Platelet Tyrosine Kinases and Fibrinogen Receptor Activation

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Abstract Platelet adhesion and aggregation during hemostasis and thrombosis are usually limited to sites where the integrity of the vessel wall is disrupted. The high concentration of platelet agonists within these sites represents a putative control mechanism for targeting platelet activation. Although much has been learned about the intracellular signaling systems controlling platelet activation, our understanding of the connection between signaling molecules and platelet aggregation remains limited. Tyrosine kinases are important signaling enzymes in cells and are abundant in platelets. Previous reports indicate that binding of glycoprotein IIb-IIIa (GPIIb-IIIa) to fibrinogen can induce the tyrosine phosphorylation of specific substrates. We show that, in turn, protein tyrosine kinase activity is necessary for agonist-induced activation of GPIIb-IIIa. Genistein and the tyrphostin AG-18 are two specific tyrosine kinase inhibitors, and the former has been shown to inhibit platelet aggregation. We use genistein and AG-18 in the present study to demonstrate that aggregation inhibition is due to suppression of GPIIb-IIIa activation. In contrast, genistin, an isoflavone compound related to genistein, and acetylsalicylic acid do not affect the tyrosine kinase-signaling pathway, nor do they inhibit GPIIb-IIIa activation induced by strong agonists. On identifying prominent tyrosine kinase substrates in activated platelets, we confirm that several substrates correspond to proteins associated with the cytoskeleton: the 85-kD subunit of phosphatidylinositol 3-kinase, the SH3-containing and actin-associating p55, pp60^c-src, and pp125^FAK. Our data showing that tyrosine kinase activity is required for GPIIb-IIIa activation, together with previous studies indicating that fibrinogen binding to its receptor results in tyrosine phosphorylation of platelet substrates, suggest that a dual regulatory mechanism allows for full platelet response only at sites where both pathways are activated, namely disrupted vessel walls. (Circ Res. 1994;75:172-180.)

Key Words • cytoskeleton • glycoprotein IIb-IIIa • tyrosine kinases • integrins • platelets

Platelet aggregation occurs when the adhesive molecule fibrinogen binds to its receptor, the integrin α6β3, a complex made of two glycoproteins, IIb (GPIIb) and IIIa (GPIIIa), on the surface of platelets.1,2 Soluble fibrinogen only binds to the activated conformation of GPIIIa-IIIIa, which is induced by platelet agonists. Much is known about the molecular biology and the biochemistry of the fibrinogen receptor, but little information is available on the mechanism(s) limiting the activation of GPIIb-IIIa on the surface of circulating platelets. In particular, it is not clear why this activation seems to be limited to sites of disrupted vessel walls, where immediate return of vessel integrity is needed. Recent advances in the understanding of agonist-induced platelet activation focused on the role of integrins as receptors for extracellular matrix proteins3 and on protein tyrosine kinases (PTKs) as transducers of signals generated by activated integrins.4

Integrins are heterodimeric integral membrane glycoproteins that mediate in a ligand-specific manner cell-cell and cell–extracellular matrix interactions.5 GPIIb-IIIa is the most abundant integrin on the surface of platelets,1,2 and it binds fibrinogen, fibronectin, von Willebrand factor, vitronectin, and thrombospondin on the surface of activated thrombocytes. In unactivated platelets, GPIIb-IIIa does not bind to circulating fibrinogen.1,2 Activation of platelets results in an uncharacterized change in GPIIb-IIIa structure, leading to lowering of the dissociation constant (Kd) for its ligands.5 This structural change appears to be intrinsic to the receptor and most likely involves the participation of specific intracellular signaling pathways and possibly cytoskeletal structures.7

