Differential Translocation of Phospholipase C Isozymes to Integrin-mediated Cytoskeletal Complexes in Thrombin-stimulated Human Platelets*

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To investigate a role of phospholipase C (PLC) isozymes in the integrin $\alpha_{IIb}\beta_3$-mediated signaling, their location was examined in thrombin-activated human platelets, revealing different regulation of their translocation to the cytoskeleton (CSK). In resting platelets, the major PLCs such as PLC$\beta_2$, PLC$\beta_3$a (155 kDa), and PLC$\gamma_2$ and the minor PLCs (PLC$\beta_1$ and PLC$\gamma_1$) were located in the Triton X-100-soluble (Tx-Sol) fraction and the membrane skeleton, whereas PLC$\beta_3$b (140 kDa) was present only in Tx-Sol fraction when examined by Western immunoblotting. Thrombin stimulation caused a rapid and transient translocation of PLC$\beta_3$a and PLC$\beta_3$b and a slower accumulation of PLC$\beta_2$ and PLC$\gamma_2$ in the reorganized CSK. The translocation to CSK of both PLC$\beta_3$a and PLC$\beta_3$b, but not PLC$\beta_2$, was dependent on integrin $\alpha_{IIb}\beta_3$-mediated aggregation. Furthermore, an actin polymerization inhibitor, cytochalasin D, or a protein tyrosine kinase inhibitor, genestein, abolished the CSK association of $\alpha_{IIb}\beta_3$, PLC$\beta_3$a, and PLC$\beta_3$b. In the genestein-pretreated platelets, pp60$^{src}$, G$\beta_3$, and protein kinase C$\alpha$ were no longer able to associate with CSK. In contrast, these agents had no or marginal inhibitory effects on the CSK association of PLC$\beta_2$ and G$\gamma_2$. The late diacylglycerol generation induced by thrombin stimulation was significantly reduced by the genestein treatment. These results suggest that the integrin $\alpha_{IIb}\beta_3$-mediated cytoskeletal association of PLC$\beta_3$ is regulated by protein tyrosine kinase and also that the activation of the relocated PLC may play a role in the late platelet-to-platelet aggregation in thrombin-stimulated human platelets.

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†The abbreviations used are: PLC, phospholipase C; RGDS, Arg-Gly-Asp-Ser; PAGE, polyacrylamide gel electrophoresis; PI, phosphatidylinositol; DAG, diacylglycerol; G$\alpha$ and G$\beta\gamma$, the $\alpha$ and the $\beta\gamma$ subunit, respectively, of GTP binding proteins; Me$_2$SO, dimethyl sulfoxide, mAb-1N5b, monoclonal antibody raised against $\beta_3$ of integrin $\alpha_{IIb}\beta_3$; M5K, membrane skeleton; CSK, cytoskeleton; Tx-Sol, Triton X-100-soluble.
EXPERIMENTAL PROCEDURES

Materials—Monoclonal antibody to integrin β3 (mAb119.9), B6A3 was kindly supplied from Yamanouchi Pharmaceutical Co., Ltd. (Tsukuba, Japan). Calpain inhibitor, calpeptin, was kindly provided by Dr. J. Un-ichi Kambayashi (Osaka University Medical School, Japan). Polyclonal antibodies to PLC isozymes (α1, α2, β, γ, γ2, δ1, δ2, Gα, PKCα, Gβ, and Gγ) were purchased from Santa Cruz Biotechnology (San Francisco, CA). Anti-Gα2 antibody was from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Polyclonal antibodies to PLCβ3 (Ab-PLCβ3C and Ab-PLCβ3M) were prepared as described previously (21). The horseradish peroxidase-conjugated secondary antibodies were from Amersham Corp. Thrombin, Arg-Gly-Asp-Ser (RGDS), and genistein were purchased from Sigma.

