Integrin-Dependent Phosphorylation and Activation of the Protein Tyrosine Kinase pp125FAK in Platelets

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Abstract. We have investigated mechanisms involved in integrin-mediated signal transduction in platelets by examining integrin-dependent phosphorylation and activation of a newly identified protein tyrosine kinase, pp125FAK (FAK, focal adhesion kinase). This kinase was previously shown to be localized in focal adhesions in fibroblasts, and to be phosphorylated on tyrosine in normal and Src-transformed fibroblasts. We show that thrombin and collagen activation of platelets causes an induction of tyrosine phosphorylation of pp125FAK and that pp125FAK molecules isolated from activated platelets display enhanced levels of phosphorylation in immune-complex kinase assays. pp125FAK was not phosphorylated on tyrosine after thrombin or collagen treatment of Glanzmann's thrombasthenic platelets deficient in the fibrinogen receptor GPIIb-IIIa, or of platelets pretreated with an inhibitory monoclonal antibody to GPIIb-IIIa. Fibrinogen binding to GPIIb-IIIa was not sufficient to induce pp125FAK phosphorylation because pp125FAK was not phosphorylated on tyrosine in thrombin-treated platelets that were not allowed to aggregate. These results indicate that tyrosine phosphorylation of pp125FAK is dependent on platelet aggregation mediated by fibrinogen binding to the integrin receptor GPIIb-IIIa. The induction of tyrosine phosphorylation of pp125FAK was inhibited in thrombin- and collagen-treated platelets preincubated with cytochalasin D, which prevents actin polymerization following activation. Under all of these conditions, there was a strong correlation between the induction of tyrosine phosphorylation of pp125FAK in vivo and stimulation of the phosphorylation of pp125FAK in vitro in immune-complex kinase assays. This study provides the first genetic evidence that tyrosine phosphorylation of pp125FAK is dependent on integrin-mediated events, and demonstrates that there is a strong correlation between tyrosine phosphorylation of pp125FAK in platelets, and the activation of pp125FAK-associated phosphorylating activity in vitro.

The interactions of cells with extracellular adhesion molecules play critical roles in regulating the morphology, proliferation, migration, and differentiation of cells. One family of receptors for extracellular adhesion molecules, called integrins, is comprised of heterodimeric transmembrane proteins (1, 17, 19, 20). Although the extracellular interactions between adhesion molecules and their integrin receptors have been well characterized, the integrin interactions with cytoplasmic targets and their role in signaling pathways that are responsible for adhesion-induced changes in cell behavior are poorly understood (5).

Platelets provide a very useful model system for investigating the mechanisms involved in integrin-induced events. The best characterized platelet integrin receptor, GPIIb-IIIa, is required for two essential functions of platelets in hemostasis—platelet-to-platelet aggregation and the spreading of platelets on blood vessel subendothelium (23, 25, 26, 33, 34). The interactions of GPIIb-IIIa with adhesion molecules are also important for activation of intracellular pathways that regulate several processes, including the activation of the Na+ /H+ antiporter and the Ca2+-dependent protease, calpain (2, 12). In platelets treated with weak agonists, such as ADP, aggregation mediated by fibrinogen binding to GPIIb-IIIa is also required for the stimulation of arachidonate metabolism and dense granule secretion (2, 18, 29, 30).

The mechanisms involved in signaling through GPIIb-IIIa remain elusive. Recent studies have shown that thrombin-induced tyrosine phosphorylation of several proteins is dependent on platelet aggregation mediated by fibrinogen binding to GPIIb-IIIa (11, 14). These results suggested that tyrosine phosphorylation might be involved in GPIIb-IIIa-regulated cellular processes. Furthermore, in NIH-3T3 cells and human epidermal carcinoma KB cells, engagement of integrins by receptor cross-linking or spreading on a fibronectin matrix induced tyrosine phosphorylation of several

