Adhesive Ligand Binding to Integrin \( \alpha_{\text{IIb}}\beta_3 \) Stimulates Tyrosine Phosphorylation of Novel Protein Substrates before Phosphorylation of \( \text{pp125}^{\text{FAK}} \)

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Abstract. Tyrosine phosphorylation of multiple platelet proteins is stimulated by thrombin and other agonists that cause platelet aggregation and secretion. The phosphorylation of a subset of these proteins, including a protein tyrosine kinase, \( \text{pp125}^{\text{FAK}} \), is dependent on the platelet aggregation that follows fibrinogen binding to integrin \( \alpha_{\text{IIb}}\beta_3 \). In this report, we examined whether fibrinogen binding, per se, triggers a process of tyrosine phosphorylation in the absence of exogenous agonists. Binding of soluble fibrinogen was induced with Fab fragments of an anti-\( \beta_3 \) antibody (anti-LIBS6) that directly exposes the fibrinogen binding site in \( \alpha_{\text{IIb}}\beta_3 \). Proteins of 50–68 kD and 140 kD became phosphorylated on tyrosine residues in a fibrinogen-dependent manner. This response did not require prostaglandin synthesis, an increase in cytosolic free calcium, platelet aggregation or granule secretion, nor was it associated with tyrosine phosphorylation of \( \text{pp125}^{\text{FAK}} \). Tyrosine phosphorylation of the 50–68-kD and 140-kD proteins was also observed when (a) fibrinogen binding was stimulated by agonists such as epinephrine, ADP, or thrombin instead of by anti-LIBS6; (b) fragment X, a dimeric plasmin-derived fragment of fibrinogen was used instead of fibrinogen; or (c) \( \alpha_{\text{IIb}}\beta_3 \) complexes were cross-linked by antibodies, even in the absence of fibrinogen. In contrast, no tyrosine phosphorylation was observed when the ligand consisted of monomeric cell recognition peptides derived from fibrinogen (RGDS or \( \gamma^{400-411} \)). Fibrinogen-dependent tyrosine phosphorylation was inhibited by cytochalasin D. These studies demonstrate that fibrinogen binding to \( \alpha_{\text{IIb}}\beta_3 \) initiates a process of tyrosine phosphorylation that precedes platelet aggregation and the phosphorylation of \( \text{pp125}^{\text{FAK}} \). This reaction may depend on the oligomerization of integrin receptors and on the state of actin polymerization, organizational processes that may juxtapose tyrosine kinases with their substrates.

INTEGRINS are a family of heterodimeric (\( \alpha\beta \)) adhesion receptors that mediate cell–cell and cell–matrix interactions (32, 53). Their expression on human platelets is critical for hemostasis because they mediate the formation of platelet plugs at sites of vascular injury (28, 35, 43, 45). One of these integrins, \( \alpha_{\text{IIb}}\beta_3 \), can recognize several adhesive ligands containing the arginine-glycine-aspartic acid (RGD) cell recognition sequence (e.g., fibrinogen, von Willebrand factor, fibronectin, vitronectin). Ligand binding to \( \alpha_{\text{IIb}}\beta_3 \) promotes two key aspects of platelet plug formation. First, the binding of plasma fibrinogen or von Willebrand factor is essential for platelet-to-platelet aggregation (3, 12). Second, interaction of \( \alpha_{\text{IIb}}\beta_3 \) with immobilized von Willebrand factor or fibrinogen results in platelet spreading on the adhesive surface (46). Platelet spreading is associated with the assembly of actin filaments and other cytoskeletal proteins beneath the plasma membrane (25, 40). These assemblies may be analogous to the focal adhesions observed in cultured fibroblasts, sites at the cell-substratum interface where integrins cluster and link extracellular adhesive ligands with the actin-rich cytoskeleton (58).

Integrin \( \alpha \) and \( \beta \) subunits each contain a large, globular extracellular domain, a single \( \alpha \)-helical membrane-spanning domain and a relatively short cytoplasmic tail (31). In the case of \( \alpha_{\text{IIb}} \) and \( \beta_3 \), 95% of each subunit is extracellular, while the cytoplasmic tails contain only 20 and 41 amino acids, respectively (18, 44). The cytoplasmic tails of \( \alpha_{\text{IIb}}\beta_3 \) and other integrins may play pivotal roles in adhesion by (a) regulating the ligand-binding function of the extracellular domains through responses to intracellular signals ("inside-out signaling"), which are important for platelet function (25, 40, 58).
Outside-in signaling has profound implications for circulating platelets since only platelets activated by agonists, such as thrombin, are able to bind soluble fibrinogen or von Willebrand factor (3, 43).