Preparation of Washed Platelets—Washed human platelets were prepared by the method described previously (22). Human blood was drawn by venipuncture, and platelet-rich plasma was obtained. Platelets were isolated by gel filtration on a Sepharose 2B column. Washed platelets were resuspended at a concentration of 1 x 10^8 platelets/ml in Hapes-Tyrode's buffer (129 mM NaCl, 8.9 mM NaHCO3, 0.8 mM KH2PO4, 1.8 mM CaCl2, 0.8 mM MgCl2, 5.6 mM dextrose, and 10 mM Hapes, pH 7.4).

Platelet Actin and Aggregation—Washed platelet suspension (0.5 ml) was incubated with stirring at 37°C in an aggregometer (Frenius, Bad Homburg, Germany). CaCl2 (1 mM) was added 30 s prior to addition of 1 unit/ml thrombin. Aggregation was measured by percentage change in light transmission. In some experiments, as indicated, platelets were preincubated for 15 min with synthetic peptide consisting of the sequence RGDS (0.5 mM) or with mAb-β3/γ2 (50 μg/ml). In some experiments, platelets were pretreated with calpain inhibitor calpeptin (30 μM), cytochalasin D (20 μg/ml), or genistein (100-150 μM), or Me2SO (0.2%, control) for 5-20 min at 37°C prior to addition of 1 unit/ml thrombin.

Determination of Diacylglycerol Content—Washed platelet suspension (1 x 10^9/ml) were pretreated with Me2SO (0.3%, control) or genistein (150 μM) at 37°C for 20 min and stimulated with thrombin (1 unit/ml) for 2 min and terminated by adding 2 ml of chloroform/methanol (1:2, v/v). Lipids were extracted by the method of Bligh and Dyer (22). Diacylglycerol (DAG) mass content was by the conversion of DAG into 32P-labeled phosphatidic acid by Escherichia coli DAG kinase in the presence of [γ-32P]ATP, according to the method as described previously (23).

Isolation and Immunoblotting Analysis of Detergent Lysates of Platelets—Platelets were lysed by addition of an equal volume of ice-cold 2% Triton lysis buffer containing 50 mM Hapes-HCl, pH 7.4, 10 mM EGTA, leupeptin (20 μg/ml), 2 mM phenylmethylsulfonfluoride, 20 μg/μl aprotinin, and 2% Triton X-100 and left on ice for 30 min. The Triton X-100-soluble (TxSol) and -insoluble fractions (cytoskeleton; CSK and membrane skeleton; MSK) were isolated as described (24). The CSK and MSK fractions were washed once in 1% Triton X-100 lysis buffer.

For immunoblotting with anti-PLC antibodies, the CSK and MSK fractions were resuspended in 200 and 150 μl of LIPA buffer (1% Triton X-100, 0.5% SDS, 10 mM EGTA, 10 mM EDTA, 1 mM phenylmethylsulfonfluoride, 10 μg/ml leupeptin), respectively, plus 50 μl of 5 x sample buffer (250 mM Tris-HCl, 82% SDS, 40% glycerol, 0.05% bromphenol blue, 5% 2-mercaptoethanol, pH 6.8), and heated at 95°C for 10 min. The TxSol fraction was mixed at a ratio of 4:1 with 5 x sample buffer and boiled.

Proteins were separated on SDS-polyacrylamide gels containing 6% polyacrylamide, transferred to the polyvinylidene difluoride membrane, and then incubated with antibodies against PLC isozymes. Antibody-antigen complexes were detected with the chemiluminescence (ECL) method (Amersham Corp.). The intensities of the bands were quantitated by densitometry (ATTO Densitograph AE-6900M) and quantitation was performed within the linear range. To know actin amounts in the CSK fraction, proteins were separated by SDS-polyacrylamide gel electrophoresis (10%), followed by staining of the gel with 0.2% Coomassie Brilliant Blue, and the bands corresponding to actin were measured by the densitometry.

Protein concentrations were determined using the Bio-Rad protein assay with bovine serum albumin as standard.