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The Journal of Cell Biology, Volume 119, Number 4, November 1992 905-912 905
115-130-kD proteins (16, 24). These results strongly implicate tyrosine phosphorylation of cellular proteins in intracellular events triggered by integrin receptors. A candidate protein tyrosine kinase that may be involved in integrin-regulated tyrosine phosphorylation has been identified. This protein, denoted pp125FAK (FAK, focal adhesion kinase), was first identified using monoclonal antibodies (mAbs) raised against phosphotyrosine-containing proteins isolated from pp60^sy^ transformed cells (22). In normal fibroblasts, pp125FAK is localized in focal adhesions, and displays a low level of tyrosine phosphorylation (22, 28; B. S. Cobb and J. T. Parsons, unpublished results). Tyrosine phosphorylation of this protein is enhanced three- to fivefold in Rous sarcoma virus infected cells expressing pp60^sy^ (22). The cDNA encoding pp125FAK contains sequences that are highly homologous to those found in the catalytic domains of protein tyrosine kinases, and expression of this cDNA in Escherichia coli causes an induction of tyrosine phosphorylation of many bacterial proteins. In addition, a TrpE-fusion protein containing the sequences corresponding to the catalytic domain of this cDNA was shown to autophosphorylate in immune-complex kinase assays (28). This protein tyrosine kinase has been implicated in integrin-signaling pathways by the evidence that it represents at least one of the 115- to 130-kD proteins that are phosphorylated on tyrosine following cross-linking of integrin receptors on KB cells (L. J. Kornberg and R. L. Juliano, personal communication) or following plating of NIH-3T3 cells or chicken embryo fibroblasts (CEF) on fibronectin matrices (J. L. Guan, I. Chickalaparampl, and D. Shalloway, personal communication; M. D. Schaller, B. S. Cobb, and J. T. Parsons, unpublished results).

We have examined integrin-mediated regulation of tyrosine phosphorylation and activity of pp125FAK in platelets. Both thrombin and collagen-stimulated platelet activation led to an induction of tyrosine phosphorylation of pp125FAK. In addition, we show that pp125FAK phosphorylating activity in vitro strongly correlates with tyrosine phosphorylation of pp125FAK in vivo. Furthermore, we have taken advantage of the availability of platelets that are genetically deficient in GP Ib-IIIa to show that thrombin- or collagen-induced tyrosine phosphorylation of pp125FAK, and stimulation of its phosphorylating activity in vitro, are dependent on fibrinogen binding to GP Ib-IIIa. These results provide the first genetic evidence linking integrin receptors with tyrosine phosphorylation of pp125FAK, and the first demonstration that tyrosine phosphorylation of pp125FAK in vivo correlates with activation of the phosphorylating activity of this protein tyrosine kinase in vitro.

**Identification of Phosphotyrosine-Containing Proteins by Immunoblotting**

Platelet lysates or immunoprecipitates (from 8 to 10 x 10^9 cells) were subjected to SDS-PAGE on 7.5% gels. Proteins were transferred to nitrocellulose and the blot was incubated for 16 h in blocking solution (5% Cohn crystallized BSA [ICN Biomedicals, Inc., Costa Mesa, CA], 170 mM NaCl, 0.2% NP-40, 50 mM Tris, pH 7.5). Immunoblots were probed with a mixture of two anti-phosphotyrosine mAbs in buffer A (3% BSA, 170 mM NaCl, 0.2% NP-40, 50 mM Tris-HCl, pH 7.5). The antibodies were mAb 4G10 (1 μg/ml) (10), kindly provided by Tom Roberts (Dana Farber Cancer Research Institute, Boston, MA), and mAb PY20 (1 μg/ml) (ICN Biomedicals, Inc., Costa Mesa, CA). Nitrocellulose filters were washed four times in buffer B (buffer A without BSA), incubated with buffer A for 30 min, and then for 2 h with a 1:250 dilution of horseradish peroxidase-conjugated goat-anti-mouse IgG (Bio-Rad Laboratories, Richmond, CA) in buffer A. Immunoreactivity was determined using the ECL chemiluminescence reagent (Amer sham Corp., Arlington Heights, IL).