Tyrosine kinases are important signal transducers in many cell types.8 Several transmembrane receptors have tyrosine kinase activity (receptor tyrosine kinases). Platelets do not possess the receptor type of tyrosine kinases but do contain intracellular PTKs. Agonist-induced platelet activation results in tyrosine phosphorylation of numerous platelet proteins.9,10 Receptor occupancy of the activated conformation of GPIIb-IIIa is necessary for functioning of certain PTKs.11 It was previously shown that on the surface of aggregating platelets, the GPIIb-IIIa receptor is able to modulate the activity of cytoplasmic tyrosine kinases, in particular pp125^{FAK} and a yet-uncharacterized PTK upstream from pp125^{FAK}.13 Therefore, it is postulated that an active connection exists between the fibrinogen receptor and signaling pathways, particularly those involving tyrosine kinases and their substrates.13 Because agonist-induced activation results in enhanced tyrosine kinase activity and these kinases are signal transducers, we hypothesized that PTKs are necessary for the conformational changes seen in GPIIb-IIIa on activation. Therefore, we investigated further the role of tyrosine kinase–regulated pathways in the expression of the activated form of GPIIb-IIIa on the surface of platelets.
Using two specific tyrosine kinase inhibitors, genistein (which belongs to the isoflavone family) and AG-18, we have shown that PTKs are necessary for agonist-induced platelet aggregation and expression of the activated conformation of GPIIb-IIIa to proceed at a physiological rate. In addition, we analyzed substrates for PTKs in activated platelets, which represent potential regulators of the biochemical reactions leading to GPIIb-IIIa activation.

**Materials and Methods**

**Materials**

Thrombin, bovine serum albumin (BSA), 4,2-(aminoethyl)-benzene sulfonylfluoride HCl (AEBSF), and genistein were obtained from CalBiochem. AG-18, a tyrosine kinase inhibitor of the tyrophostin family, was a generous gift of Dr. Alexander Levitzki, Hebrew University of Jerusalem, Israel. Chymostatin, leupeptin, antipain, pepstatin, collagen solution (type I), 0.1% in 0.1N acetic acid, fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG, prostaglandin E1 (PGE1), ADP, and collagen solution (type I) were from BACHEM Bioproducts, Swiss. Trypsin 136 was purchased from ICN and the monoclonal antibody to p85-PI3-kinase (PI3-kinase), AG-18 stock solution was also suspended in dimethyl sulfoxide, and further dilution of the stock solution in Tyrode’s buffer required sonication (use of Branson Sonifier probe at levels 3 through 5 until a clear solution was obtained). The platelet kinase inhibitors were analyzed for maximal concentrations that are theoretically achievable inside platelets.

**Preparation of Platelets**

Venipuncture was performed on normal human volunteers using a 19-gauge needle without a tourniquet, the first 5 mL of venous blood was discarded, and then a total 25.5 mL of blood was drawn into a plastic syringe containing 4.5 mL of a citrated buffer (mmol/L: citric acid 38, sodium citrate 74.8, and dextrose 136). For experiments involving the effect of aspirin (acetylsalicylic acid) on platelet tyrosine kinase, volunteers were given 325 mg of enteric-coated aspirin daily for 7 days to allow for maximal platelet cyclooxygenase inhibition.

Platelet isolation was performed at room temperature. Platelet-rich plasma was obtained by centrifugation of the citrated blood at 150g for 15 minutes. To reduce platelet activation induced by in vitro manipulations, PGE1 (1 μmol/L) was added to the platelet-rich plasma, and the platelets were pelleted by further centrifugation at 450g for 10 minutes. The platelet-poor plasma was removed, and the platelets were resuspended with Tyrode’s buffer (mmol/L: NaCl 138, KCl 2.9, NaHCO3, 12, NaHPO4 0.36, glucose 5.5, CaCl2 1.8, and MgCl2 0.49) by gently covering the platelet pellet with 3 mL of Tyrode’s buffer and aspirating the buffer without disrupting the cells. The pellet was resuspended in Tyrode’s buffer to provide the platelet concentration required for each type of experiments (10⁷ to 10⁸ cells per milliliter).

**Platelet Activation**

All platelets were studied within 2 hours of phlebotomy. At room temperature, platelets were activated with thrombin (0.25 to 0.5 U/mL), the thrombin receptor activating peptide–Ser-Phe-Leu-Arg-Asn (sflrn, 43 μmol/L), or ADP (20 μmol/L) for various periods of time as indicated. Activation was performed by adding the agonist to the sample, inverting the sample once, and letting the sample rest.

In some experiments, a tyrosine kinase inhibitor (genistein or AG-18) was added to the samples before the addition of agonist. Genistein was suspended in dimethyl sulfoxide and then diluted in Tyrode’s buffer by using sonication when indicated. The incubation time and concentration (600 μmol/L or 162 μg/mL) were selected by performing preliminary experiments with serial dilutions of unsonicated genistein. In these conditions, 600 μmol/L genistein corresponds to the lowest concentration that achieved maximum inhibition of tyrosine kinase activity without disrupting the integrity of the cells (assessed by microscopy; see below) within 90 minutes of phlebotomy. AG-18 stock solution was also suspended in dimethyl sulfoxide, and further dilution of the stock solution in Tyrode’s buffer required sonication (use of Branson Sonifier probe at levels 3 through 5 until a clear solution was obtained). The tyrosine kinase inhibitor concentrations achieved intracellularly in these conditions were not known and may vary. Therefore, the inhibitor concentrations indicated in the present study correspond to the maximal concentrations that are theoretically achievable inside platelets.

