Platelets express a single low affinity receptor for immunoglobulin, FcγRII, that triggers multiple cellular responses upon interaction with multivalent immune complexes. In this study we show that immobilized IgG is also a potent stimulant of platelet activation triggering adhesion, aggregation, massive dense granule secretion, and thromboxane production. Platelet adhesion to IgG was blocked by the FcγRII receptor-specific monoclonal antibody, IV.3. Pretreatment of the platelets with cytochalasin D to inhibit actin polymerization similarly prevented cell binding to IgG having no effect on platelet binding to fibrinogen. Platelet adhesion to IgG also led to the induction of tyrosine phosphorylation of multiple proteins including pp125FAK and p72SYK. These proteins were also tyrosine-phosphorylated in αIbβ3-deficient IgG-adherent platelets from patients with Glanzmann’s thrombasthenia. These data demonstrate that FcγRII mediates pp125FAK phosphorylation and platelet adhesion to IgG independent of the integrin αIbβ3. Treatment of the platelets with bisindolylmaleimide to inhibit protein kinase C prevented phosphorylation of pp125FAK as well as several other proteins, but not p72SYK phosphorylation. This study establishes that the FcγRII receptor mediates pp125FAK phosphorylation via protein kinase C.

The mechanism by which Fcγ receptors trigger SYK phosphorylation and activation is not fully understood. Based on model(s) proposed for multichain immune recognition receptors such as the T cell antigen receptor, TCR (3, 9, 11–13), FcγRII ligation most likely affects several sequential events. First among these is an association between FcγRII with a Src family member that triggers transient FcγRII receptor phosphorylation. An association between FcγRII and the Src family member p56/53Lck was detected in monocytic THP-1 cells (14) and human B lymphocytes (15, 16), whereas in natural killer cells Fcγ receptor ligation initiated activation of Lck followed by Syk phosphorylation (17). In platelets activated by immune complexes, FcγRII appeared associated with Src (6) but since FcγRII phosphorylation was observed in Src-deficient mice (18) another kinase may also affect FcγRII phosphorylation. Phosphorylation may enable the FcγRII receptor to recruit the p72SYK kinase, through an interaction between the FcγRII-ARAM motif and the p72SYK SH2 binding domain, as a result of which p72SYK will become phosphorylated and active (3).

Another yet unresolved issue is the identification of downstream effector targets of p72SYK. This issue is rather complex because distinct cellular functions are likely to be defined by substrate specificity. We are interested in substrates that are potentially involved in the regulation of cell shape and spreading. Both of these functions are frequently regulated by members of the integrin adhesion receptor family. Integrins are transmembrane heterodimers that interact both with extracellular matrix and cytoskeletal proteins (for reviews see Refs. 19–21). Recent studies have implicated p72SYK in signal transduction downstream from integrins. Induction of p72SYK phosphorylation was detected upon adhesion of THP-1 cells to fibrinectin (22). Platelet interaction with fibrinogen or collagen, mediated by the integrin receptors αIbβ3 (GP IIb-IIIa) and αIIbβ3, respectively, similarly triggered p72SYK phosphorylation (23).

In most cell systems studied thus far, integrin receptor ligation triggers the induction of tyrosine phosphorylation of a 125-kDa protein, itself a tyrosine kinase localized in focal adhesion plaques, pp125FAK (for review see Ref. 24). It is not clear at present whether there is a direct connection between p72SYK phosphorylation and these downstream functions of pp125FAK.
and pp125FAK phosphorylation and activation. In the THF-1 cells, inhibition of actin polymerization by cytochalasin D prevented pp125FAK phosphorylation but not p72SYK phosphorylation (22). Treatment of the platelets with cytochalasin D similarly prevented pp125FAK (23) but not p72SYK phosphorylation (2, 23). In addition, both in THF-1 cells and platelets, integrin receptor ligation effectively triggered p72SYK phosphorylation but not pp125FAK phosphorylation (18, 23). These data suggest that if the two events are sequentially linked, p72SYK activation may be an earlier event than pp125FAK phosphorylation.

In the present study we examined whether FcγRII receptors-mediated signals in platelets involve pp125FAK phosphorylation. Our data demonstrate that FcγRII mediates platelet adhesion to immobilized IgG and the induction of pp125FAK phosphorylation independent of the integrin αIIbβ3. We have also found that protein kinase C regulates pp125FAK phosphorylation, but not p72SYK phosphorylation downstream from FcγRII.