Evidence for outside-in signaling by integrin receptors is accumulating in several cell systems, including platelets (31, 33). For example, the adhesion of fibroblasts to fibronectin or fibronectin fragments has been shown to stimulate protein tyrosine phosphorylation, an increase in intracellular pH, and upregulation of genes that control the GI/S cell cycle and the synthesis of metalloproteinases (24, 48, 56, 60, 61). However, unlike growth factor receptors, which initiate signaling through the intrinsic protein tyrosine kinase activity of their cytoplasmic tails, integrins contain no such activity. Thus, integrins must initiate the process of protein tyrosine phosphorylation indirectly, perhaps by modifying the activity or subcellular localization of non-receptor tyrosine kinases, tyrosine phosphatases, or their substrates. In this context, fibroblasts and other cell types have been shown to contain a novel protein tyrosine kinase, ppl25FAK, which localizes to focal adhesions and becomes activated when the α2β1 fibronectin receptor is engaged by fibronectin or cross-linked by antibodies (7, 8, 23, 34, 47, 62). Although the substrates for ppl25FAK have not been identified, it is hypothesized that this enzyme plays a role in linking adhesive events at the cell surface with intracellular events required for cell function.

Platelets are an excellent model system to study some of the functional implications of integrin-mediated signaling since their physiology is well understood and they have not been exposed to the perturbations of tissue culture. Activation of platelets by thrombin or ADP results in the phosphorylation of numerous proteins ranging in size on SDS gels from 30–140 kD, including ppl25FAK (17, 21, 36). Moreover, the phosphorylation of ppl25FAK and at least two other proteins with molecular masses of ~100 kD is dependent on platelet aggregation mediated by fibrinogen and Ωmβ3 (16, 22, 36). Therefore, platelets have been used in the present study to better define the sequence of events between ligand binding to an integrin and tyrosine phosphorylation of ppl25FAK. Our approach was to ask whether the binding of soluble fibrinogen to Ωmβ3 was sufficient to trigger protein tyrosine phosphorylation in the absence of a platelet agonist and without platelet aggregation. Fab fragments of an antibody specific for the β3 integrin subunit were used to induce fibrinogen binding without antecedent platelet activation. The results indicate that occupancy of Ωmβ3 by fibrinogen indeed initiates a process of tyrosine phosphorylation in platelets that precedes phosphorylation of ppl25FAK. This response may require fibrinogen-induced oligomerization of Ωmβ3 receptors and it may depend on the act of polymeerization within the cell.

Materials and Methods

Antibodies and Other Reagents

All monoclonal antibodies were IgGκ and were purified by Staph A column chromatography (MAPS II; Bio-Rad Labs., Richmond, CA), except for PAC1 (IgMε), which was purified by boric acid precipitation and Sepharose 4B chromatography (51). All antibodies demonstrated a single band on non-reduced SDS gels and two bands, representing the heavy and light chains, on reduced gels. Fab fragments were prepared using immobilized papain (Immunopure Fab Preparation Kit, Pierce, Rockford, IL). Anti-LIBS6 is an antibody specific for the β3 integrin subunit that induces exposure of the fibrinogen binding site on αmβ3 (19). Antibody SASS is specific for β3 but does not expose the fibrinogen receptor (1). PAC1 is specific for an activated conformation of Ωmβ3 (53); 7E3 (a gift from Robert Jordan, Centocor, Inc., Malvern, PA) and A2A9 are specific for the Ωmβ5 complex (2, 11); S12, a gift from Rodger McEver (Oklahoma Medical Research Foundation), recognizes the α-granule membrane protein, P-selectin (37); and 4G10 (13) and PY20 are specific for phosphotyrosine residues and were obtained from Tom Roberts (Dana Farber Cancer Institute) and ICN Biomedicals, Inc. (Costa Mesa, CA), respectively. UP28 is an affinity-purified rabbit polyclonal antibody specific for phosphotyrosine residues (30). Polyclonal antisera specific for ppl25FAK was a gift from Tom Parsons, University of Virginia (47). The F(ab')2 fragment of a goat antibody specific for mouse immunoglobulin heavy and light chains was from Zymed Labs., Inc., South San Francisco, CA.

Fibrinogen was purified from a commercial source (Kabi, Stockholm, Sweden) (3). Analysis of purified fibrinogen on SDS-polyacrylamide gels under reducing conditions showed the characteristic Aa, Bβ, and γ chains and no contamination with von Willebrand factor or other high molecular weight proteins (38). Fibrinogen fragment X, a gift from Zaverio Ruggeri (Scripps Research Institute, La Jolla, CA), was prepared and purified as described previously (39, 54). It migrated as a single band with a molecular mass of 250 kD on nonreduced SDS gels. Peptides RGDS and "44°°-4H" (HHLGQAQQAGDV, single letter amino acid code) were from Peninsula Laboratories, Belmont, CA. Cytochalasin D was from Aldrich Chem. Co., Milwaukee, WI. All agonists and platelet inhibitors were from Sigma Chem. Co., St. Louis, MO, except for bovine thrombin (Calbiochem, San Diego, CA).