**Immunoprecipitation and Kinase Assays**

RIPA extracts were clarified at 25,000 g for 30 min. This and all subsequent steps were carried out at 4°C. For immunoprecipitation of phosphotyrosine-containing proteins, lysates were incubated with polyclonal rabbit anti-phosphotyrosine antibodies (from rabbit UP28), that had been affinity-purified on a phosphotyrosine column (32). Control samples were incubated with 1 μg/ml of rabbit anti-mouse immunoglobulin (RAM). For immunoprecipitation of pp125FAK, RIPA lysates were precleared twice with Pansorbin by sedimentation at 16,000 g for 15 min and then incubated with mAb 2A7 (10 μg/ml) plus RAM, or a polyclonal antiserum to pp125FAK (28). Immune complexes were incubated for 30 min with 30 μl of Pansorbin or Pansorbin precoated with 1 μg/ml of RAM. Immunoprecipitates were washed three times in RIPA buffer. To examine tyrosine phosphorylation of platelet proteins or pp125FAK, immunoprecipitates were eluted from Pansorbin, separated by SDS-PAGE, and immunoblotted onto nitrocellulose filters which were then probed with anti-phosphotyrosine mAbs, 4G10 or PY20 as described above. To examine the autophosphorylating activity of pp125FAK, immunoprecipitates were washed twice in RIPA buffer, once with low salt buffer (100 mM NaCl, 10 mM Tris, pH 7.5, 5 mM MnCl2) and incubated in 30 μl of kinase reaction mixture containing 10 mM Tris, pH 7.4, 5 mM MnCl2, 1 μM ATP, and 10 μCi[32P]ATP [1 Ci=37 GBq, ICN] for 5 min at room temperature. The kinase reaction was stopped by the addition of 1 ml of RIPA buffer at 4°C. The phosphorylated proteins were eluted from the Pansorbin, and separated by electrophoresis on SDS/7.5% polyacrylamide gels as described. Phosphorylated proteins were detected by autoradiography. Gels containing 32P-labeled proteins were rehydrated in destaining solution (10% methanol, 5% acetic acid), and then treated with 1 M KOH for 2 h at 56°C to remove alkali-labile phosphate (9).
Results

Tyrosine Phosphorylation of pp125FAK in Thrombin-Treated Platelets

Thrombin treatment of platelets isolated from peripheral blood initiates a cascade of changes in cell physiology that mimic many of the events associated with platelet activation in vivo, including activation of intracellular signaling pathways leading to the secretion of alpha and dense granules (23). Fibrinogen molecules released from alpha granules bind to GP IIb-IIIa, and subsequent interactions of platelet-bound fibrinogen molecules lead to platelet aggregation (23).

To determine if pp125FAK is phosphorylated on tyrosine in activated platelets, pp125FAK was immunoprecipitated from lysates of resting or thrombin-treated platelets, transferred to nitrocellulose, and probed with mAbs specific for phosphotyrosine residues (Fig. 1). The pp125FAK immunoprecipitates from untreated platelets did not show any reactivity with the mAbs to phosphotyrosine. However, a 125,000 Mₐ phosphotyrosine-containing protein was immunoprecipitated from all thrombin-treated platelet lysates (lanes 5–7). This protein displayed the predicted electrophoretic mobility of pp125FAK, and was not detected in control antibody immunocomplexes from thrombin-treated platelets (lane 2). Preincubation of these blots with 200 μM phosphotyrosine completely blocked immunoreactivity with the mAb to phosphotyrosine. The antiphosphotyrosine immunoreactivity of pp125FAK reached its highest level within 1 min after thrombin treatment, and was maintained at this level throughout the time period examined (5 min) (data not shown). Under our conditions of platelet isolation and thrombin treatment, dense granule secretion occurs within seconds of thrombin stimulation, and aggregation follows after 30–60 s. Thus, the time course of pp125FAK phosphorylation suggested that tyrosine phosphorylation of pp125FAK might occur after platelet aggregation.