MATERIALS AND METHODS

Reagents—Rabbit polyclonal antisera BC3 and BR12 were used to immunoprecipitate pp125FAK and p72SYK, respectively. A rabbit polyclonal antisera to p72SYK was used for immunoblotting. Monoclonal antibody IV.3(1) was from Meredex Inc. Monoclonal antibody 4G10 was from Upstate Biotechnology Inc. Bisindolylmaleimide (BIS; G 1092003X) (25) and thapsigargin were from Calbiochem. Fibrinogen, apyrase, PGI2, indomethacin, and BSA were from Sigma. Human IgG was purchased from Sigma and Pierce. BAPTA-AM and Fura-2-AM were purchased from Molecular Probes. Cytochalasin D (CD) was from Aldrich. Bisindolylmaleimide, BAPTA-AM, thapsigargin, and CD were dissolved in Me2SO. The final Me2SO concentration was 0.5%. Untreated polyalbumin; mAb, monoclonal antibody; BAPTA, 1,2-bis(2-aminophen基ether)-N,N,N’-tetraacetic acid; Me2SO, dimethyl sulfoxide.

Platelet Preparation—Human platelets were isolated by gel filtration from freshly drawn citrated whole blood using a histopaque gradients. Packed platelet volume was estimated by measuring platelet counts and 1 ml of Nihon formula A acid-citrate-dextrose solution supplemented with 1 μM PGE1, and 1 unit/ml apyrase as described previously (26). Platelet concentration was adjusted to 2–5 × 10^9 platelets per ml in an incubation buffer containing 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl2, 5.6 mM glucose, 1 mg/ml BSA, 3.3 mM NaH2PO4, and 20 mM HEPES, pH 7.4. Platelet adhesion to IgG (50 μg/ml), Fc-1gG (50 μg/ml), or FBGN (100 μg/ml) was studied in polystyrene plates precoated with the specific protein and blocked with BSA as described previously (26). Platelets were added to the IgG-, FBGN-, Fc-1gG-, or BSA-coated plates for 60 min at room temperature. Adherent cell morphology was examined by scanning electron microscopy (27).

To examine the effect of the inhibitor on platelets, gel-filtered platelets were incubated with 12 μM Bis (1 h), 12 μM CD (10 min), 1 μM prostaglandin I2 (PGI2) (1 min), 10 μM indomethacin (10 min), or with 0.5% Me2SO alone (Me2SO vehicle) (1 h). When the effect of BAPTA-AM was studied, platelets in plasma were incubated with 100 μM BAPTA-AM at 37°C for 30 min and gel-filtered. To examine the effect of BAPTA-AM on intracellular Ca2+ plateau in plasma were loaded in some experiments with 100 μM Fura-2-AM for 30 min at 37°C followed by BAPTA-AM or Me2SO alone for 30 min before gel filtration (28). Changes in Ca2+ were monitored at a wavelength pair of 340 nm/510 nm in a Perkin-Elmer LS-58 Luminescence spectrophotofluorimeter. To specifically effect the Ca2+ concentration, the endomembranous Ca2+-ATPase, thapsigargin which rapidly induces calcium mobilization from the platelet intracellular stores (29) and 1 mM CaCl2 were sequentially added to the platelet suspension, and recording was continued after each addition. Treatment of the platelets with thapsigargin triggered an abrupt increase in [Ca2+], that was further augmented with the addition of CaCl2. This biphasic response was not detected in the BAPTA-AM-loaded platelets demonstrating that BAPTA was able to prevent [Ca2+]i (data not shown).

Identification of Phosphotyrosine-containing Proteins—Protein tyrosine phosphorylation was studied as described previously (26).

1 The abbreviations used are: BIS, bisindolylmaleimide; PGI2, prostaglandin I2; FBGN, fibrinogen; CD, cytochalasin D; BSA, bovine serum albumin; mAb, monoclonal antibody; BAPTA, 1,2-bis(2-aminophen基ether)-N,N,N’-tetraacetic acid; Me2SO, dimethyl sulfoxide.

RESULTS

Effect of Immobilized IgG on Platelets—Unstimulated gel-filtered platelets were exposed to IgG coated on a polystyrene surface. Suspended platelet aggregates of various sizes were observed within 10 min of platelet exposure to the IgG-coated surfaces. Adherent platelet aggregates were observed shortly thereafter (Fig. 1A). Binding to IgG was prevented by the FcγRII receptor mAb, IV.3(1). Adherent platelet aggregates were also observed on surfaces coated with Fc-IgG at the same concentration (data not shown).