Preparation of Platelets and Induction of Fibrinogen Binding to Ωmβ3

Human platelets were isolated by gel-filtration from freshly drawn blood anticoagulated with 0.15 vol NIH formula A acid-citrate-dextrose solution supplemented with 1 μM PGE1 and 1 U/ml arsyrase (57). Unless stated otherwise, platelet concentration was adjusted to 2 × 1010 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, 33 mM NaH2PO4, 20 mM Hepes, pH 7.4, and 10 μM indomethacin. To initiate fibrinogen binding to Ωmβ3, fibrinogen (typically 0.25 mg/ml) and anti-LIBS6 Fab (0.15 mg/ml) were added to the unstirred platelet suspension final concentration 1–5 × 109/ml) for up to 5 min at room temperature in a final vol of 0.3 ml. In some cases, fibrinogen binding was stimulated by addition of 10 μM epinephrine, 10 μM ADP, or 0.1 U/ml thrombin, instead of by anti-LIBS6 Fab. Unless specified otherwise, platelets stimulated by thrombin were stirred at 1,000 rpm to cause platelet aggregation. All reactions were stopped by lysing the platelets for 30 min at 4°C in radiimmunoprecipitation (RIP) buffer containing 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 158 mM NaCl, 10 mM Tris-HCl, pH 7.2, 1 mM sodium EDTA, 1 mM PMNS, 1 mM NaVO4, and 100 kallikrein inactivator U/ml of Trasylol (FBA Pharmaceuticals, West Haven, CT). Lysates were then analyzed for phosphotyrosine-containing proteins.

Identification of Phosphotyrosine-containing Proteins

All steps were at 4°C. RIPA extracts of platelets were clarified at 15,000 g for 30 min and immunoprecipitated with either the polyclonal anti-phosphotyrosine antibody (UP28) (30) or the polyclonal anti-ppl25FAK antibodies (36) using Pansorbin (Calbiochem, La Jolla, CA). Rabbit anti-mouse IgG was used as a control. The immunoprecipitates were then washed three times in RIPA buffer, eluted from the Pansorbin in SDS sample buffer, separated by SDS-PAGE, and transferred to nitrocellulose filters (36). The filters were then probed for phosphotyrosine-containing proteins with a combination of the monoclonal antibodies, 4G10 and PY20 (1 μg/ml each), and protein bands containing the bound antibodies were detected with a horseradish peroxidase-conjugated goat anti-mouse IgG and the ECL chemiluminescence reaction (Amersham Corp., Arlington Heights, IL) (36).
Antibody-Cross-Linking of αβ₃

In some experiments, αβ₃ complexes on gel-filtered platelets were cross-linked with primary and secondary antibodies. Gel-filtered platelets were resuspended to 1.9 × 10⁶/ml in incubation buffer and incubated for 15 min at room temperature with 20 μg/ml of SSA6, an anti-β₃ monoclonal antibody. Unbound SSA6 was then removed by washing as previously described (50), and the platelet (now 1.5 × 10⁵/ml) were incubated for 20 min at 37° with 25 μg/ml of a goat F(ab')₂ fragment specific for mouse immunoglobulin heavy and light chains. Reactions were stopped with RIPA buffer and the lysates analyzed for phosphotyrosine-containing proteins as described above.

Analyses of Platelet Function

After the incubation of gel-filtered platelets with fibrinogen and/or anti-LIBS6 Fab, duplicate or triplicate 5-μl samples were processed for flow cytometry to monitor various aspects of platelet function. Activation of integrin αβ₃ was quantitated by platelet binding of FITC-labeled PAC1 (49). Platelet α-granule secretion was monitored by surface expression of P-selectin using FITC-S12 (37). The presence of platelet aggregates was assessed by phase-contrast microscopy, by platelet aggregometry, and by analyzing the forward light scatter profile of platelet particles in the flow cytometer. Dense granule secretion was monitored by measurement of ATP release in the forward light scatter profile of platelet particles in the flow cytometer. Dense granule secretion was monitored by measurement of ATP release in the forward light scatter profile of platelet particles in the flow cytometer. Dense granule secretion was monitored by measurement of ATP release in the forward light scatter profile of platelet particles in the flow cytometer. Dense granule secretion was monitored by measurement of ATP release in the forward light scatter profile of platelet particles in the flow cytometer.

Results

Fibrinogen Binding to Integrin αIbb₃ Triggers Protein Tyrosine Phosphorylation in Platelets

Previous studies have shown that at least three proteins become phosphorylated on tyrosine residues when platelets undergo aggregation in response to agonists: p125FAK, a 125-kD protein tyrosine kinase, and two uncharacterized proteins with mobilities on SDS gels of ~100 kD (16, 22, 36). Platelet aggregation requires both fibrinogen binding to αIbb₃ and stirring to enhance cell-cell contact. In the present study, we asked whether fibrinogen binding to αIbb₃ stimulates a process of tyrosine phosphorylation before platelet aggregation and the phosphorylation of p125FAK. Fibrinogen binding to platelets is typically stimulated by addition of an agonist, such as thrombin. However, agonists can initiate platelet activation through multiple signaling pathways and can stimulate cellular responses in addition to aggregation, thus potentially complicating interpretation of protein phosphorylation experiments. Therefore, fibrinogen binding was induced by the Fab fragment of an antibody (anti-LIBS6) that binds to the β₃ integrin subunit and directly exposes the fibrinogen binding site in αIbb₃. Unlike conventional agonists, anti-LIBS6 Fab, by itself, does not cause detectable platelet activation (19). To further insure that the gel-filtered platelets used here were initially in a resting state, they were obtained from fresh blood in the presence of PGE₁ and aprotase, and all studies were carried out in the presence of 10 μM indomethacin to inhibit prostaglandin synthesis.