RESULTS

Distribution of PLC Isozymes in Human Platelets—We have previously demonstrated that human platelets contained two PLCβ isoforms that are different in molecular mass (PLCβ1, 155 kDa and PLCβ3, 140 kDa) when analyzed by using the Ab-PLCβ3C against the C-terminal (amino acids 1202-1217) of PLCβ3 and Ab-PLCβ3M against the 550-561 amino acid residue of PLCβ3 (21). The PLCβ3b was only detected with Ab-PLCβ3M but not Ab-PLCβ3C, suggesting that there is C-terminal proteolysis. As indicated in Fig. 1, the distribution of these two PLCβ3 forms was different in the resting platelets; PLCβ3a was present in the TxSol and MSK, whereas PLCβ3b was only present in TxSol fraction. PLCβ3a, PLCβ3b, and PLCγ2 were present in both TxSol and MSK fractions but absent in CSK fraction. A small amount of PLCγ1 was present and PLCβ1 was hardly detectable. PLCβ4, PLCδ1, and PLCδ2 were unable to be detected under the conditions used here. The same volume (20 μl) from each fraction (TxSol, 1.0 ml; MSK, 0.15 ml; and CSK, 0.2 ml) was subjected to densitometric analysis of immunoblots (n = 4). It has been shown that 90% of PLCβ2, 87% of PLCβ3a, and 90% of PLCγ2 were located in the TxSol fraction, whereas 9, 10, and 8% of each PLC isozyme were in the MSK fraction. In contrast, PLCβ3b was entirely distributed in the TxSol fraction.

Upon platelet stimulation with 1 unit/ml thrombin for 30 s, all PLC isozymes appeared in the CSK at different levels. The extents of CSK association of PLCβ3a and PLCβ3b were increased 24- and 80-fold, respectively, while those of PLCβ2 and PLCγ2 were 3- and 10-fold. At 30 s after stimulation with thrombin, approximately one-fourth of total PLCβ3b was translocated to CSK. The time course of translocations of PLC isozymes in thrombin-stimulated platelets displayed decreases of PLCβ3a (a, b) in the TxSol fraction (Fig. 2, left panels) and increases in the CSK fraction, reaching a maximum at 30 s after stimulation (Fig. 2, right panels). No significant changes of PLCs were seen in the MSK fraction (Fig. 2, middle panels).

These results suggested that the PLCs translocated to CSK were originated from TxSol but not from MSK. The marked decreases of PLCβ3a (a, b) in the CSK after 30 s stimulation were compatible with our previous observations that PLCβ3 (a, b) were proteolytically degraded to 100 kDa by the activation of calpain in thrombin-stimulated platelets (21). This truncated form (100 kDa) was detected in CSK (data not shown).

Although PLCβ2 and PLCγ2 were also translocated to CSK from the TxSol fraction by thrombin stimulation, the extents were much smaller compared with those of PLCβ3. Interestingly, unlike PLCβ3, both PLCβ2 and γ2 underwent progres-
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Fig. 2. Time course of thrombin-induced translocation of PLC isozymes. Washed platelets were stimulated with 1 unit/ml thrombin for the indicated times under stirring conditions. The platelets were lysed in 1% Triton X-100-containing lysis buffer and stood on ice for 30 min. The TxSol (1.0 ml), MSK (0.15 ml), and CSK (0.2 ml) fractions were prepared as described in Fig. 1. A, 20 μl were subjected to 6% SDS-PAGE and immunoblotted by anti-PLC isozyme antibodies. Result shown is a representative experiment of three independent experiments. B, the changes of PLC isozyme levels in the different fractions were quantified by scanning densitometry and mass of all fractions (TxSol, MSK, and CSK fractions) of each PLC isozyme was designated as 100%. Values shown are means from three independent experiments.