After incubation, the cells were immediately lysed by either boiling in sodium dodecyl sulfate (SDS) buffer (2% SDS, 80 mmol/L Tris [pH 6.8], 10% glycerol, 5% 2-mercaptoethanol, trace amount of bromophenol blue, and 1 mmol/L sodium vanadate) or in an ice-cold Triton X-100 buffer (L buffer, containing 145 mmol/L NaCl, 0.1 mmol/L MgCl2, 15 mmol/L HEPES [pH 7.0], 10 mmol/L EGTA, 1 mmol/L sodium vanadate, 1% Triton X-100, and the protease inhibitors AEBSF [25 γL], leupeptin [50 μg/mL], chymostatin [25 μg/mL], antipain [25 μg/mL], and pepstatin [25 μg/mL]). Triton X-100 lysates were sonicated for 15 seconds with a Branson Sonifier probe (at level 2).

**Immunoblotting and Immunoprecipitation**

The Triton X-100–lysed samples normalized for total protein (3 g/L) were incubated with an antibody (monoclonal antibodies anti-phosphotyrosine, anti-PI3-kinase, anti-pp60src, anti-pp125src, and anti-p85-SH3) at 0.1 to 0.2 mg/mL for 5 minutes and then incubated with saturating amounts of protein A–Sepharose beads (50 to 100 μL of pelleted beads per milliliter). Anti-phosphotyrosine immunoprecipitates were obtained using a monoclonal antibody covalently attached to agarose beads (UBI). Samples were gently rotated for 90 minutes at 4°C. The beads were washed four times in L buffer without Triton X-100 or protease inhibitors and then boiled in 60 μL of SDS buffer for 5 minutes. The samples were analyzed by SDS-polyacrylamide gel electrophoresis by use of 4% to 20% gradient gels. The proteins were transferred onto nitrocellulose membrane, and immunoblots were reacted with specific antibodies to 0.2 mg/L in Tris-buffered saline (TBS, containing 20 mmol/L Tris base [pH 7.6] and 137 mmol/L NaCl) and then with a peroxidase-labeled goat anti-mouse antibody (1 mg/L) in TBS, as previously described. Immunoblots were developed using the enhanced chemiluminescence system (Amersham Corp.). The intensity of individual bands on chemilumino grams was quantified by densitometry.

**Platelet Aggregation**

We measured platelet aggregation in whole blood by use of a single-channel impedance meter (Chronolog) and a sample volume of 0.5 mL stirred at 10⁷ rpm. Baseline readings were obtained for 20 minutes to document the quiescent state of the platelets, and agonists were then swiftly pipetted into the
samples. To study the effect of genistein on platelet aggregation, we measured platelet aggregation in response to 0.5 U/mL thrombin in the presence and absence of a 45-minute pretreatment with 600 μmol/L genistein. For each platelet sample, the effect of genistein was expressed as the ratio of the change in impedance, observed 15 minutes after agonist stimulation of a blood sample pretreated with genistein, to the change in impedance of a nonpretreated sample.

Measurement of Activated GPIIb-IIIa Density

Fibrinogen-Binding Assay

This assay is based on the unique ability of activated GPIIb-IIIa to bind fibrinogen in solution on the surface of platelets. Labeling of fibrinogen with FITC, quantification of fibrinogen in solution, and determination of the fluorescein to fibrinogen ratio were performed as reported previously. Platelets (10^7 cells per milliliter) were suspended in Tyrode’s buffer (500 μL) and incubated with FITC-fibrinogen (0.1 mg/mL) with or without an agonist or a tyrosine kinase inhibitor. Samples were analyzed in a fluorescence-activated cell sorter (FACScan, Becton Dickinson), which was calibrated daily for fluorescence by use of Cytoflour fluorescent beads. Data acquisition and processing from 10^5 cells were carried out on a Hewlett Packard computer with FACScan research software (version B). The median channel number was used as the measure of platelet fluorescence intensity. Four concentrations of FITC calibration beads were used to generate a standard curve, which provided the median channel number as a function of fluorescein equivalents present per bead, which are directly proportional to the number of FITC molecules. With this standard curve and the known fluorescein-to-fibrinogen ratio, we were able to determine the number of FITC-fibrinogen molecules bound per platelet: FITC-fibrinogen per platelet=fluorescein equivalents per platelet/fibrinogen equivalents per fibrinogen molecule.

