both A and B, the arrows on the right indicate the positions of Syk.

**Response to F(ab)\(^{2}\)-anti-p62**, then gradually became dephosphorylated as platelets aggregated. Concurrent with this enhanced tyrosine phosphorylation, the kinase activity of Syk as measured by histone phosphorylation also increased 4–5-fold when compared to that of unstimulated platelets. The maximal tyrosine phosphorylation and activation of Syk were observed at 10–30 s following F(ab)\(^{2}\)-anti-p62 stimulation, preceding the peak appearance of tyrosine-phosphorylated proteins in whole

**Protein-tyrosine Phosphorylation via Platelet GPVI**

To evaluate the involvement of PTK activity in the induction of protein-tyrosine phosphorylation, pretreatment with tyrphostin A47, a protein kinase C inhibitor, chelerythrine chloride, then the various concentrations of the PTK inhibitor, tyrphostin A47, or the protein kinase C inhibitor, chelerythrine chloride, then the effects of these inhibitors on F(ab)\(^{2}\)-anti-p62-stimulated platelet aggregation and protein-tyrosine phosphorylation were examined (Fig. 3). Me\(_2\)SO, which did not significantly alter the profile of tyrosine-phosphorylated protein upon F(ab)\(^{2}\)-anti-p62 stimulation, was used as a vehicle to deliver these inhibitors. In contrast to chelerythrine, which was less potent in inhibiting F(ab)\(^{2}\)-anti-p62-induced platelet aggregation and protein-tyrosine phosphorylation, pretreatment with tyrphostin A47 abrogated F(ab)\(^{2}\)-anti-p62-induced aggregation in parallel with a dose-dependent inhibition of protein-tyrosine phosphorylation. These results suggested that F(ab)\(^{2}\)-anti-p62-stimulated platelet activation was considerably dependent on activation of PTKs in platelets.

**Effect of tyrphostin A47 and chelerythrine on F(ab)\(^{2}\)-anti-p62-induced platelet aggregation and protein-tyrosine phosphorylation.** Panel A, washed platelets were preincubated with 0.5% (v/v) dimethyl sulfoxide (Me\(_2\)SO) (a), chelerythrine (b, 20 \(\mu\)M), or tyrphostin A47 (c, 50 \(\mu\)M; d, 200 \(\mu\)M), and stimulated with 150 \(\mu\)g/ml of F(ab)\(^{2}\)-anti-p62 under stirring conditions. Platelet aggregations were monitored and their representative tracings were shown. Panel B, washed platelets were preincubated with 0.5% Me\(_2\)SO (lanes 1 and 2), tyrphostin A47 (A47) (lanes 3–5, 50, 100, and 200 \(\mu\)M, respectively), or chelerythrine (CLR) (lane 6, 10 \(\mu\)M; lane 7, 20 \(\mu\)M), and unstimulated (lane 1) or stimulated for 1 min with 150 \(\mu\)g/ml of F(ab)\(^{2}\)-anti-p62 under stirring conditions (lanes 2–7). Platelets were lysed in SDS sample buffer and subjected to anti-phosphotyrosine immunoblotting as described in the legend to Fig. 1. The positions of molecular weight standards are indicated on the right.

**Time course of F(ab)\(^{2}\)-anti-p62-stimulated tyrosine phosphorylation and activation of Syk in normal, PGI\(_2\)-treated, and Glanzmann’s thrombasthenic platelets.** Panel A, washed platelets from a donor with Glanzmann’s thrombasthenia (GT) were stirred and stimulated with 150 \(\mu\)g/ml of F(ab)\(^{2}\)-anti-p62 for the times indicated below each lane. Anti-Syk immunoprecipitates were prepared and analyzed as described above. In both A and B, the arrows on the right indicate the positions of Syk.