Fig. 3. Effects of RGDS and mAb-INβ3 on the protein content in CSK and aggregation in thrombin-stimulated platelets. Washed platelets were pretreated with RGDS (0.5 mg/ml) or mAb-INβ3 (50 μg/ml) for 5 min at 37°C and then stimulated with thrombin (1 unit/ml) for the indicated times. Platelet aggregation was measured as described under “Experimental Procedures.” A, the protein content in the CSK fraction was isolated from 10^9 platelets and expressed in mean ± S.D. from three different experiments. B, aggregation was measured in changes of light transmission and the value at 5 min was designated as 100%.

Fig. 4. Effects of RGDS and mAb-INβ3 on CSK association of PLC isozymes and integrin αIIbβ3 in thrombin-stimulated platelets. Washed platelets were pretreated with RGDS (0.5 mg/ml) or mAb-INβ3 (50 μg/ml) and then stimulated by thrombin (1 unit/ml) for 30 s. Control platelets without the pretreatments were stimulated with thrombin for the indicated times. The CSK fractions were prepared, lysed, and subjected to SDS-PAGE and immunoblotted as described in Fig. 1. Data are representative of three different experiments.

Regulation of Cytoskeletal Association of PLC Isozymes—Aggregation requires fibrinogen binding to the activated integrin αIIbβ3 in thrombin-stimulated platelets (2–5). To examine involvement of the fibrinogen binding to the integrin in the CSK association of PLCs, platelets were pretreated with the fibrinogen antagonist peptide RGDS or with the inhibitory monoclonal integrin β3 antibody (mAb-INβ3) (25). It was demonstrated that the thrombin stimulation caused an increase of protein content in CSK in a time-dependent manner and reached a plateau at 30–60 s after stimulation (Fig. 3). The increased proteins contain mainly polymerized actin as well as MSK proteins (3). The enhancement of actin polymerization was prevented by RGDS or mAb-INβ3, thereby leading to considerable inhibition of thrombin-induced aggregation. Under these conditions we have examined the CSK association of different PLC isoforms. It has been known that integrin αIIbβ3 is located in MSK in resting platelets and then translocated to the reorganized actin-rich CSK by platelet aggregation (3, 24). As shown in Fig. 4, the integrin αIIbβ3 appeared in CSK at 15 s after thrombin stimulation and reached the maximal level at 120 s when platelets were nearly fully aggregated. The relocation of αIIbβ3 to the actin-rich CSK was inhibited by RGDS or mAb-INβ3. The time course of PLC translocation to CSK showed a good correlation with that of the integrin αIIbβ3 translocation. Interestingly, the pretreatment of platelets with RGDS or mAb-INβ3 caused differential inhibition in CSK association of PLC isoforms (Table I). The CSK association of
PLCβ3(a, b) was almost completely prevented by either RGDS or mAb-INβ3, but the association of PLCγ2 and β2 was much less affected. The PLC isozymes, PLCβ2, PLCβ3a, and PLCγ2, in MSK of resting platelets were unaffected with these treatments (data not shown). These results suggested the differential regulation in the integrin αIIbβ3-mediated cytoskeletal association of PLC isozymes.

As seen in Figs. 2 and 4, during a concomitant increase in aggregation, there were decreases in CSK association of PLCβ3(a, b) after the peak at 30 s after stimulation. In our previous study, similar decreases of these PLCβ3(a, b) enzymes have been observed in intact platelets stimulated with thrombin, which were thought to be induced by calpain activation (21). Then we examined whether the translocation of PLCs to CSK is associated with calpain activation. As shown in Fig. 5, A and B, calpeptin, a specific calpain inhibitor, did not interfere with relocation of all PLCs to CSK, thus indicating that once translocated to the reorganized CSK, PLCβ3(a, b) were proteolytically modified by calpain.