PACI-Binding Assay

PACI is a monoclonal IgM specific for the activated conformation of GPIIb-IIIa. To detect activated GPIIb-IIIa on the surface of platelets, we used fluorescence-activated flow cytometric analysis of PACI binding to platelets. Platelets (~10^7 cells per milliliter) were incubated in Tyrode’s buffer in the presence or absence of a tyrosine kinase inhibitor. After this incubation, the platelets were activated in the presence of a saturating concentration of FITC-PACI (20 to 30 mg/L) and analyzed on a FACScan flow cytometer. A total of 10^5 cells were counted for each experiment. Quantification of PACI binding resulting from platelet activation was performed by subtracting the median of the fluorescence signal corresponding to nonactivated platelets (nonspecific binding) from the signal obtained after agonist activation of platelets in the presence or the absence of genistein.

Immunocytochemistry

Washed platelet suspensions (~10^7 cells per milliliter) were prepared in Tyrode’s buffer as described above. Collagen-coated glass coverslips were prepared by loading a coverslip on top of a drop (200 to 500 μL) of a collagen solution (type I, 0.1% in TBS) spread on a flat paraffin (American National Can) surface for 10 seconds and then air-dried overnight. Platelets were gently pipetted onto the coated side of the coverslip and allowed to adhere for 30 minutes, and the excess cell suspension was removed by gently blotting the edge of the coverslip onto filter paper. Genistein (600 μmol/L) in a volume of 300 μL was then added to cover the adhered platelets on the coverslip for 45 minutes, and the solution was removed by gently blotting the edge of the coverslip onto filter paper. Next, platelets were covered with 300 μL of thrombin (0.25 U/mL) and then fixed after 1 minute by immersion in a solution of 3.7% formaldehyde in phosphate-buffered saline (PBS) for 30 minutes. The excess fixing solution was removed by blotting the edge of the coverslip onto filter paper, and the coverslips were washed by immersing them in a solution of BSA (1.0 g) in 100 mL PBS (BSA-PBS) for 10 minutes with gentle rocking.

To stain cells for intracellular proteins, the cell membranes were permeabilized by incubating the coverslips in 0.2% Triton X-100 in PBS for 10 minutes. Nonspecific binding was blocked with BSA-PBS for 30 minutes, and coverslips were reacted with the primary antibody (monoclonal anti-phosphotyrosine, 2.5 mg/L in PBS) for 60 minutes. The coverslips were washed with PBS for 30 minutes as above, reacted with FITC-labeled goat anti-mouse IgG (5 mg/L) for 60 minutes, and then washed in BSA-PBS for 30 minutes as above. The coverslips were mounted onto glass slides using Vectashield mounting medium (Vector Laboratories), and photomicrographs of representative fields were obtained using a Zeiss microscope and Kodak Ektachrome 400X films.

Results

Effect of Genistein on Platelet Protein Tyrosine Phosphorylation

To test our hypothesis that tyrosine kinase-regulated pathways are necessary for GPIIb-IIIa activation, we began our studies by assessing the ability of genistein to block tyrosine phosphorylation of platelet proteins. Platelets preincubated for 45 minutes with genistein contained markedly reduced concentrations of tyrosine-phosphorylated substrates at both baseline (Fig 1, lane 2) and after activation with thrombin (Fig 1, lane 4), confirming the strong inhibitory activity of genistein for platelet tyrosine kinases. In the absence of genistein, thrombin stimulation of platelets resulted in the tyrosine phosphorylation of several platelet proteins (Fig 1, lane 3). The pattern seen on Western blots developed with anti-phosphotyrosine antibody was similar to that observed by others and to the pattern that we observed after stimulation with a high concentration of ADP (20 μmol/L, not shown). The rather high
concentration of genistein (600 μmol/L) was required to maximally inhibit platelet tyrosine kinases in these experiments within 45 minutes of incubation. However, this concentration of genistein was not lytic to the cells, nor did it inhibit platelet adhesion to collagen, as assessed by microscopic analysis of the cells on collagen-coated coverslips, and further functional studies also supported that the integrity of the platelet was not disrupted (see below).