**Fig. 1.** The next question to be addressed was what PTKs are involved in cAMP-insensitive protein-tyrosine phosphorylation stimulated by F(ab)\(^{2}\)-anti-p62. Among several PTKs so far identified in platelets, it is only Syk that has been reported to become directly activated by clustering of platelet surface glycoproteins (31, 34, 35). Therefore we first analyzed tyrosine phosphorylation and the activation state of Syk in F(ab)\(^{2}\)-anti-p62-stimulated platelets (Fig. 4A). In the absence of PGI\(_2\), Syk became rapidly and transiently tyrosine-phosphorylated in response to F(ab)\(^{2}\)-anti-p62, then gradually became dephosphorylated as platelets aggregated. Concurrent with this enhanced tyrosine phosphorylation, the kinase activity of Syk as measured by histone phosphorylation also increased 4–5-fold when compared to that of unstimulated platelets. The maximal tyrosine phosphorylation and activation of Syk were observed at 10–30 s following F(ab)\(^{2}\)-anti-p62 stimulation, preceding the peak appearance of tyrosine-phosphorylated proteins in whole

**Fig. 2.** Effects of RGDS and PGI\(_2\) on platelet ATP release, aggregation, and protein-tyrosine phosphorylation induced by F(ab)\(^{2}\)-anti-p62. Panels A and B, platelet-rich plasma (3.0 × 10\(^{9}\) platelets/ml) was incubated with 1 mM RGDS tetrapeptide (A) or 3 \(\mu\)M PGI\(_2\) (B) and stimulated with 150 \(\mu\)g/ml of F(ab)\(^{2}\)-anti-p62 under stirring conditions. Representative tracings of platelet aggregation and ATP release were shown. Panels C and D, washed platelets (5.0 × 10\(^{9}\)/ml) were incubated with 1 mM RGDS (C) or 3 \(\mu\)M PGI\(_2\) (D) and stimulated with 150 \(\mu\)g/ml of F(ab)\(^{2}\)-anti-p62 under stirring conditions for the times indicated below each lane. Platelets were lysed in SDS sample buffer and subjected to anti-phosphotyrosine immunoblotting as described in the legend to Fig. 1. The positions of molecular weight standards are shown on the right.
Effects of PGI$_2$, and RGDS on F(ab)$_2$-anti-p62-stimulated subcellular redistribution and phosphorylation state of Syk in the 1% Triton X-100-insoluble fraction. Panel A, washed platelets were stirred and stimulated with 150 μg/ml of F(ab)$_2$-anti-p62 for the times indicated between each lane in the absence (αp62) or presence of 3 μM PGI$_2$, (PGI$_2$/αp62) or in the presence of 1 mM RGDS (RGDS/αp62). Platelets were lysed and fractionated into 1% Triton X-100-soluble (SOL) and -insoluble cytoskeleton (CSK) fractions. The Triton-soluble and insoluble fractions from 1 × 10$^7$ and 4 × 10$^7$ cells, respectively, were resolved by 10% SDS-polyacrylamide gel electrophoresis and immunoblotted with an anti-Syk mAb. Panel B, the cytoskeleton fractions prepared as described above were resolubilized in 0.5 μN NaCl, precipitated with anti-Syk serum, resolved by 8% SDS-polyacrylamide gel electrophoresis, and analyzed on anti-phosphotyrosine immunoblotting (αPY) followed by reprobing with an anti-Syk mAb (αSyk). In both A and B, the arrows on the right indicate the positions of Syk.

Platelet lysates (see Fig. 1B). Notably, even in the presence of PGI$_2$, F(ab)$_2$-anti-p62 stimulated tyrosine phosphorylation of Syk and an increase in its kinase activity up to 5-fold, which was comparable to that of PGI$_2$-untreated platelets. The striking difference between the PGI$_2$-treated and -untreated platelets was that tyrosine phosphorylation and activation of Syk was sustained in the presence of PGI$_2$. GPIIb-IIIa-deficient thrombathenic platelets (Fig. 4B) or RGDS-treated platelets (data not shown) also showed a persistent increase in Syk activity when stimulated with F(ab)$_2$-anti-p62 in the absence of PGI$_2$, suggesting that the deactivation of Syk was dependent on GP Ib-IIIa-mediated events.