It has been demonstrated that the extracellular triggering of integrin response involves hierarchies of protein interactions upon tyrosine phosphorylation and actin polymerization (26). To examine whether the CSK association of PLCs is dependent on actin integrity or protein tyrosine phosphorylation, platelets were pretreated with cytochalasin D, an inhibitor of actin polymerization, or genistein, a protein tyrosine kinase inhibitor. As indicated in Fig. 5B, an increase of polymerized actin in CSK fraction was reduced to 27% by cytochalasin D pretreatment. The same treatment caused the decreased CSK association of PLCβ3(a, b) to 23 and 14% of the untreated control, respectively (Fig. 5A, A and B). In contrast, no inhibition by cytochalasin D was observed in the CSK association of PLCγ2. These results indicated that actin polymerization is involved in the CSK association of PLCβ3(a, b).

Experiments with tyrosine kinase inhibitor, herbimycin A, has indicated that tyrosine phosphorylation of various proteins is required for the cytoskeletal attachment of integrin αIIbβ3 (15). In the present study, we have examined the effect of genistein on the cytoskeletal association of PLC enzymes in thrombin-stimulated platelets. Genistein (100 μM) pretreatment of platelets caused a marked reduction (80%) in the thrombin-induced CSK association of αIIbβ3 (Fig. 5, A and B). Similar inhibitory effects were observed in PLCβ3(a, b), although genistein had no effect on actin polymerization. There was no inhibition for PLCβ2 translocation. Moreover, it was shown that pp60c-src and protein kinase Cα were translocated to the CSK fraction in thrombin-stimulated platelets (Fig. 6). However, this event was blocked by pretreatment of genistein. Heterotrimeric GTP-binding proteins, Gαi2 and Gαq, as detected by specific antibodies, were also translocated to the reorganized CSK in thrombin-stimulated platelets. The translocation to CSK was regulated differently; the CSK association of Gq was inhibited by treatment of genistein but that of Gi2 was not. These findings that genistein selectively inhibited the αIIbβ3-mediated cytoskeletal association of signaling molecules raise the possibility that tyrosine phosphorylation is involved in their differential translocation to CSK.

Possible Roles of Cytoskeletal Association of PLCβ3 in Platelet Aggregation—Our previous study indicated that thrombin stimulation of human platelets induced a biphasic accumulation of DAG by PLC activation; the early phase showed a sharp

### Table I

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| PLC Isozyme | RGDS | mAb-INβ3 |
|-------------|------|----------|
| PLCβ2      | 181 ± 3.3 | 16.1 ± 2.3 |
| PLCβ3a     | 84.7 ± 8.5 | 80.3 ± 6.5 |
| PLCβ3b     | 90.6 ± 5.9 | 88.6 ± 4.9 |
| PLCγ2      | 64.8 ± 5.6 | 40.5 ± 7.6 |

**Fig. 5.** Effects of calpeptin, cytochalasin D, and genistein on the CSK association of PLC isozymes and integrin αIIbβ3 in thrombin-stimulated platelets. Washed platelets were preincubated with either 0.2% Me2SO (control), calpeptin (Calp, 30 μM), cytochalasin D (CD, 20 μM), and genistein (Gen, 100 μM) at 37°C for 5–20 min and then stimulated by thrombin (1 unit/ml) for 30 s. A, CSK fractions were subjected to SDS-PAGE and immunoblotted with PLC isozymes, integrin β3, and actin in the Coomassie Blue-stained gel. The results are mean ± S.D. from three different experiments.

**Fig. 6.** Effects of genistein on the CSK association of various signaling molecules in thrombin-stimulated platelets. Washed platelets were preincubated with either 0.3% MeSO (--) or genistein (150 μM) at 37°C for 20 min and then stimulated by thrombin (1 unit/ml) for 2 min. CSK fractions were subjected to SDS-PAGE and immunoblotted with anti-PLCβ3, mAb-INβ3, c-Src, Gαi2, Gαq, and PKCα antibodies. Data show a representative of three different experiments.
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Fig. 7. Effects of genistein on the DAG mass changes in thrombin-stimulated human platelets. Washed platelets were incubated with either 0.3% Me2SO (control) or genistein (150 μM) at 37°C for 20 min and then stimulated by thrombin (1 unit/ml) for 2 min. Lipids were extracted and the DAG content was determined as described under "Experimental Procedures." The results are mean ± S.D. from triple determinations of two different experiments.