Inhibition of Platelet Aggregation by Genistein

Genistein and other tyrosine kinase inhibitors such as the tyrophostins are strong inhibitors of platelet aggregation.\(^\text{23,24}\) To correlate the biochemical observation of tyrosine phosphorylation inhibition with intact platelet responsiveness, we analyzed whole-blood aggregation with and without preincubation with genistein (600 μmol/L, Fig 2). A total of 10 normal subjects were studied, and the extent of platelet aggregation for each subject without genistein (1.0) was compared with the extent of aggregation after genistein treatment. For each individual, the presence of genistein strongly reduced the aggregation of platelets. Even in the presence of superphysiological thrombin concentrations (0.5 U/mL), the reduction of platelet aggregation was substantial and averaged 59±13% (mean±SD). This inhibition was nearly as strong as that seen with saturating concentrations of direct GPIIb-IIIa blockers such as the Arg-Gly-Asp (RGD)-like cyclic heptapeptides or inhibitory antibodies (7E3)\(^\text{25,26}\) and was much stronger than the aggregation/inhibition obtained with acetylsalicylates (data not shown). Genistein, therefore, represents a potent inhibitor of platelet aggregation. The mechanism allowing this tyrosine kinase inhibitor to reduce platelet aggregation is unknown.

Effect of Genistein on GPIIb-IIIa Activation

Because aggregation requires activated GPIIb-IIIa receptors on the surface of platelets, we asked whether the antiaggregant effect of genistein could result from the inhibition of GPIIb-IIIa activation. We analyzed the effect of genistein on the activation of GPIIb-IIIa by measuring the binding to platelet surface of the anti-GPIIb-IIIa antibody PAC1, which recognizes specifically the activated conformation of GPIIb-IIIa.\(^\text{20}\) By use of FACScan analysis of PAC1 binding to platelets, genistein resulted in a marked reduction of the expression of activated GPIIb-IIIa receptor (Fig 3a, panel IV). However, this reduction was overcome with time, as progressive recovery of PAC1 binding was observed over the 30 minutes following thrombin stimulation of genistein-treated platelets (Fig 3a, panel V). The effect of genistein on PAC1 binding was concentration dependent and was more pronounced when the thrombin receptor-activating peptide was used instead of thrombin itself (Fig 3b).

To elucidate the mechanism by which the genistein inhibition of activated GPIIb-IIIa was lost over time, we studied the kinetics of the tyrosine phosphorylation reaction triggered by thrombin in platelets pretreated with genistein (Fig 4). Immunoblot analysis demonstrated that the reexpression of the activated conformation of GPIIb-IIIa detected by PAC1 assay (Fig 3a) correlated with the progressive resumption in tyrosine phosphorylation of platelet proteins (Fig 4). Prolonging the duration of platelet exposure to thrombin resulted in an increase in the amount of phosphotyrosine proteins seen in platelet extracts. After 30 minutes of agonist exposure, the amount of phosphotyrosine seen in genistein-treated platelets (Fig 4, lane 6) approached levels (69% according to densitometric analysis) that are seen in non-genistein-treated maximally activated platelets (0.25 U/mL thrombin for 1 minute; Fig 4, lane 7).

In particular, the tyrosine phosphorylation of substrates p110, p95, and p85-SH3 at 30 minutes was nearly as marked as in thrombin-activated platelets in the absence of genistein. Densitometric analysis of the bands corresponding to these three substrates revealed that >75% of the phosphotyrosine signal was recovered at 30 minutes (Fig 4, lane 6), which represents a 5.1- to 10.4-fold increase in tyrosine phosphorylation of these substrates compared with the corresponding signals obtained in genistein-treated unstimulated platelets (Fig 4, lane 1). Thus, the kinetics of tyrosine kinase activity, and in particular the tyrosine phosphorylation of p110, p95, and p85-SH3, closely paralleled the kinetics of the expression of activated GPIIb-IIIa. These data also indicated that the effect of genistein was not to disrupt platelet integrity but instead to reversibly inhibit platelet tyrosine kinase activities and their pathways.