Fibrinogen binding to GP IIb-IIIa and subsequent platelet aggregation are necessary for tyrosine phosphorylation of several proteins in thrombin-treated platelets (11, 14). We examined whether tyrosine phosphorylation of pp125FAK was dependent on fibrinogen binding to GP IIb-IIIa by analyzing tyrosine phosphorylation of pp125FAK in thrombin-treated platelets from GP IIb-IIIa-deficient donors with Glanzmann's thrombasthenia (Fig. 1, lanes 8–14). Glanzmann's platelets undergo normal secretion of alpha and dense granules after thrombin stimulation, but are unable to aggregate (34). There was no detectable phosphorylation of pp125FAK in Glanzmann's platelets (lanes 10–14), however, we detected normal levels of pp125FAK in these platelets in immunoblot assays (data not shown). These results indicate that tyrosine phosphorylation of pp125FAK is dependent on fibrinogen binding to GP IIb-IIIa.

pp125FAK Phosphorylation in Collagen-Treated Platelets

Tyrosine phosphorylation of pp125FAK was also examined in platelets treated with collagen, which interacts with another integrin receptor, GP Ia-IIa (α6β1) (Fig. 2) (27). Like thrombin, collagen causes full activation of platelet secretion and aggregation (3). Collagen binding to platelets is inhibited by antibodies to GP Ia-IIa (8), yet collagen-induced platelet aggregation is dependent on fibrinogen interaction with GP IIb-IIIa. In normal platelets, collagen induced tyrosine phosphorylation of pp125FAK at levels similar to those induced by thrombin (lanes 2 and 3). As in thrombin-treated platelets, the induction of tyrosine phosphorylation of pp125FAK by collagen was dependent on GP IIb-IIIa because pp125FAK was not detectably phosphorylated in collagen-treated Glanzmann's platelets (lane 8). Thus, agonist engagement of the integrin collagen receptor is not sufficient to induce tyrosine phosphorylation of pp125FAK in platelets deficient in GP IIb-IIIa, suggesting that fibrinogen binding to GP IIb-IIIa and/or platelet aggregation might be required for the induction of pp125FAK phosphorylation.

Stimulation of pp125FAK Protein Tyrosine Kinase Activity

pp125FAK was recently shown to be capable of autophosphorylation when expressed as a TrpE fusion protein in E. coli (28). To examine whether the catalytic activity of pp125FAK is stimulated by thrombin, pp125FAK immunoprecipitates from untreated or thrombin-treated platelets were...
incubated with [γ-32P]ATP (Fig. 3 A). Under these conditions, only a low level of phosphate was incorporated into a 125-kD protein in anti-pp125FAK immunoprecipitates from untreated platelets (lane 7). However, platelet activation by thrombin resulted in enhanced 32P-incorporation into this protein (lanes 2 and 3). Control immunoprecipitates did not contain a similar phosphorylated protein (lane 4). Several other 32P-labeled proteins of Mr 56,000, 62,000, and 73,000 were detected in these assays. Since the proteins of Mr 73,000 and 62,000 were also observed in the control immunoprecipitations (as assayed by reprobing with the polyclonal anti-pp125FAK serum, data not shown), these results suggest that thrombin stimulates the autophosphorylating activity of pp125FAK, though we cannot absolutely rule out the involvement of another kinase which is activated by thrombin.