Anti-LIBS6 Fab caused exposure of fibrinogen receptors on platelets in a dose-dependent fashion, as determined by platelet binding of FITC-PAC1, an antibody specific for the fibrinogen binding site in αIbb₃ (51) (Fig. 1). 150 μg/ml of anti-LIBS6 Fab stimulated FITC-PAC1 binding 15-fold, and this concentration of anti-LIBS6 Fab was used in further studies. When unstimred and unstimulated platelets were incubated at 1.5 × 10⁵/ml for 5 min at 22°C, and then analyzed on immunoblots for phosphotyrosine-containing proteins, there was basal phosphorylation of proteins at 180, 120, 96 and 65, and 48 kD, although the intensity of these bands varied from experiment to experiment. Similar results were obtained when platelets were incubated with anti-LIBS6 Fab alone or 0.25 mg/ml fibrinogen alone (Fig. 2, lanes 1–3). In contrast, when platelets were incubated with both anti-LIBS6 Fab and fibrinogen, additional protein bands with molecular masses of 50–68 kD and 140 kD became phosphorylated (Fig. 2, lanes 4–6).

Phosphorylation of these proteins was maximal at 0.25 mg/ml of fibrinogen, a concentration of ligand known to saturate platelet fibrinogen receptors (3). This phosphorylation was prevented by an anti-αIbb₃ antibody (7E3 Fab) known to inhibit fibrinogen binding (Fig. 2, lane 7) (11). None of the phosphoprotein bands induced by fibrinogen binding comigrated with either purified fibrinogen or anti-LIBS6 Fab fragments on reduced SDS gels (not shown).

Several additional characteristics of the phosphorylation response to fibrinogen binding were studied. The 50–68-kD and 140-kD proteins became phosphorylated on tyrosine residues within 30 s of addition of anti-LIBS6 Fab and fibrinogen to the platelets. The extent of phosphorylation increased over 5 min (Fig. 3). This delay in phosphorylation was at least partially due to the time required for antibody and fibrinogen binding. In platelets from an individual with homozygous Glanzmann's thrombasthenia (which expressed ~5% the normal amount of αIbb₃), the phosphorylation response was barely detectable, with only minimal phosphorylation after a 5-min incubation (Fig. 3).

Figure 1. Effect of anti-LIBS6 Fab on platelet fibrinogen receptor expression. Gel-filtered platelets were prepared as described in Materials and Methods and incubated at 10⁵/ml in duplicate 50-μl aliquots with the indicated amounts of anti-LIBS6 Fab for 15 min at room temperature in the presence of 40 μg/ml of FITC-PAC1. Each sample was then diluted with 200 μl of PBS and 10,000 platelets were analyzed by flow cytometry. PAC1 binding, expressed as the mean platelet fluorescence intensity in arbitrary fluorescence units, reflects exposure of the fibrinogen binding site in αIbb₃. Each data point represents the mean ± SEM of three separate experiments.
Fibrinogen binding to αcβ3 induces tyrosine phosphorylation in platelets. Unstirred gel-filtered platelets were incubated for 5 min at room temperature in the absence of fibrinogen or antibody (lane 1), in the presence of 0.25 mg/ml fibrinogen (lane 2), or in the presence of 0.15 mg/ml anti-LIBS6 Fab (lane 3). In lanes 4–6, platelets were incubated with the anti-LIBS6 Fab in the presence of increasing amounts of fibrinogen (0.05 mg/ml, 0.25 mg/ml, and 1.0 mg/ml, respectively). In lane 7, anti-LIBS6 Fab and 0.25 mg/ml fibrinogen were present along with 20 μg/ml of antibody 7E3 Fab, an anti-αcβ3 antibody that inhibits fibrinogen binding. In lane 8, platelets were aggregated by stirring for 5 min in the presence of 0.1 U/ml thrombin. After each incubation, platelets were lysed in RIPA buffer and immunoprecipitated with an affinity-purified polyclonal anti-phosphotyrosine antibody (UP 28). Immunoprecipitated proteins were separated by SDS-PAGE and probed on immunoblots with two monoclonal antibodies specific for phosphotyrosine residues, as described in Materials and Methods. In this and some subsequent figures, an arrow identifies a 140-kD phosphoprotein and a bracket identifies 50–68 kD phosphoproteins.

Several observations indicated that this tyrosine phosphorylation response of normal platelets was independent of platelet aggregation. First, the extent of tyrosine phosphorylation was unaffected by varying the platelet concentration over a 10-fold range (10^8–10^9/ml) during the incubation with fibrinogen. Second, platelet aggregation was avoided by not stirring, and no aggregation could be seen by phase-contrast microscopy, platelet aggreometry, or flow cytometry. Third, phosphorylation of the 50–68-kD and 140-kD proteins was observed even in the presence of 5 μM prostacyclin, an inhibitor of agonist-mediated platelet activation and aggregation (Fig. 4). Fourth, the previously described pair of phosphoproteins of ~100 kD which are phosphorylated after thrombin-induced platelet aggregation were not observed in unstirred platelets treated with anti-LIBS6 Fab and fibrinogen (compare Fig. 2, lanes 4–6 with platelets in lane 8 that were aggregated in response to thrombin and stirring).