We assumed that platelet aggregation on IgG was indicative of granule secretion and/or activation of the cytoxogenease pathway leading to thromboxane production. To assess dense granule secretion, [14C]serotonin-loaded platelets were exposed to IgG-, BSA-, or FBGN-coated surfaces, and serotonin release into the medium was monitored after 1 h. Platelets adherent to IgG released 4.1 ± 1.33 times more [14C]serotonin than platelets exposed to a BSA-coated surface but, as previously shown (26), platelet adherence to FBGN did not cause detectable serotonin release (1.26 ± 0.34 relative to platelets exposed to
BSA-coated surfaces). When compared with the release triggered by pretreatment of the platelets with 1 unit/ml thrombin for 1 min before exposure to the IgG-coated surface, the untreated IgG-adherent platelets released 59% ± 10% (n = 4) of their dense granule content.

To measure thromboxane production supernatants of untreated platelets exposed for 1 h to IgG-, FBGN-, or BSA-coated surfaces were collected and assayed for thromboxane B2, a stable analogue of thromboxane A2 (30). Platelets pretreated with indomethacin, a cyclooxygenase inhibitor, and exposed to the BSA-coated surfaces for 1 h served as controls. Untreated platelet interaction with BSA or adherence to FBGN caused no detectable thromboxane release. In contrast, platelet adhesion to IgG-coated surfaces triggered massive thromboxane production (308 ± 143 relative to platelets exposed to BSA-coated surfaces) that was completely inhibited by indomethacin (1 unit/ml thrombin relative to platelets exposed to BSA-coated surfaces). Platelets adherent to IgG released on the average 10 times more thromboxane than FBGN-adherent platelets prestimulated with 1 unit/ml thrombin for 1 min. These results indicate that immobilized IgG cannot only support platelet adhesion but also is a potent stimulant of granule secretion and thromboxane production.

Intracellular Signaling Components and/or Pathways That Regulate Platelet Adhesion to IgG—For these studies, platelets were pretreated with several specific and well characterized inhibitors that include bisindolylmaleimide (BIS), a specific protein kinase C inhibitor (25), indomethacin, PGI2, a prostacyclin that increases cAMP and blocks activation in response to a variety of agonists (31), and cytochalasin D (CD) which blocks actin polymerization in agonist-stimulated platelets and spreading on FBGN (26). As shown in Fig. 2, pretreatment of the platelets with indomethacin, PGI2, or BIS caused a partial yet statistically significant decrease in platelet binding to IgG (65% ± 5%, 69% ± 4%, and 56% ± 4%, respectively). Bisindolylmaleimide, but not indomethacin or PGI2, blocked aggregation (Fig. 1B) and reduced secretion by more than 50%. These results are consistent with the previously observed effects of BIS on platelet aggregation and secretion (25). Treatment of the platelets with CD caused an even greater decrease in platelet binding to IgG (65% ± 5% and 69% ± 4%, and 56% ± 4%, respectively). Bisindolylmaleimide, but not indomethacin or PGI2, blocked aggregation (Fig. 1B) and reduced secretion by more than 50%. These results are consistent with the previously observed effects of BIS on platelet aggregation and secretion (25). Treatment of the platelets with CD caused an even greater decrease in platelet binding to IgG (65% ± 5% and 69% ± 4%, and 56% ± 4%, respectively). Bisindolylmaleimide, but not indomethacin or PGI2, blocked aggregation (Fig. 1B) and reduced secretion by more than 50%. These results are consistent with the previously observed effects of BIS on platelet aggregation and secretion (25). Treatment of the platelets with CD caused an even greater decrease in platelet binding to IgG (65% ± 5% and 69% ± 4%, and 56% ± 4%, respectively). Bisindolylmaleimide, but not indomethacin or PGI2, blocked aggregation (Fig. 1B) and reduced secretion by more than 50%.
were exposed for 1 h to BSA-, IgG-, or FBGN-coated surfaces. To examine whether pp125FAK was phosphorylated in platelets adherent as compared to the FBGN-adherent platelet lysates (Fig. 3, A–C). A higher intensity of the tyrosine-phosphorylated p72SYK band was consistently detected in the IgG-adherent platelets, these specific proteins were immunoprecipitated from BIS- or BAPTA-AM-pre-treated platelets. Treatment with BIS prevented the detection of a phosphorylated pp125FAK protein but did not affect p72SYK phosphorylation while BAPTA-AM had no effect on tyrosine phosphorylation of either pp125FAK or p72SYK (Fig. 5). Treatment of the platelets with indomethacin or PGI2 also had no effect on pp125FAK phosphorylation. These data indicated that p72SYK phosphorylation may be necessary but it is certainly not sufficient to trigger pp125FAK phosphorylation.