The activation of pp125FAK phosphorylation in vivo and in vitro was examined under several additional experimental conditions. To determine whether tyrosine phosphorylation of pp125FAK was dependent on fibrinogen binding to GP Ib-IIIa, platelets were preincubated with a GP Ib-IIIa-specific mAb (7E3) that prevents fibrinogen binding to this receptor. This treatment was used to prevent aggregation as an alternative for the use of Glanzmann's platelets because the availability of these genetically defective platelets is limited. A polyclonal rabbit antiserum to pp125 was employed in these immunoprecipitations. Pretreatment with mAb 7E3 inhibited tyrosine phosphorylation of pp125FAK in thrombin-treated platelets (Fig. 4 A) and prevented thrombin-induced pp125FAK phosphorylation in vitro (Fig. 4 B). Pretreatment with mAb 6F1, specific for collagen receptor GP Ia-IIa (8) did not reduce the thrombin-induced phosphorylation of pp125FAK in vivo (Fig. 4 A, lane 5) or in vitro (Fig. 4 B, lane 5). However, this antibody prevented collagen stimulation of pp125FAK phosphorylation in vivo. There were no detectable differences in the levels of pp125FAK in these immunoprecipitations (as assayed by reprobing with the polyclonal anti-pp125FAK serum, data not shown). A 62-kD protein was also detected in the in vitro kinase assays of anti-pp125FAK immunoprecipitates from thrombin- and collagen-treated cells; however, the phosphorylation of this protein did not correlate with pp125FAK phosphorylation. These results indicate the stimulation of the phosphorylation activity of pp125FAK is dependent on fibrinogen-binding to GP Ib-IIIa in both thrombin- and collagen-treated platelets, and demonstrate a correlation between tyrosine phosphorylation of pp125FAK in vivo and activation of the in vitro phosphorylation activity of pp125FAK.

To examine whether fibrinogen binding to GP Ib-IIIa is sufficient to stimulate pp125FAK phosphorylation, or whether subsequent platelet aggregation is required for this event, platelets were activated by thrombin or ADP and epinephrine in the absence of stirring (Fig. 5). Under these condi-

Figure 2. Induction of pp125FAK tyrosine phosphorylation in thrombin- or collagen-treated platelets. Gel-filtered platelets from a normal donor (lanes 1-3) or from a donor with Glanzmann's thrombasthenia (lanes 6-10) were stirred for 2 min in the absence of agonist (UN; lanes 1 and 6), with thrombin (THR; lanes 2, 5, 7, and 10), or with collagen (COL; lanes 3-4 and 8-9), and lysed in RIPA buffer. Lysates were incubated with anti-pp125FAK mAb 2A7 plus RAM (pp125FAK; lanes 1-3 and 6-8), or with RAM alone (RAM; lanes 4, 5, 9, and 10). Immunoprecipitated proteins were analyzed on immunoblots and probed with mAbs specific for phosphotyrosine residues.

Figure 3. Stimulation of pp125FAK in vitro tyrosine kinase activity. (A) Gel-filtered platelets were stirred in the absence of agonist (lane 1), or in the presence of thrombin (THR; 1 U/ml) for 1 min (lane 2) or 4 min (lanes 3 and 4) and lysed in RIPA buffer. Lysates were incubated with anti-pp125FAK mAb 2A7 plus RAM (pp125FAK; lanes 1-3) or with RAM alone (RAM; lane 4). The immunocomplexes were incubated with [γ-32P]ATP for 5 min to allow for in vitro phosphorylation. Phosphorylated proteins were separated on SDS/7.5% polyacrylamide gels. (B) For preferential detection of phosphotyrosine-containing proteins, the gel shown in A was treated with 1 M KOH at 56°C for 2 h (9) to preferentially hydrolyze phosphoserine and phosphothreonine.
Figure 4. Activation of the in vitro tyrosine kinase activity associated with pp125FAK is dependent on the integrin GP IIb-IIIa. Gel-filtered platelets unstimulated (UN; lane 2), or stimulated with thrombin (THR; lanes 1, 3, 4, and 5) or collagen (COL; lanes 6, 7, and 8) were lysed in RIPA buffer. Platelets were preincubated for 20 min with mAb 7E3 that interacts with GP IIb-IIIa (7E3/THR, lanes 4 and 8) or with mAb 6F1, which interacts with GP Ia-IIa (6F1/COL; lanes 5 and 7). Lysates were incubated with anti-pp125FAK polyclonal rabbit serum or normal rabbit serum (NRS) as indicated. The immunoprecipitates were either probed on immunoblots with mAbs specific for phosphotyrosine residues (A) or incubated with 10 μCi [γ-32P]ATP as described in Materials and Methods (B) to allow for in vitro phosphorylation. The gel shown in B was treated with alkali (9).