rapid peak at 10 s after stimulation, and the subsequent second phase DAG formation is slow and sustained, reaching a maximum at 2 min, where platelets are fully aggregated (23). Since genistein prevented the translocation to CSK of PLCβ3(a, b) (Fig. 5), effect of genistein on DAG formation was examined at 2 min after thrombin stimulation. As shown in Fig. 7, a significant reduction of DAG formation was observed in genistein-preincubated platelets. Similar inhibition of DAG formation was also caused by another tyrosine kinase inhibitor, herbimycin A (data not shown). These results suggested that the late DAG formation could be partly due to PI hydrolysis via the PLCβ3(a, b) translocated to the reorganized CSK.

At the present time, we do not have relevant interpretation for the role of PLCβ3 activation at the reorganized CSK in the late stage of platelet aggregation (platelet-to-platelet aggregation). However, a recent report has shown that the αIIbβ3-mediated signaling regulated by protein tyrosine phosphorylation participates in formation of fibrin polymers (15). In fact, we also observed the inhibition of the thrombin-induced dot retraction in genistein-treated platelets (data not shown).

DISCUSSION

Several lines of evidence indicate that integrins are involved in bi-directional signaling across the plasma membrane (1). In platelets, the agonist stimulation causes activation of αIIbβ3 integrin by unique signal transduction (inside-out signaling), and the subsequent fibrinogen binding to the αIIbβ3 integrin, leading to cytoskeletal rearrangement and platelet aggregation, causes tyrosine kinase activation including pp125<sup>FAK</sup> and pp60<sup>src</sup> (outside-in signaling) (1, 3, 4, 27). The ability of αIIbβ3 to undergo cytoskeletal rearrangement is blocked by the monoclonal antibody to integrin β3 or the fibrinogen antagonist, RGDS (27). Furthermore, recent study has shown that protein tyrosine kinase inhibitors, genistein or herbimycin A, and actin polymerization inhibitor, cytochalasin D, prevent the association of various signaling molecules with integrin (26). These observations suggest that the integrin αIIbβ3 is anchored to underlying cytoskeletal molecules and that the association of αIIbβ3 with cytoskeletal proteins is regulated by activation of protein tyrosine kinases.

In the present study, we have demonstrated that in thrombin-stimulated human platelets, the PLCβ3(a, b) were preferentially relocated to the detergent-insoluble actin-rich CSK (Figs. 1 and 2). This translocation was dependent on the integrin αIIbβ3 activation (Fig. 4 and Table I). Furthermore, it is inhibited by either cytochalasin D or genistein (Figs. 5 and 6), suggesting that the translocation of PLCβ3(a, b) to CSK is mediated by formation of microfilament-dependent complexes containing αIIbβ3 protein tyrosine kinase and their substrates in aggregated platelets.

The decreases in the PLCβ3(a, b) level were observed in CSK after the peak at 30 s of thrombin stimulation (Fig. 2, right panels). In our earlier report it was indicated that PLCβ3 is one of the substrates for calpain in thrombin-stimulated human platelets (21). Thus it was conceivable that the decreased PLCβ3 level reflects its cleavage by calpain. In fact, calpeptin, a calpain inhibitor, blocked the cleavage of PLCβ3(a, b), although the inhibitor did not inhibit their translocation to CSK mediated by αIIbβ3 activation. In addition to the PLCβ3, some other proteins also are cleaved by calpain in the late aggregation phase, and their products are located in CSK (28, 29). The calpain activation has been shown to be dependent on αIIbβ3-mediated signaling. In fact, the fibrinogen antagonist RGDS or antibody mAb 1B9β3 could prevent the cleavage of PLCβ3 (data not shown). Therefore, it can be assumed that the cleavage by calpain of PLCβ3(a, b) may be associated with the integrin activation.