Previous reports have indicated that activated Syk became incorporated into the 1% Triton X-100-insoluble cytoskeleton fraction in thrombin-stimulated platelets (31, 39). Since F(ab)$_2$-anti-p62 elicited cAMP-insensitive activation of Syk, we examined if the elevation of cAMP would affect subcellular redistribution of Syk in F(ab)$_2$-anti-p62-stimulated platelets. The Triton X-100-soluble and -insoluble fractions were prepared from resting or F(ab)$_2$-anti-p62-activated platelets, and subcellular distribution of Syk in these two fractions was analyzed on anti-Syk immunoblots (Fig. 5A). In the absence of PGI$_2$, increasing amounts of Syk became relocalized to the Triton-insoluble cytoskeleton fraction in a time-dependent manner with a concomitant decrease in Syk recovered in the Triton-soluble fraction. In contrast, F(ab)$_2$-anti-p62-induced association of Syk with the cytoskeleton was not observed in either PGI$_2$- or RGDS-pretreated platelets. We further studied the tyrosine phosphorylation state of Syk in the cytoskeleton fraction (Fig. 5B). Tyrosine-phosphorylated Syk was found in the cytoskeleton at 45 s following F(ab)$_2$-anti-p62 stimulation but significantly decreased at 90 s, although a comparable amount of Syk was recovered at both time points. This observation was consistent with the tyrosine phosphorylation state of Syk in the whole cell lysates prepared in RIPA buffer (Fig. 4A). These results indicated that although F(ab)$_2$-anti-p62-induced activation of Syk was cAMP-insensitive, subsequent cytoskeletal association of Syk and its dephosphorylation on tyrosine were cAMP-sensitive or dependent on GPIIb-IIIa-mediated aggregation.

Effects of PGI$_2$ on F(ab)$_2$-anti-p62-stimulated kinase activity of c-Src and tyrosine phosphorylation of FAK. Panel A, washed platelets were stirred and stimulated with 150 μg/ml of F(ab)$_2$-anti-p62 for the times indicated below each lane in the absence (PGI$_2$-) or presence (PGI$_2$+) of 3 μM PGI$_2$. Platelets were lysed in 1% Triton X-100-containing buffer and precipitated with an anti-Src mAb. The anti-Src immunoprecipitates were divided into two aliquots; one was analyzed on a quantitative immunoblotting with anti-Src (αSrc), and the other was subjected to in vitro kinase assay using histone as an exogenous substrate. Autoradiographies of c-Src autophosphorylation (c-Src) and histone phosphorylation (Histone) are shown. The arrow on the right indicates the position of FAK. The comparable amount of FAK in each lane was verified on the reprobing of the same membrane with anti-FAK mAb (data not shown).

F(ab)$_2$-anti-p62 also stimulates an increase in c-Src activity in a PGI$_2$-insensitive manner—The other cytosolic PTKs implicated in early events in platelet activation are the five members of the Src family kinases. We then assessed whether the activity of the Src-related PTKs would be altered following F(ab)$_2$-anti-p62 stimulation. When we used RIPA buffer for the solubilization and subsequent immunoprecipitation of the Src-related kinases, we could not observe any increase in their activity in response to F(ab)$_2$-anti-p62 (data not shown), as was consistent with previous reports (25, 26). However, when we used 1% Triton X-100-containing buffer without ionic detergents, we could observe a 2-2.5-fold increase in c-Src activity as measured by histone phosphorylation or c-Src autophosphorylation (Fig. 6A). On the other hand, even using the same buffer, we could not detect any change in the activity of the other four Src family kinases except c-Src in platelets stimulated by F(ab)$_2$-anti-p62 (data not shown). The activity of c-Src in the Triton X-100 soluble fraction was increased as early as 10 s following F(ab)$_2$-anti-p62 stimulation and appeared to diminish thereafter to a basal level. In addition, even in the presence of PGI$_2$, F(ab)$_2$-anti-p62 could stimulate an increase in the activity of c-Src to a similar degree, indicating that F(ab)$_2$-anti-p62 induces activation of not only Syk but also c-Src in a PGI$_2$-insensitive manner. The previous report using thrombin stimulation disclosed that c-Src relocates into the Triton X-100-insoluble cytoskeleton fraction depending on GPIIb-IIIa-mediated aggregation (29). This same phenomenon was also confirmed by studying subcellular redistribution of c-Src following F(ab)$_2$-anti-p62 stimulation (data not shown). Therefore, although we found that PGI$_2$-treated platelets showed a persistent increase in c-Src activity after F(ab)$_2$-anti-p62 stimulation while PGI$_2$-untreated platelets showed a transient one, this apparent difference in the kinetics of c-Src activity between PGI$_2$-treated and -untreated platelets could be explained by the cytoskeletal association of the activated c-Src depending on GPIIb-IIIa-mediated aggregation as suggested by Clark and Brugge (26).
PGI₂ inhibits F(ab)₂-anti-p62-induced Tyrosine Phosphorylation of FAK—Later events in platelet activation are usually thought to accompany tyrosine phosphorylation and activation of another cytosolic PTK, FAK, which is implicated in platelet spreading or aggregation (21, 30). We then asked whether F(ab)₂-anti-p62-stimulated cAMP-insensitive signalings include tyrosine phosphorylation of FAK. As shown in Fig. 6B, F(ab)₂-anti-p62 induced tyrosine phosphorylation of FAK in PGI₂-unstimulated platelets but not in PGI₂-treated ones. In addition, F(ab)₂-anti-p62 was incapable of inducing tyrosine phosphorylation of FAK in RGDS-treated platelets or in thrombastic platelets (data not shown). These findings indicated that tyrosine phosphorylation of FAK in F(ab)₂-anti-p62-stimulated platelets was also dependent on GP1b-IIIa-mediated events.