Figure 5. Platelet aggregation is required for stimulation of tyrosine phosphorylation of pp125FAK. Gel-filtered platelets unstimulated (UN; lane J) or stimulated with thrombin (THR; lanes 2, 3, and 6) or a combination of ADP, epinephrine, and fibrinogen (ADP/EPI; lanes 4 and 5) were either left unstimred (lanes 2 and 4) or stirred (lanes 1, 3, 5, and 6) for 1 min before lyzing in RIPA buffer. Platelet aggregation and secretion of ATP were monitored by lumiaggregometry. Lysates were incubated with the polyclonal rabbit anti-pp125FAK serum or normal rabbit serum (NRS). (A) Immunoprecipitated proteins were analyzed on immunoblots probed with mAbs specific for phosphotyrosine residues. (B) The immune complexes were incubated with 10 μCi [γ-32P]ATP as described in Materials and Methods to allow for in vitro phosphorylation. Phosphorylated proteins were separated on an SDS/7.5% polycrylamide gel and the gel was alkali-treated as described in Materials and Methods. The level of ATP secreted from dense granules of unstirred platelets treated with thrombin or collagen was equivalent to that detected in stirred samples, indicating that the level of thrombin activation of platelet secretion was not affected by platelet stirring.

In platelets treated with thrombin, granule secretion takes place in the absence of stirring ([14] and as monitored in this experiment by a luciferase assay of ATP secretion using the lumiaggregometer). In the absence of stirring, we did not detect any tyrosine phosphorylation of pp125FAK in platelets treated with the strong agonist, thrombin, or the combination of relatively weak agonists, GP IIb-IIIa is activated and is capable of binding to fibrinogen (4, 14); however, no aggregation takes place (as previously reported [14] and as monitored in this experiment by aggregometry, data not shown).
phosphorylation of pp125 FAK.

In addition, there was only a minimal level of pp125 FAK phosphorylation in vitro in pp125 FAK immunoprecipitates extracted from unstirred, agonist-treated platelets (Fig. 5 B). These results strongly suggest that fibrinogen binding to GP IIb-IIIa is not sufficient to induce pp125 FAK phosphorylation, and that subsequent events that occur during the process of aggregation are necessary to activate tyrosine phosphorylation of pp125 FAK.

pp125 FAK is localized in focal adhesions in CEFs (28). These cellular structures include the integrin receptors and associated cytoskeletal proteins, including actin. Since actin polymerization is induced after thrombin treatment of platelets (6, 7, 13, 21), we examined whether the phosphorylation of pp125 FAK is affected by agents that disrupt the actin cytoskeleton. Platelets were pretreated with 10 μM cytochalasin D (CD) which blocked agonist-induced actin polymerization in platelets (6, 7, 13, 21), but did not inhibit thrombin- or collagen-induced platelet aggregation (data not shown). Pretreatment with CD completely blocked tyrosine phosphorylation of pp125 FAK in thrombin or collagen stimulated platelets (Fig. 6 A). In addition, CD pretreatment of platelets incubated with collagen prevented the stimulation of pp125 FAK phosphorylating activity as assayed in these immunocomplex kinase assays (Fig. 6 B). There was no increase in tyrosine phosphorylation of pp125 FAK after longer periods of thrombin treatment (data not shown). These results suggest that cytochalasin D disrupts actin-dependent cytoskeletal interactions that are necessary for tyrosine phosphorylation of pp125 FAK and activation of its phosphorylating activity.

Discussion

Previous studies in platelets provided the first evidence that integrin-mediated intracellular signaling may involve protein tyrosine phosphorylation (11, 14). The phosphorylation of several proteins induced by thrombin was inhibited under conditions where fibrinogen binding to its integrin receptor, GP IIb-IIIa, was blocked (11, 14). These results raised the possibility that fibrinogen-dependent engagement of GP IIb-IIIa, and subsequent platelet aggregation, results in the activation of a protein tyrosine kinase. A candidate kinase, pp125 FAK, was originally identified in studies of a phosphotyrosine-containing protein from normal and Src-transformed CEFs (22). The evidence that this protein is localized in focal adhesions (28), and that it is phosphorylated on tyrosine after cross-linking integrins on human carcinoma cells (L. J. Kornberg and R. L. Juliano, personal communication) or after plating NIH-3T3 cells or CEFs on fibronectin matrices (J. L. Guan, I. Chackalaparampil, and D. Shalloway, personal communication; M. D. Schaller, B. S. Cobb, and J. T. Parsons, unpublished results), suggested that engagement of integrin molecules could potentially alter the activity of this kinase.