We have previously shown in in vitro experiments that the cleavage of purified PLCβ3a by calpain produced a 100 kDa PLC and that this truncated PLC was, to a greater extent, activated by Gβγ subunits than its original forms (30). The present study using intact platelets indicated that heterotrimeric G proteins (G<sub>q</sub> and G<sub>βγ</sub>) involving PLCβ3 activation were translocated to the reorganized CSK (Fig. 6). The location of the heterotrimeric G protein in the cytoskeleton was described in a previous report that demonstrated that G<sub>βγ</sub> was co-localized with vinculin in association with focal adhesions (31). Moreover, it was recently shown that G<sub>(q/11)</sub>γ<sub>2</sub> was recovered in the actin-rich fraction in agonist-stimulated rat mammary tumor (WRK) cells and that cytochalasin D inhibited G protein-induced PLC activation, suggesting an important role of the actin-rich cytoskeleton in the G protein-regulated PLC activation (32). Accordingly, PLCβ3(a, b) translocated to CSK was assumed to be activated by the G proteins in thrombin-stimulated platelets. Thus it was DAG production with a peak at 2 min after stimulation. This late DAG formation was reduced by genistein treatment which blocked the translocation of PLCβ3 and G<sub>q</sub> (Figs. 6 and 7). Our previous study has suggested that thrombin stimulates a biphasic accumulation of DAG, with an early phase reaching a peak at 10 s and a later phase at 2–3 min, and that the second phase of DAG accumulation may be derived from PIs in human platelets (23). These results lead us to speculate that the integrin-ligand interaction induces translocation to the actin-rich CSK of PLCβ3(a, b) and G<sub>q</sub>, resulting in DAG accumulation by activated PLCβ3 in the late aggregation step.

Several lines of evidence suggest that protein tyrosine kinases such as pp60<sup>src</sup> are required for the cytoskeletal attachment of integrin αIIbβ3 (9, 26, 33) and also for the retraction of fibrin polymers in thrombin-stimulated platelets (15). We have demonstrated here that the treatment of platelets with genistein prevented the CSK association of various signaling molecules such as PLCβ3(a, b), pp60<sup>src</sup>γ<sub>2</sub>, PKCα, and αIIbβ3<sub>γ2</sub>, thus leading to inhibition of the PLC activation. These findings have led us to postulate that the translocation of PLCβ3 to CSK which was controlled by protein tyrosine kinase may play a role in the late aggregation (formation of large platelet-to-platelet aggregation).

It has been known that platelets have at least two adhesive glycoprotein receptors, integrin αIβ1 (GPⅠa-IIa) and αIIbβ3 (GPⅠb-Ⅲa), which mediate transmembrane signaling (3–5, 35). Platelet αIβ1 acting as a receptor for collagen mediates...
activation of tyrosine kinases including pp125Fak (36, 37). The β1 or β2 family of integrins induces activation of the tyrosine kinase PLCγ1 pathway (37, 38). Recently, PLCγ2, but not γ1, was shown to be activated via tyrosine phosphorylation in platelets stimulated by collagen but not by thrombin (39, 40). Our data obtained in this study demonstrated that in the cytoskeletal association of thrombin-stimulated platelets, PLCγ2 is distinct from PLCβ3 in that PLCβ3 is entirely integrin αIIbβ3-dependent and inhibited by cytochalasin D. Thus, PLCγ2 appears to play a role in the transmembrane signaling initiated through αIIbβ3, rather than αIIbβ3 in platelet aggregation. In addition, PLCβ2, which is most abundant in platelets, also becomes associated with CSK by aggregation with thrombin, but its association was independent of αIIbβ3 and genistein, suggesting that PLCβ2 participates in the early stage of agonist-induced signaling but not in the integrin-mediated signaling pathway in human platelets. However, the activation mechanisms and exact roles of different PLC isozymes in these two pathways remain to be disclosed.

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