Collagen but Not Thrombin Also Evokes PGI₂-insensitive Activation of c-Src and Syk—Collagen has been reported to be an agonist that causes cAMP-insensitive platelet activation including the induction of tyrosine phosphorylation of multiple proteins (14, 15). For comparison with F(ab)₂-anti-p62-stimulated cAMP-insensitive signaling, we examined the effects of PGI₂ on collagen-stimulated activation or tyrosine phosphorylation of c-Src, Syk, and FAK. Although PGI₂ inhibited collagen-induced platelet aggregation (data not shown), it did not prevent collagen-stimulated activation of c-Src and Syk (Fig. 7). Moreover, collagen was capable of inducing PGI₂-insensitive tyrosine phosphorylation of FAK (Fig. 6B) in agreement with a previous report (30). Furthermore, we examined the effects of PGI₂ on thrombin-induced activation of c-Src and Syk. In contrast to collagen or F(ab)₂-anti-p62, thrombin failed to activate either c-Src or Syk in PGI₂-treated platelets (Fig. 7).

F(ab')₂-anti-p62 as Well as Collagen Stimulates cAMP-insensitive Tyrosine Phosphorylation of PLC-γ In a recent series of reports that collagen stimulates the tyrosine phosphorylation of PLC-γ₂, but not PLC-γ₁, in platelets presumably by non-G-protein-coupled receptors (17, 18). This prompted us to examine whether either of the PLC-γ isoforms was included in the proteins phosphorylated on tyrosine by the stimulation of F(ab')₂-anti-p62. As shown in Fig. 8, F(ab')₂-anti-p62 did not induce tyrosine phosphorylation of PLC-γ₁ but did for PLC-γ₂. Furthermore, F(ab')₂-anti-p62-stimulated tyrosine phosphorylation of PLC-γ₂ was observed also in PGI₂-treated platelets, indicating that GPVI-mediated signaling is coupled to cAMP-insensitive tyrosine phosphorylation of PLC-γ₂. Interestingly, we found that collagen-stimulated tyrosine phosphorylation of PLC-γ₂ was also PGI₂-insensitive (data not shown).
In confirmation of these previous reports, we observed that cAMP increasing agents abrogated protein-tyrosine phosphorylation induced by thrombin or STA2, but did not with those induced by collagen. Although the mechanisms for cAMP-sensitive or -insensitive protein-tyrosine phosphorylation are to be investigated further, the unique nature that F(ab')2-anti-p62-stimulated platelet activation is dependent on cAMP-insensitive induction of protein-tyrosine phosphorylation may support the concept that activation of PTKs is one of the earliest signaling responses coupled to GPVI engagement.

In search for responsible PTKs for cAMP-insensitive protein-tyrosine phosphorylation, we found that the effects of PGI2 on the activation of c-Src, Syk, and FAK varied between agonists employed in this study. While F(ab')2-anti-p62 and collagen stimulated activation of c-Src and Syk in a cAMP-insensitive fashion, activation of these kinases by thrombin was inhibited in the presence of PGI2. Furthermore, collagen did stimulate tyrosine phosphorylation of FAK in PGI2-treated platelets, but F(ab')2-anti-p62 did not. These results indicate that both cAMP-sensitive and -insensitive activation mechanisms exist for such PTKs as c-Src, Syk, and FAK, and that either of them is operating depending on the nature of each different receptor-coupled signal.