In this report we demonstrate that pp125 FAK is phosphorylated on tyrosine after thrombin or collagen treatment of platelets and that pp125 FAK molecules isolated from agonist-treated platelets display elevated levels of tyrosine phosphorylation in vitro in immune-complex kinase assays. These results show a correlation between tyrosine phosphorylation in vivo and the activation of the in vitro phosphorylating activity associated with pp125 FAK. In addition, we demonstrate that activation of tyrosine phosphorylation of pp125 FAK both in vivo and in vitro is dependent on events induced by fibrinogen binding to GP IIb-IIIa. pp125 FAK was not phosphorylated on tyrosine after thrombin or collagen treatment of GP IIb-IIIa-deficient platelets from donors with Glanzmann's thrombasthenia, and there was no detectable stimulation of pp125 FAK-associated phosphorylating activity in vitro in platelets pretreated with a mAb to GP IIb-IIIa to block fibrinogen binding. In addition, tyrosine phosphorylation of pp125 FAK in vivo and in vitro required platelet stirring, suggesting that fibrinogen binding to GP IIb-IIIa alone is not sufficient to induce pp125 FAK phosphorylation and that subsequent integrin-dependent events that accompany platelet aggregation are required for pp125 FAK phosphorylation. These results strongly support the possibility that tyrosine phosphorylation of pp125 FAK occurs downstream of platelet aggregation mediated by fibrinogen binding to GP IIb-IIIa, and thus implicate pp125 FAK as a candidate kinase that may be responsible for GP IIb-IIIa-dependent tyrosine phosphorylation events.

These results extend the preliminary studies performed in other cell types which showed that engagement and clustering of integrin-family fibronectin receptors (by macrocrossing or binding to a fibronectin matrix) causes an induction of tyrosine phosphorylation of pp125 FAK (J. L. Guan, I. Chackalaparampil, and D. Shalloway, personal communica-
It is important to establish the mechanisms involved in coupling integrin receptors with protein tyrosine kinases. The induction of tyrosine phosphorylation of pp125FAK by collagen in solution was dependent on fibrinogen binding to GP IIb-IIIa, suggesting that pp125FAK phosphorylation occurs "downstream" from platelet aggregation (mediated by fibrinogen binding to GP IIb-IIIa), and that engagement of the collagen receptor, GP Ia-IIa, is not sufficient for pp125FAK phosphorylation.

Interactions between integrins and cytoskeletal protein complexes that include actin microfilaments might form the framework upon which tyrosine kinases and other signaling enzymes associate at the plasma membrane. This possibility is consistent with recent evidence indicating that GP IIb-IIIa as well as several enzymes, including phosphatidylinositol 3'-kinase, diacylglycerol kinase, and pp60c-src, associate with a detergent-insoluble cell fraction following platelet activation (15, 35). Furthermore, we have found that the association of pp60c-src with this fraction is dependent on GP IIb-IIIa because this kinase is not found in the Triton-insoluble fraction of thrombin-treated platelets from GP IIb-IIIa-deficient patients with Glanzmann's thrombasthenia (E. Clark, L. Lipfert, and J. Brugge, unpublished data).