The absence of effect of genistein (a compound related to genistin but without tyrosine kinase–inhibitory activity) on GPIIb-IIIa activation was consistent with our hypothesis that suppression of GPIIb-IIIa activation by genistein was mediated by the tyrosine kinase–inhibitory effect of this molecule (Fig 3b). To test further the specificity of the effect of tyrosine kinase inhibition on activation of GPIIb-IIIa, we used another tyrosine kinase inhibitor belonging to the tyrophostin-family, AG-18, and measured the activation of GPIIb-IIIa by quan-
Identifying the number of FITC-fibrinogen molecules bound to platelets in the presence and the absence of AG-18 and ADP stimulation (20 μmol/L). AG-18 was selected for this study because of its relative specificity for the nonreceptor type of tyrosine kinases.\(^{25}\) Concentration-dependent inhibition of FITC-fibrinogen binding to platelets was observed with AG-18, even when platelets were activated with saturating concentration of ADP (Fig 5).

Identification of Major Substrates for Platelet Tyrosine Kinase

In most cells, tyrosine kinases, whether receptor or cytoplasmic, are known to regulate both growth rate and cytoskeletal organization.\(^{8,28}\) In anucleated cells like platelets, which have lost their potential for proliferation, tyrosine kinases are likely to function as regulators of the actin response to agonists and may affect GPIIb-IIIa activation through changes in the actin superstructure. To test this hypothesis, we analyzed by immunoprecipitation the major substrates for PTK in activated platelets.

We used a battery of antibodies against known platelet proteins that are involved in cytoskeletal reorganization to test whether they correspond to tyrosine kinase substrates in thrombin-activated platelets; these substrates potentially mediate the downregulation of GPIIb-IIIa activation induced by tyrosine kinase inhibitors, pp60\(^{src}\),\(^{29}\) the most abundant PTK in platelets, and pp125\(^{Src}\) are both associated with focal adhesion actin networks and are clearly phosphorylated on tyrosine after thrombin (or ADP) activation (Fig 6). This phosphorylation was inhibited by genistein. PI\(_3\)-kinase has also been suspected to participate in actin cytoskeleton reorganization, and its activity greatly increases in response to agonist stimulation of platelets.\(^{31,32}\) The docking p85 subunit of PI\(_3\)-kinase was phosphorylated on tyrosine in response to agonists, and this phosphorylation was inhibited by genistein. Similar results were seen for p85-SH3, a protein whose function is unknown but which contains a putative actin cytoskeleton–binding SH3 domain and colocalizes with actin in cells.\(^{33}\) In contrast, when GPIIb-IIIa itself was immunoprecipi-
tated from resting and activated platelets, direct phosphorylation on tyrosine of the receptor subunits could not be detected, nor could phosphoproteins be coprecipitated with the receptor (data not shown).

Fig 4. Time course of tyrosine phosphorylation of proteins in platelets pretreated with genistein. Platelet samples matching those analyzed by FACSscan (Fig 3a) were analyzed for their phosphotyrosine content. Conditions for Western blots were as described for Fig 1. Note that genistein inhibition is progressively overcome in activated platelets with increasing time of exposure to agonist. The increase in protein phosphorylation in activated cells treated with genistein matches the time course of activation of glycoprotein Ib-IIIa as measured by monoclonal antibody PAC1–fluorescence-activated cell sorter assay.

Fig 5. Bar graph showing inhibition of fluorescein isothiocyanate (FITC) fibrinogen binding to ADP-activated platelets with the tyrophostin AG-18. FITC-fibrinogen binding assay was used to measure the effect of AG-18, a tyrosine kinase inhibitor of the tyrophostin family, on the activation of glycoprotein Ib-IIIa. Washed platelets (105 cells) were exposed to serial dilutions of AG-18 in Tyrode’s buffer for 15 minutes in the presence of FITC-fibrinogen (0.1 mg/mL) before stimulation with ADP (20 μmol/L for 15 minutes). The data correspond to the mean (n=3) number of FITC-fibrinogen molecules bound per platelet in each condition. AG-18 inhibited FITC-fibrinogen binding to platelets maximally activated with ADP, and this inhibition is concentration dependent.