The tyrosine phosphorylation response induced by anti-LIBS6 Fab and fibrinogen was not associated with bulk secretion from platelet dense granules, as monitored by ATP release, or secretion from α-granules, as monitored in flow cytometry by surface expression of the α-granule membrane protein, P-selectin (not shown). In addition, phosphorylation could not be attributed to the release of ADP, an endogenous agonist, from platelet dense granules because it occurred even in the presence of apyrase or pyruvate kinase/phosphoenolpyruvate, enzyme systems that remove ADP (Fig. 4). Tyrosine phosphorylation in response to anti-LIBS6 Fab and fibrinogen was not associated with a measurable increase in cytoplasmic free calcium, as detected with the calcium-sensitive fluorescent probe, Fura-2, either in the presence or absence of 1 mM CaCl₂ (not shown). Finally, phosphorylation was not uniquely dependent on the method of activation of αcβ3 since it was also observed when fibrinogen binding to unstirred platelets was induced by conventional agonists, such as epinephrine, ADP, or thrombin instead of by anti-LIBS6 Fab (Fig. 5). Taken together, these results indicate that tyrosine phosphorylation of the 50–68-kD and 140-kD proteins in platelets requires fibrinogen binding to αcβ3, but it does not require platelet aggregation or secretion.

Integrin-mediated Tyrosine Phosphorylation Is Initiated before Phosphorylation of pp125FAK

In fibroblasts or human carcinoma cells spread on fibronectin, pp125FAK localizes to integrin-rich focal adhesions and...
becomes phosphorylated on tyrosine residues, thus implicating this tyrosine kinase in integrin-mediated signaling events (7, 23, 33, 34, 47). pp125Fak also becomes phosphorylated on tyrosine residues and activated after agonist-induced platelet aggregation or after spreading of platelets on a fibrinogen matrix (36) (24a). Fig. 6 shows that phosphorylation of pp125Fak did not occur in unstirred platelets under conditions where anti-LIBS6 Fab and fibrinogen caused phosphorylation of the 50–68-kD and 140-kD proteins. This finding suggests that pp125Fak is not the kinase responsible for phosphorylation of the 50–68-kD and 140-kD substrates. As expected, pp125Fak became phosphorylated when platelets were stirred and allowed to form large aggregates in response to thrombin (Fig. 6) or to maximal concentrations of ADP or epinephrine (not shown). In contrast, when platelets were stirred in the presence of anti-LIBS6 Fab and fibrinogen, they formed only small aggregates, and phosphorylation of pp125Fak was not observed unless the platelets were simultaneously treated with sub-threshold concentrations of a conventional agonist, such as epinephrine (not shown).

These experiments suggest that fibrinogen binding to $\alpha_{\text{IIb}}\beta_3$ results in sequential tyrosine phosphorylation: "early" reactions occur directly upon ligation of the fibrinogen receptor and include phosphorylation of 50–68 kD and 140 kD substrates; later reactions are associated with platelet aggregation or spreading and include phosphorylation of pp125Fak and a pair of substrates of $\sim$100 kD. Although phosphorylation of pp125Fak is dependent on fibrinogen-mediated platelet aggregation, additional agonist-induced platelet reactions appear to be required.

**Integrin-mediated Tyrosine Phosphorylation Depends on the State of Actin Polymerization**

Thrombin-induced platelet aggregation leads to actin polymerization and to enrichment of $\alpha_{\text{IIb}}\beta_3$ within the actin-rich, Triton X-100-insoluble, "cytoskeletal" fraction of platelets (63). Also enriched within this fraction are structural proteins, such as vinculin, and potential regulatory proteins, such as PI3-kinase and pp60Src (10, 29, 63). Although fibrinogen binding to $\alpha_{\text{IIb}}\beta_3$ presumably represents a more
subtle stimulus to platelets than does thrombin, we asked whether the state of actin polymerization might influence \(\alpha_{\text{mB}}\) mediated tyrosine phosphorylation. Fibrinogen binding was studied in the presence of 10 \(\mu\)M cytochalasin D, which reduces the state of actin polymerization in the cell. Cytochalasin D markedly inhibited phosphorylation of the 50–68 kD and 140 kD substrates when fibrinogen binding was stimulated by either anti-LIBS6 Fab or thrombin (illustrated in Fig. 7 for the 140-kD substrate). In platelets aggregated by thrombin, cytochalasin D also inhibited phosphorylation of the pair of ~100 kD “aggregation-dependent” substrates (Fig. 7). Cytochalasin D at this concentration inhibited the binding of fibrinogen to platelets treated with thrombin or anti-LIBS6 Fab by 25–30% (not shown). However, this partial inhibition of ligand binding could not explain the almost complete inhibition of tyrosine phosphorylation by cytochalasin D, thus implicating actin polymerization in the process of tyrosine phosphorylation.