The results presented in this report show a strong correlation between tyrosine phosphorylation of pp125FAK in vivo and enhanced phosphorylation of pp125FAK in immune complex kinase assays in vitro. In each assay in which duplicate immunoprecipitates were assayed for in vivo tyrosine phosphorylation and in vitro phosphorylation activity (Figs. 4-6), conditions that prevented tyrosine phosphorylation of pp125FAK in vivo also inhibited the phosphorylation of pp125FAK in vitro. These results suggest that tyrosine phosphorylation of pp125FAK by another kinase or by autophosphorylation might stimulate the phosphorylating activity of this kinase. Alternatively, autophosphorylation of pp125FAK in vivo may not affect its catalytic activity, but merely be a consequence of its "activated" state. Further studies to determine whether pp125FAK is phosphorylated in vivo by autophosphorylation, and whether this phosphorylation is necessary for its in vitro phosphorolytic activity are in progress.

Although we can not rule out the possibility that pp125FAK is phosphorylated in incomplexes. Formed with mAb 2A7, we detected a 56-kD alkali-stable phosphorylated protein whose phosphorylation correlated with pp125FAK phosphorylation (Fig. 3). However, this protein was not detected in any of the in vitro kinase assays using the polyclonal rabbit anti-pp125FAK serum. A 62-kD protein was detected in the in vitro kinase assay using the polyclonal rabbit anti-pp125FAK immunoprecipitates; however, the phosphorylation of this protein did not correlate with pp125FAK phosphorylation in vitro (Figs. 4 and 5) and, whereas pp125FAK phosphorylating activity could be removed by preclearing with anti-pp125FAK serum, this treatment did not diminish the 62-kD protein phosphorylation (data not shown). The identity of these coprecipitating proteins and the basis for their precipitation by the antibodies to pp125FAK are not known.

In thrombin-treated platelets, tyrosine phosphorylation of several proteins is dependent on aggregation mediated by fibrinogen binding to GP IIb-IIIa (11, 14). Any of these proteins are candidate targets of pp125FAK kinase activity. In contrast, spreading of NIH-3T3 cells on a fibrinogen matrix leads to a much more limited tyrosine phosphorylation response (16). In these cells, only a diffuse band of Mr 115,000-130,000, and a few minor phosphotyrosine-containing proteins were detected in immunoblots probed with antibodies to phosphotyrosine. Although this diffuse Mr 115,000-130,000 immunoreactive band could contain other phosphotyrosine-containing proteins in addition to pp125FAK, these results suggest that there are only a few candidate targets of pp125FAK activity in these cells.

In platelets, we have found that the protein tyrosine kinases Src, Yes, Fyn, and Lyn redistribute to the triton-insoluble, cytoskeleton-rich cell fraction in an aggregation-dependent manner (E. Clark, L. Lipfert, and J. Brugge, unpublished observations). These protein tyrosine kinases could also be responsible for the phosphorylation of substrate proteins following aggregation due to their redistribution to this cytoskeletal fraction. The differences in integrin-regulated tyrosine phosphorylation of cellular proteins in platelets and other cell types suggest that there are cell-type or integrin-specific targets of adhesion-regulated tyrosine kinases.

The platelet proteins that are phosphorylated on tyrosine in an aggregation-dependent fashion could be responsible for the activation of intracellular signaling pathways necessary for cellular processes that are dependent on platelet aggregation (i.e., activation of the Na⁺/H⁺ pump or calpain activation). Further studies are necessary to determine whether there is a cause-effect relationship between these events and to determine the function of proteins that are phosphorylated on tyrosine following platelet aggregation mediated by fibrinogen binding to GP IIb-IIIa.

We thank Dr. Sandy Shattil for many fruitful discussions and critical review of this manuscript; Ed Clark and Dr. Charles Abrams for critical review of this manuscript; Robert Abel (Christiana Medical Center, Newark, DE) for providing blood from donors with Glanzmann's thrombasthenia, Dr. Barry Coller for mAb 6F1; Dr. Robert Gordon for his generous supply of mAb 7E3; Dr. Thomas Roberts for mAb 4G10; and Cass Lutz for secretarial assistance.

This work was supported by Howard Hughes Medical Institute and grants from the National Institutes of Health (CA40042 and CA29243 to J. T. Parsons). M. D. Schaller is supported by a postdoctoral fellowship from the National Cancer Institute of Canada.

Received for publication 20 April 1992 and in revised form 8 August 1992.
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