Fig 6. Identification of major substrates for protein tyrosine kinases in activated platelets by immunoprecipitation. Platelets were pretreated in Tyrode’s solution with and without genistein (800 μmol/L) for 45 minutes and then activated with thrombin (0.25 U/mL) for 1 minute. At the end of this incubation, the cells were lysed in Triton X-100 buffer. The extracts were incubated with the indicated monoclonal antibodies, and immune complexes (corresponding to 50 μg of total platelet proteins) were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blotting. Note that the tyrosine phosphorylation of pp125Fak, the p85 subunit of phosphatidylinositol 3-kinase (PI3-kinase), SH3-p85 and pp60src is increased in thrombin-activated platelets. This phosphorylation can be prevented by genistein pretreatment.

Detection of Tyrosine Kinase Substrates in Permeabilized Platelets

Immunohistochemistry was performed on platelets adhering to collagen-coated coverslips with and without thrombin activation and genistein inhibition to detect the proteins phosphorylated by platelet PTKs (Fig 7). Thrombin activation resulted in the formation of cellular extensions,34 and these extensions were rich in tyrosine-phosphorylated substrates, considering the relatively small volume of these extensions and the intensity of their staining for phosphotyrosine. These experiments indicated that genistein at the concentration used (600 μmol/L, which was enough to block the phosphorylation on tyrosine of platelet substrates) did not detectably alter platelet integrity, since the adhesion of platelets to collagen-coated coverslips was not abolished by genistein pretreatment. It is possible that extension formation, which requires cytoskeletal reorganization,34 was inhibited by genistein. This effect correlates with the decrease in tyrosine phosphorylation of platelet proteins (Fig 7). However, immunocytochemical data are semiquantitative, and additional studies will be necessary to conclude that genistein inhibited the actin response to agonists.

Discussion

We provide evidence that phosphorylation of specific tyrosine kinase substrates in activated platelets is necessary to allow the rapid conformational change of GPIIb-IIIa to occur. Change in GPIIb-IIIa conformation on the platelet surface is necessary for binding to soluble fibrinogen, thus allowing fibrinogen-mediated bridge formation between thrombocytes. Besides being an adhesive molecule, GPIIb-IIIa receptor also functions as a signal modulator. Recent publications suggest that GPIIb-IIIa cross-linking by fibrinogen (one fibrin-
ogen molecule linking two molecules of GPIIb-IIIa within the same cell) may induce the activation of a specific tyrosine kinase functioning upstream from pp125c-src. Thus, receptor occupancy of GPIIb-IIIa may be necessary for PTK-induced signal transduction.

Our data suggest a reverse role for tyrosine kinases in fibrinogen-receptor functioning. The correlation between the time course of GPIIb-IIIa activation and tyrosine phosphorylation of platelet proteins provides further support for the concept that PTKs are intricately involved in GPIIb-IIIa functioning. This observation is consistent with data observed with tyrophostin-23 on C.b-9-permeabilized platelets. Although it is unlikely that genistein and AG-18, or any of the tyrosine kinase inhibitors, are entirely specific for tyrosine kinases, the following facts support the concept that the mechanism of inhibition corresponds to tyrosine kinase downregulation: (1) GPIIb-IIIa activation inhibition was obtained in two separate groups of experiments using two different tyrosine kinase inhibitors and different assays for measuring GPIIb-IIIa activation. (2) Genistein, a compound which resembles to genistein but has no tyrosine kinase activity, did not suppress GPIIb-IIIa activation.

Both platelet activation and GPIIb-IIIa activation are linked to cytoskeletal changes, and PTKs appear to play a pivotal role in these changes. A fraction (20% to 30%) of platelet GPIIb-IIIa has been shown to form a stable complex with the Triton X-100-insoluble actin cytoskeleton in activated platelets. We confirm that two thrombin-activated platelet PTK substrates are themselves PTKs, pp125c-src and pp60c-src. These two PTKs have been found in many cells to be associated with focal adhesions. Focal adhesions correspond to specialized areas of contact between the plasma membrane and the extracellular matrix, which results from the interaction of an integrin (such as GPIIb-IIIa) with a protein of the extracellular matrix (like fibrinogen). Focal adhesions also contain proteins that connect the integrin with actin stress fibers at the cytoplasmic aspect of the membrane. pp60c-src is the most abundant PTK in platelets and is constitutively phosphorylated on tyrosine. Phosphorylation of tyrosine residue 527 (Tyr-527) of Src is known to inhibit pp60c-src tyrosine kinase activity. It is likely that the activity of pp60c-src is regulated by the balance between specific PTKs and tyrosine phosphatases. Whether pp60c-src (or isoenzymes thereof) represents the PTK mediating the tyrosine phosphorylation of specific substrates in response to fibrinogen cross-linking of GPIIb-IIIa has yet to be demonstrated.