Platelet adhesion to the IgG-coated surface stimulated tyrosine phosphorylation of multiple proteins, most of which displayed an electrophoretic mobility similar to that of proteins detected in lysates of FBGN-adherent platelets (Fig. 6). However, tyrosine phosphorylation of a 72-kDa protein, migrating with an electrophoretic mobility similar to p72SYK, a 47- and 44-kDa doublet, and a broad 39–42-kDa band was predominantly observed in the IgG-adherent platelets (Fig. 6). Tyrosine phosphorylation of proteins of 105 and 101 kDa and of proteins smaller than 47 kDa was sensitive to pretreatment of the platelets with BIS (Fig. 6, lanes 3 and 7). Pretreatment of the platelets with indomethacin or PGI2 had no detectable effects on the induction of protein tyrosine phosphorylation (Fig. 6, lanes 6 and 7, respectively). These data suggested that adhesion to IgG triggers protein tyrosine phosphorylation partially dependent on protein kinase C activation but independent of thromboxane production.

**DISCUSSION**

Platelet adhesion to IgG-coated surfaces triggered tyrosine phosphorylation of p72SYK and pp125FAK. These specific pro-

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![Figure 3](image-url)  
**Fig. 3.** Induction of tyrosine phosphorylation of pp125FAK and p72SYK in platelets adhering to IgG matrix. Gel-filtered platelets were exposed for 1 h to BSA-, IgG-, or FBGN-coated surfaces. In A, the platelets (nonadherent to BSA or adherent to IgG or FBGN) were lysed in radiimmunoprecipitation buffer, and lysates were incubated with a polyclonal antiserum against p72SYK (lanes 2, 4, and 6), pp125FAK (lanes 3, 5, and 7), or normal rabbit serum (NRS) (lanes 1 and 8). In B and C, platelets adherent to IgG (lanes 1 and 3) or to FBGN (lane 2) were lysed, and the lysates were incubated with a normal rabbit serum (lane 1) or a polyclonal antiserum against p72SYK (lanes 2 and 3). Immunoprecipitated proteins were analyzed by immunoblotting with mAb4G10 (A and B) or a polyclonal antiserum against p72SYK (C). Molecular weight markers are marked on the left. Arrowheads indicate the location of pp125FAK and p72SYK.

![Figure 4](image-url)  
**Fig. 4.** Induction of tyrosine phosphorylation of pp125FAK and p72SYK in normal and α₁β₃-deficient platelets adhering to IgG. Gel-filtered platelets from a normal (N) donor (lanes 1–3) or a patient with Glanzmann’s thrombasthenia (G) (lanes 4–6) were exposed to collagen (COL) (lanes 1 and 6) or IgG (lanes 2–5)–coated surfaces for 1 h. Adherent platelets were lysed in radiodimmunoprecipitation buffer, and lysates were incubated with a polyclonal antiserum against pp125FAK (A), lanes 1, 2, 4, and 6) or p72SYK (B and C, lanes 3 and 5). Immunoprecipitated proteins were analyzed by immunoblotting with mAb4G10 (A and B) or a polyclonal antiserum against p72SYK (C). Molecular weight markers are marked on the left.
Fig. 5. Effect of bisindolylmaleimide and BAPTA-AM on the induction of pp125FAK and pp72SYK tyrosine phosphorylation. Platelets were treated for 30 min with 0.5% Me2SO (lanes 1-5) or 100 µM BAPTA-AM (lanes 6 and 7) and gel-filtered. The platelets were next treated for 1 h with 0.5% Me2SO (lanes 3 and 7) or 12 µM BIS (lanes 4 and 5) and then exposed to IgG-coated surfaces for 1 h. Adherent platelets were lysed in radiimmune precipitation buffer, and lysates were incubated with a polyclonal antiserum against pp72SYK (lanes 3, 5, and 7), pp125FAK (lanes 2, 4, and 6), or normal rabbit serum (NRS) (lane 1). Immunoprecipitated proteins were analyzed by immunoblotting with mAb 4G10. Molecular mass markers are marked on the left. The arrowheads indicate the location of pp125FAK and pp72SYK.