That fibrinogen binding might be influencing the state of actin polymerization in the platelet was suggested further by experiments evaluating the reversibility of ligand binding to \(\alpha_{\text{mB}}\). When platelets are activated by agonists, a fraction of the fibrinogen (or antibody PAC1) which becomes bound cannot be displaced from the cells by addition of 10 mM EDTA. This process of irreversible binding is progressive over time and appears to require actin polymerization (41). When FITC-PAC1 binding to platelets was stimulated with anti-LIBS6 Fab, the amount of irreversibly bound PAC1 increased significantly over time (P < 0.01) (Fig. 8). Taken together with the cytochalasin D results, this suggests that tyrosine phosphorylation in platelets may be influenced by the state of actin polymerization after fibrinogen binding to \(\alpha_{\text{mB}}\).

Integrin-mediated Tyrosine Phosphorylation Requires Fibrinogen Receptor Occupancy by a Multimeric Ligand

Fibrinogen is a dimeric molecule with at least three potential \(\alpha_{\text{mB}}\) recognition sites: two RGD-containing sequences in the Aot chain at positions 95–97 and 572–575, and a short linear sequence at the carboxy terminus of the \(\beta\)-chain (\(\gamma_{\text{t}}\)44-45) (43). Because fibrinogen is multivalent, we asked whether tyrosine phosphorylation triggered by the binding of this ligand might require the cross-linking of \(\alpha_{\text{mB}}\) complexes. As an initial approach to this question, the ability of fibrinogen fragment X to support tyrosine phosphorylation was studied. Fragment X is a dimeric, 250 kD plasmin-derived fragment of fibrinogen that lacks Aot572-575 but still...
contains the \( \alpha_{95-97} \) and \( \gamma_{404-411} \) cell recognition sites (9). Addition of fragment X at 0.5 mg/ml to gel-filtered platelets did not stimulate tyrosine phosphorylation, presumably because it did not bind to these resting cells. However, when the binding of fragment X to \( \alpha_{95\beta_3} \) was stimulated by anti-LIBS6 Fab, tyrosine phosphorylation of the 50–68-kD and 140-kD proteins was observed (Fig. 9).

In contrast to the results with fragment X, two short monomeric peptides derived from the cell recognition sequences in fibrinogen, RGDS, and \( \gamma_{404-411} \), failed to stimulate tyrosine phosphorylation, either in the absence or presence of anti-LIBS6 Fab (Fig. 10). This result could not be explained by a lack of binding of these peptides to \( \alpha_{95\beta_3} \) since each peptide inhibited tyrosine phosphorylation induced by fibrinogen (Fig. 10). Inasmuch as platelet aggregation did not occur in these experiments, the requirement for a dimeric ligand for cell signaling may reflect the need for ligand-induced oligomerization of \( \alpha_{95\beta_3} \) complexes within individual platelets. Consistent with this interpretation, when \( \alpha_{95\beta_3} \) was cross-linked by a primary \( \beta_3 \) mouse monoclonal antibody (SSA6) and the F(\( \alpha\beta \)) fragment of a secondary goat antibody specific for mouse immunoglobulins, tyrosine phosphorylation of the 50–68-kD and 140-kD proteins was observed, even when one mM RGDS was added to prevent fibrinogen binding (not shown). A F(ab')\( \alpha\beta \) fragment was used as the secondary antibody in this experiment to prevent signaling through the platelet Fc\( \gamma RI \) receptor. The lack of tyrosine phosphorylation of this 40-kD receptor confirms that it was not involved in any of the foregoing results (30).

**Discussion**

The present studies demonstrate that a process of protein tyrosine phosphorylation is triggered in platelets when fibrinogen, a dimeric and multivalent adhesive ligand, binds to integrin \( \alpha_{95\beta_3} \). The initial phosphorylation is restricted to substrates with mobilities on reduced SDS gels of 50–68 kD and 140 kD. It occurs within seconds of fibrinogen binding, and it is observed whether ligand binding is stimulated by a conventional agonist or by the Fab fragment of a \( \beta_3 \) antibody (anti-LIBS6) that causes direct exposure of the platelet fibrinogen receptor. In contrast to tyrosine phosphorylation of the focal adhesion kinase, pp125\( \text{FAK} \), phosphorylation of the 50–68 kD and 140 kD substrates does not require platelet aggregation, nor is it dependent on prostaglandin synthesis, a change in the cytosolic free calcium concentration, or granule secretion. As a result, the 50–68-kD and 140-kD substrates are phosphorylated before the phosphorylation of pp125\( \text{FAK} \), making it unlikely that this tyrosine kinase is responsible for their phosphorylation. Studies using well-characterized, plasmin-derived fragments of fibrinogen as well as peptides derived from the cell recognition domains of fibrinogen indicate further that tyrosine phosphorylation in response to the binding of soluble fibrinogen requires the dimeric form of this ligand, suggesting a role for integrin oligomerization in the signaling process. In addition, studies with cytochalasin D indicate that the earliest phases of integrin-mediated tyrosine phosphorylation in platelets may depend on the state of actin polymerization in the cell.