Although c-Src and Syk have been reported to become activated by a variety of platelet agonists, the present work is the first report demonstrating activation of c-Src through clustering of platelet surface glycoproteins. Furthermore, GPVI is newly identified as a member of platelet membrane glycoproteins whose engagement leads to tyrosine phosphorylation and activation of Syk. In other cells, it has been reported that the Src family and Syk/ZAP-70 family kinases are recruited to the phosphorylated tyrosine-based activation motif-containing receptors and synergistically involved in generating cellular responses (44–46). Although we tried several immunoprecipitation analyses under various lysis conditions, we could not reproducibly detect association of GPVI with c-Src or Syk, and did not observe tyrosine phosphorylation of GPVI through the time course of F(ab')2-anti-p62-stimulated platelet activation. However, it appears that the possibility of interaction of PTK with GPVI cannot be ruled out, because activated GPVI may be poorly recovered by intact anti-p62 IgG in the presence of an excess amount of its F(ab')2 fragments. In fact, we could observe coprecipitation of Syk with GPVI when we stimulated platelets with anti-p62 IgG instead of F(ab')2-anti-p62 and directly collected GPVI-anti-p62 IgG complex. Very recently, Ozaki et al. (35) have reported that both persistent activation of Syk and association of c-Src with Syk are evoked by antibody-induced clustering of CD9 but are not functional per se with respect to tyrosine phosphorylation of substrate proteins and the full picture of platelet aggregation, although the recruitment of these PTKs to CD9 remains to be clarified (35). Clearly, their work is in sharp contrast to our novel observations that clustering of GPVI induces functional activation of Syk and c-Src leading to platelet activation as well as cAMP-insensitive tyrosine phosphorylation of many cellular substrates.

Clark et al. (31) reported that the agonist-induced activation of Syk was enhanced by GPIIb-IIIa-dependent mechanisms, implying the possible role of Syk not only in the "early" phase but in the aggregation-dependent protein-tyrosine phosphorylation in platelets. On the contrary, in agreement with another recent report (35), our results showed that activity of Syk was rather negatively regulated also in an aggregation-dependent fashion, because tyrosine-phosphorylated Syk was rapidly dephosphorylated on tyrosine and deactivated as platelets aggregated. This was not explained solely by the translocation of activated enzyme to the cytoskeleton fraction which was true for c-Src (26), because such deactivation of Syk was also observed in RIPA lysates containing the solubilized cytoskeleton fraction. We further observed that the cytoskeletal association of Syk following GPVI cross-linking was GPIIb-IIIa-dependent and demonstrated for the first time that relocated Syk subsequently became dephosphorylated on tyrosine in the cytoskeleton fraction. These findings indicate that cytoskeletal reorganization is involved in dephosphorylation of Syk through the GPIIb-IIIa-dependent mechanisms. The dual regulation, that is, GPIIb-IIIa-mediated activation and deactivation, may differentially modulate the activity of Syk in such a way as to depend on each platelet agonist or ligand. We have recently provided evidence that dephosphorylation of tyrosine-phosphorylated proteins in platelets occurs mostly on the cytoskeleton by the action of protein-tyrosine phosphatases regulated by GPIIb-IIIa-mediated aggregation (38). The same mechanisms could be applied to the deactivation of Syk by the interaction with protein-tyrosine phosphatase in the cytoskeleton.

Although some clinical reports from our laboratory and others have implicated the possible involvement of GPVI in collagen-induced platelet activation, no biochemical evidence has been provided to date for this concept. It is noteworthy that signaling through GPVI resembles collagen stimulation in the sense that both induce cAMP-insensitive activation of c-Src and Syk accompanied by tyrosine phosphorylation of PLC-γ2. In order to obtain more conclusive evidence for the involvement of GPVI in collagen-platelet interaction, it should be of great value to study collagen-induced signaling events in the GPVI-deficient platelets. Interestingly enough, our preliminary results showed that the GPVI-deficient platelets from the few patients so far reported specifically lacked activation of Syk but possessed normal activation of c-Src when stimulated by collagen, although both of these kinases became normally activated when these platelets were stimulated by other agonists including thrombin. Given the fact that F(ab')2-anti-p62-induced clustering of GPVI evoked activation of c-Src and Syk as was shown in this study, a model is emerging that GPVI-mediated signaling may be essential for collagen-stimulated activation of Syk while c-Src could be activated not only through GPVI but also presumably via GP Ib-IIa in collagen-platelet interaction. Based on the present work, our efforts are currently directed to more complete understanding of the mechanisms for collagen-induced platelet activation which possibly involve coordinated signalings from different membrane receptors including GPVI.

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