pp60c-src has been shown to be associated with another cytoskeleton-associated enzyme, PI_2-kinase. We show that the p85 subunit of PI_2-kinase is tyrosine-phosphorylated on activation of platelets with thrombin and that this phosphorylation is inhibited with genistein. PI_2-kinase phosphorylates inositol phospholipids on the third carbon of the inositol ring. In other systems, the activity of this enzyme has been connected to actin cytoskeleton reorganization. The mechanism mediating PI_2-kinase control on the actin cytoskeleton is not yet understood but is likely to involve the known interaction of actin-regulatory proteins with inositol phospholipids. It was shown that genistein also inhibits the genesis of membrane phosphoinositides such as phosphatidylinositol 4,5-diphosphate and prevents specific actin functions. Together, these data suggest that genistein and AG-18 inhibition of GPIIb-IIIa activation may be mediated by the alteration of the metabolism of membrane inositol phospholipids after agonist stimulation of platelets and possibly by the perturbation of the actin cytoskeleton reorganization resulting from the abnormal metabolism of membrane phospholipids.

SH3 domains, like the one found on p85-SH3, have been shown to target proteins to specific cellular sites, including actin filaments. Accordingly, the distribution of p85-SH3 has been shown to match the distribution of filamentous actin. By immunoprecipitation, we found...
that p85-SH3 represents a major substrate for PTKs in thrombin-activated platelets. Although it is not known whether p85-SH3 serves as a regulatory protein for the stability and/or the cross-linking of actin filaments, its strong tyrosine phosphorylation on platelet activation, its association with the actin cytoskeleton, and the presence of an SH3 domain within its sequence support the hypothesis that p85-SH3 plays an important role in linking the activity of agonist-activated PTKs and the actin cytoskeleton.

Although the present study provides new clues concerning the role of platelet PTKs in the expression of high-affinity fibrinogen receptors on the surface of activated platelets, key questions remain to be answered. (1) Which platelet tyrosine kinase activity/substrate represents the limiting step slowing down the expression of activated GPIIb-IIIa upon addition of genistein? pp60^src, with its high platelet concentration, focal adhesion subcellular distribution, and association with the actin cytoskeleton, appears as a likely candidate; however, transgenic mice lacking pp60^src did not display evidence of bleeding disorders nor any gross platelet abnormalities.\(^\text{45}\) Furthermore, it is noteworthy that the activity of isoenzymes of pp60^src also present in platelets may actually be able to overcome the deficient pp60^src function. Additional studies directly linking substrate phosphorylation to GPIIb-IIIa activation are under way. (2) How does the actin cytoskeleton control the expression of activated GPIIb-IIIa? We observed that cytochalasin D, a known actin inhibitor, reduces by ~50% the density of the binding site for soluble fibrinogen on the surface of maximally activated platelets (J.B. Addo, N. Faraday, D. Grigoryev, P.F. Bray, and P.J. Goldschmidt-Clermont, unpublished data). However, the molecular reactions linking the actin cytoskeleton to GPIIb-IIIa activation and their putative regulation by platelet PTKs are still unknown. Further studies on the function of p85-SH3 in actin cytoskeleton organization may provide additional clues about these reactions.

Nevertheless, it appears that platelet PTKs are modulated at the two ends of the platelet-activating effector system. Clearly, agonist stimulation results in tyrosine phosphorylation of numerous platelet proteins, leading to cytoskeletal reorganization and changes in GPIIb-IIIa conformation that allows this receptor to bind soluble fibrinogen and thereby induce the aggregation of platelets. However, binding of fibrinogen to its receptor is also necessary for additional PTK activity.\(^\text{11,13}\)

Since intricate regulation of activating signals and responsive effects is necessary for proper platelet function only at site where a platelet thrombus is needed, it is not surprising that PTKs are bidirectionally used in the signal-effector mechanism of platelet aggregation. Thus the PTK signaling system of platelets, with its dual regulation through integrins and agonist receptors, may serve to integrate stimuli and generate a thrombotic response only at sites where it is required, such as the damaged vessel wall. However, given the potential for positive reinforcement in such a signaling system, a perturbation in the tyrosine kinase/phosphatase balance may cause a lower threshold for a stimulus to lead to aggregation at sites and times where normally aggregation would not occur.

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**References**


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