Although resting platelets can adhere constitutively to immobilized fibrinogen through \( \alpha_{95\beta_3} \), unstimulated platelets cannot bind fluid-phase fibrinogen (3, 46). However, when platelets are exposed to damaged vascular surfaces, they come in contact with agonists that activate these cells and cause a conformational change in \( \alpha_{95\beta_3} \) that increases its apparent affinity for fibrinogen to a \( K_d \) of \( \sim 0.05 \) mg/ml (3, 52). This process of inside-out signaling involves the action of GTP-binding proteins, protein kinase C, and possibly protein tyrosine kinases (50). It serves to prevent the interaction of platelets with fibrinogen in the circulation, yet it provides for rapid binding of fibrinogen to platelets in the vicinity of a vascular wound. Although regulation of the affinity state of \( \alpha_{95\beta_3} \) is clearly beneficial to the organism, it presented a technical challenge to us in the present study since we wanted to focus on signaling events distal to fibrinogen binding. Had we chosen to only activate platelets with a conventional agonist in order to expose the fibrinogen receptors, any resultant changes in the levels of protein tyrosine phosphorylation might have been due to agonist activation of metabolic pathways unrelated to those triggered by fibrinogen binding.
To overcome this problem, the fibrinogen binding site in αIIbβ3 was exposed by the Fab fragment of anti-LIBS6. The Fab fragment was used instead of the intact immunoglobulin to insure that platelets were not activated by ligation of the FcγRII receptor. Ligation of this 40-kD receptor on platelets is known to be associated with its phosphorylation on tyrosine residues (30). No such phosphorylation was observed in our experiments. Anti-LIBS6 Fab caused fibrinogen receptor exposure in a dose-dependent fashion, as monitored by flow cytometry using the activation-specific anti-αIIbβ3 antibody, PAC1. Anti-LIBS6 Fab did not, however, induce platelet tyrosine phosphorylation unless fibrinogen was present. Fibrinogen-dependent phosphorylation of the 50–68-kD and 140-kD platelet proteins was not dependent on the particular method used to expose fibrinogen receptors since it was also observed when agonists, such as epinephrine, ADP, or thrombin were used instead of anti-LIBS6 Fab to activate αIIbβ3. Of note, the ligand-binding function of integrins in other cells is also subject to regulation (20, 31). Thus, development of “activating antibodies” similar in function to anti-LIBS6 may prove generally useful in studying integrin-mediated signaling reactions.

Several results indicated that the binding of a dimeric form of the fibrinogen molecule was necessary to initiate tyrosine phosphorylation through αIIbβ3. Binding of fibrinogen fragment X, which like fibrinogen is dimeric also caused tyrosine phosphorylation. In contrast, the monomeric fibrinogen-derived cell recognition peptides, RGDS and γ300-411, failed to cause phosphorylation, despite the fact that they bound to the fibrinogen receptor, as indicated by their ability to inhibit fibrinogen-dependent tyrosine phosphorylation. Each half-molecule of fibrinogen contains at least three potential recognition domains for αIIbβ3 (γ499-507 and two RGD-containing sequences at Aα95-97 and Aα572-574). In this sense, even a fibrinogen monomer may be multivalent. Why then would a fibrinogen dimer be required for signaling? A dimeric ligand might be necessary to either cross-link αIIbβ3 complexes on adjacent platelets or to induce oligomerization of such complexes on the surface of individual platelets.

It could be argued that fragment X caused signaling not because it is dimeric but because it retains both the RGD se-
Cross-linking of $\alpha_{\text{IIb}}\beta_3$ by antibodies induces protein tyrosine phosphorylation in platelets. Gel-filtered platelets were initially incubated in the presence or absence of 20 $\mu$g/ml SSA6, an anti-$\beta_3$ monoclonal antibody (labeled "1° Ab" in the figure). Platelets were then washed by centrifugation in the presence of PG12 and apyrase to remove unbound primary antibody. Some of the samples were then incubated with 25 $\mu$g/ml of goat F(ab')2 fragment specific for mouse immunoglobulins (labeled "2° Ab" in the figure) to cross-link the primary antibody. During this second incubation, some of the samples were also incubated with 0.15 mg/ml anti-LS6 Fab and 0.25 mg/ml fibrinogen. Reactions were stopped with RIPA buffer and the lysates analyzed for phosphotyrosine-containing proteins as described in Fig. 2.

![Figure 1](image_url)

**Figure 1L** Cross-linking of $\alpha_{\text{IIb}}\beta_3$ by antibodies induces protein tyrosine phosphorylation in platelets. Gel-filtered platelets were initially incubated in the presence or absence of 20 $\mu$g/ml SSA6, an anti-$\beta_3$ monoclonal antibody (labeled "1° Ab" in the figure). Platelets were then washed by centrifugation in the presence of PG12 and apyrase to remove unbound primary antibody. Some of the samples were then incubated with 25 $\mu$g/ml of goat F(ab')2 fragment specific for mouse immunoglobulins (labeled "2° Ab" in the figure) to cross-link the primary antibody. During this second incubation, some of the samples were also incubated with 0.15 mg/ml anti-LS6 Fab and 0.25 mg/ml fibrinogen. Reactions were stopped with RIPA buffer and the lysates analyzed for phosphotyrosine-containing proteins as described in Fig. 2.