Fig. 6. Induction of protein tyrosine phosphorylation in platelets adhering to IgG matrix. Gel-filtered platelets were untreated (lanes 1, 2, 4, and 5) or treated for 1 h with 12 µM BIS (lanes 3 and 7), for 10 min with 10 µM indomethacin (INDO) (lane 6), or for 1 min with 1 µM PGI2 (lane 8) and were then incubated for 1 h on FBGN (lanes 1 and 4) or IgG-coated surfaces (lanes 2-3 and 5-8). To examine the induction of protein tyrosine phosphorylation, adherent platelet lysates were analyzed by immunoblotting with mAb 4G10. Molecular masses (in kilodaltons) are denoted on the left, and the center arrows indicate the location of the 105-, 101-, 72-, 47-, 44-, and 42–39-kDa proteins, respectively. The 72-, 47-, 44-, and 42–39-kDa proteins were well resolved in some but not in other experiments (compare left and right panels). Protein tyrosine phosphorylation was also observed following treatment of fibronectin-adherent RBL-2H3 cells with FcεRII-divalent antibodies. No phosphorylation was induced, however, by the aggregation of the FcεRI receptor in suspended cells (36, 37). In mouse macrophages, FcγRIII and FcγRI receptor-mediated phagocytosis was associated with induction of p72SYK, but not pp125FAK tyrosine phosphorylation (38). The present study is thus the first to demonstrate that an immunoglobulin receptor can directly signal pp125FAK phosphorylation.

Both in platelets and in neutrophils the cytoskeleton assembly is a required component of the Fc receptor-mediated signaling pathways. Treatment of the neutrophils with CD prevented the actin filaments assembly and phagocytosis suggesting that these events may be functionally linked (39). Kang et al. (5) have similarly shown that pretreatment of platelets with cytochalasin B blocked phosphatidic acid accumulation, intracellular Ca2+ increase, p47 and p20 phosphorylation, serotonin release, and aggregation, induced by heat-aggregated IgG. Consistent with these data we observed that CD prevents platelet binding to IgG. Integrin-mediated tyrosine phosphorylation of pp125FAK in platelets is sensitive to CD treatment (26, 32). pp125FAK may regulate the cytoskeleton assembly through its interaction with actin-binding proteins or indirectly by phosphorylation of other regulatory proteins (20, 21, 24). If pp125FAK phosphorylation is essential for platelet adhesion to an IgG-coated surface, inhibitors of pp125FAK phosphorylation are expected to prevent adhesion. Our studies with BIS, however, indicate that inhibition of pp125FAK phosphorylation does not prevent platelet binding to IgG. Preliminary studies with human erythroleukemia (HEL) cells similarly suggest that pp125FAK phosphorylation is not required for cell binding to IgG. HEL cells express both the FcγRII and α1β2 receptor (40, 41). pp125FAK phosphorylation was detected in phorbol 12-myristate 13-acetate-treated HEL cells adherent to FBGN.2 In contrast, pp125FAK phosphorylation was not observed in either untreated or phorbol 12-myristate 13-acetate-treated IgG-adherent HEL cells. A tyrosine-phosphorylated pp72SYK protein was, however, detected in the same lysates. Taken together, these data suggest that adhesion to IgG is regulated in a manner independent of pp125FAK phosphorylation.

Tyrosine phosphorylation of p72SYK has been demonstrated in thrombin-stimulated platelets (33, 42), following FcγRII receptor clustering (2), or when the platelet α1β2 or α2β1 integrin receptors were engaged (23). This phosphorylation event was insensitive to the presence of CD or EGTA (2). In contrast, translocation of the tyrosine-phosphorylated p72SYK kinase to the Triton X-100-insoluble fraction that is stimulated in platelets by thrombin was affected by CD (23, 42). These data raise the possibility that p72SYK plays a role in linking the Fc receptor to the actin cytoskeleton and that severing this link with CD interferes with many of the Fc receptor-mediated functions. A second possibility, not exclusive of the former, is that once the p72SYK and Fc receptor complex is formed, additional signaling components may become activated. In mouse macrophages (43) or the human monocytic cell line THP-1 (44), for example, FcγRIIA ligation triggers tyrosine phosphorylation of several proteins including Shc, GTPase-activating protein, phospholipase C-γ2 and Vav. Shc and GTPase-activating protein activation may initiate activation of Ras-dependent pathway(s) while phospholipase C-γ1 activation can trigger diacylglycerol and inositol 1,4,5-trisphosphate formation from phosphatidylinositol 4,5-biphosphate, leading to protein kinase C activation and release of intracellular calcium (45–48). In concert, these secondary signaling mediators may affect the cytoskeleton assembly. Similar mechanisms...
may explain the Fc receptor ability to respond to immobilized IgG as an adhesion receptor to a matrix ligand.

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