The inhibition of fibrinogen-dependent tyrosine phosphorylation by cytochalasin D suggests a role for actin polymerization in early signaling events triggered through $\alpha_{\text{IIb}}\beta_3$. Resting platelets contain short actin filaments which associate via actin-binding protein to a spectrin-rich submembrane lamina (5, 26). Actin-nucleating activity increases after agonist-induced platelet activation or during platelet spreading on a surface (25). During platelet spreading, mature cytoskeletal structures analogous to focal adhesions are formed (40). Since the binding of soluble fibrinogen to platelets does not cause platelet aggregation unless stirring takes place, any actin assembly triggered by ligand binding to $\alpha_{\text{IIb}}\beta_3$ would be expected to be more subtle than that induced during aggregation or spreading. Nonetheless, it is plausible that fibrinogen binding might stimulate the formation of nascent, actin-rich structures through the interaction of the integrin $\beta_3$ subunit with actin-binding proteins such as $\alpha$-actinin or talin (58). It may be pertinent in this regard that pp60$^{c-src}$ has been shown to partition to the detergent-insoluble "cytoskeletal" fraction of platelets after thrombin treatment, but this does not occur in platelets deficient in $\alpha_{\text{IIb}}\beta_3$ (10). Thus, ligand-induced oligomers of $\alpha_{\text{IIb}}\beta_3$ may function as sites of nucleation for structural and regulatory proteins of the platelet cytoskeleton.

Although we could detect no generalized increase in platelet cytoplasmic free calcium in response to anti-LS6 Fab and fibrinogen, it is still theoretically possible that $\alpha_{\text{IIb}}\beta_3$ plays a role in cytoskeletal organization and tyrosine phosphorylation by regulating localized calcium influx (6). When heterologous cells transfected with wild-type $\alpha_{\text{IIb}}\beta_3$ become adherent to fibrinogen, they stimulate cytosolic calcium oscillations, followed by tyrosine phosphorylation of a 125-kD protein (42). Although integrin subunits do not exhibit the multiple transmembrane domains typical of many ion channel proteins, a protein with such a structure has been reported to be associated with a $\beta_3$-related integrin in leukocytes (Lindberg, E. and E. J. Brown. 1992. Mol. Biol. Cell. 3:95a).

On the basis of the existing data, we propose a model of integrin-mediated signaling in platelets in which binding of fibrinogen causes oligomerization of $\alpha_{\text{IIb}}\beta_3$ within the plane of the plasma membrane, leading to actin assembly in proximity to these oligomers. During this process, one or more protein tyrosine kinases and their potential substrates might become localized to these regions, presumably through specific protein–protein interactions. Once juxtaposed to the appropriate enzyme(s), substrates such as the 50–68-kD and 140-kD proteins would become phosphorylated on tyrosine residues. After this initial phase, additional enzymes and substrates, for example ppl25$^{FAK}$, might be drawn into the cytoskeleton during subsequent platelet aggregation or spreading. In this model, phosphorylation and activation of ppl25$^{FAK}$ would occur after other integrin-mediated fos-
phorylation reactions. This is consistent with the recent suggestion that pp125Fak represents a point of convergence for phosphorylation reactions that follow ligation of integrins and neuropeptide receptors (33, 62). It is also consistent with the finding that pp125Fak is phosphorylated after platelet adhesion to collagen in a reaction that involves the collagen receptor, α2β1, but not αmβ2 (24a).

As shown here and as demonstrated previously (36), tyrosine phosphorylation of pp125Fak occurs when platelets aggregate in response to maximal concentrations of agonists, such as thrombin. Phosphorylation of pp125Fak also was observed when platelets were allowed to aggregate in response to the combination of fibrinogen, anti-LLB6 Fab, and a subthreshold concentration of epinephrine. In contrast, no phosphorylation of pp125Fak occurred when platelets were stirred in the presence of this concentration of epinephrine alone or the fibrinogen-anti-LLB6 Fab alone. This suggests that fibrinogen- and αmβ2-mediated platelet aggregation is necessary but not sufficient for pp125Fak phosphorylation.

Several questions can now be posed based on the observation that tyrosine phosphorylation in platelets is triggered directly by binding of a soluble adhesive ligand to integrin αmβ2. Do adhesive ligands other than fibrinogen, such as fibronectin, vWF, or vitronectin, induce signaling when they bind to αmβ2? Is the tyrosine phosphorylation caused by stimulation of a tyrosine kinase or by inhibition of a tyrosine phosphatase? What are the identities and functions of the 50–68-kD and 140-kD substrates that become phosphorylated? These questions may shed additional light on the regulatory role of integrin-mediated tyrosine phosphorylation of pp125Fak in other cell types.

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