Integrin-Dependent Phosphorylation and Activation of the Protein Tyrosine Kinase pp125^{FAK} 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, pp125^{FAK} (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 pp125^{FAK} and that pp125^{FAK} molecules isolated from activated platelets display enhanced levels of phosphorylation in immune-complex kinase assays. pp125^{FAK} 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 GP IIb-IIIa. Fibrinogen binding to GP IIb-IIIa was not sufficient to induce pp125^{FAK} phosphorylation because pp125^{FAK} was not phosphorylated on tyrosine in thrombin-treated platelets that were not allowed to aggregate. These results indicate that tyrosine phosphorylation of pp125^{FAK} is dependent on platelet aggregation mediated by fibrinogen binding to the integrin receptor GP IIb-IIIa. The induction of tyrosine phosphorylation of pp125^{FAK} 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 pp125^{FAK} in vivo and stimulation of the phosphorylation of pp125^{FAK} in vitro in immune-complex kinase assays. This study provides the first genetic evidence that tyrosine phosphorylation of pp125^{FAK} is dependent on integrin-mediated events, and demonstrates that there is a strong correlation between tyrosine phosphorylation of pp125^{FAK} in platelets, and the activation of pp125^{FAK}-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 $\alpha\beta$ transmembrane proteins (1, 17, 19, 20). Although the extracellular interactions between adhesion molecules and their integrin receptors have been well characterized, the interactions of integrins 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, GP IIb-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 GP IIb-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 Ca^{2+}-dependent protease, calpain (2, 12). In platelets treated with weak agonists, such as ADP, aggregation mediated by fibrinogen binding to GP IIb-IIIa is also required for the stimulation of arachidonate metabolism and dense granule secretion (2, 18, 29, 30).

The mechanisms involved in signaling through GP IIb-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 GP IIb-IIIa (11, 14). These results suggested that tyrosine phosphorylation might be involved in GP IIb-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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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 pp125^{FAK} (FAK, focal adhesion kinase), was first identified using monoclonal antibodies (mAbs) raised against phosphotyrosine-containing proteins isolated from pp60$^{v\text{-}src}$ transformed cells (22). In normal fibroblasts, pp125^{FAK} 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$^{v\text{-}src}$ (22). The cDNA encoding pp125^{FAK} 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 able 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 (CEFs)[1] 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).

We have examined integrin-mediated regulation of tyrosine phosphorylation and activity of pp125^{FAK} in platelets. Both thrombin and collagen-stimulated platelet activation led to an induction of tyrosine phosphorylation of pp125^{FAK}. In addition, we show that p125^{FAK} phosphorylating activity in vitro strongly correlates with tyrosine phosphorylation of pp125^{FAK} in vivo. Furthermore, we have taken advantage of the availability of platelets that are genetically deficient in GP IIb-IIIa to show that thrombin- or collagen-induced tyrosine phosphorylation of pp125^{FAK}, and stimulation of its phosphorylating activity in vitro, are dependent on fibrinogen binding to GP IIb-IIIa. These results provide the first genetic evidence linking integrin receptors with tyrosine phosphorylation of pp125^{FAK}, and the first demonstration that tyrosine phosphorylation of pp125^{FAK} in vivo correlates with activation of the phosphorylating activity of this protein tyrosine kinase in vitro.

Materials and Methods

Platelet Preparation and Activation

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 prostaglandin E_1 (PGE_1) (31). Briefly, platelet-rich plasma was prepared by sedimentation of blood at 180 *g* for 20 min. The platelets were concentrated by an additional sedimentation at 900 *g* for 15 min, resuspended in 3 ml plasma supplemented with 1 μM PGE_1 and 1 U/ml apyrase, and filtered over a Sepharose-2B column equilibrated in 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 5.6 mM glucose, 1 mg/ml BSA, 3.3 mM NaH_2PO_4, and 20 mM Hepes, pH 7.4. 0.5 ml of gel-filtered platelets at a concentration of 3–6 × 10^8 platelets/ml were left untreated or incubated for the indicated time in the absence or presence of α-thrombin (1 U/ml; Chrono-Log Corp., Havertown, PA), collagen (5 μg/ml; Sigma Chemical Co., St. Louis, MO), or ADP (10 μM, Chrono-Log Corp.), epinephrine (10 μM, Chrono-Log Corp.), and fibrinogen (50 μg/ml, Chrono-Log Corp.). Unless otherwise indicated, the platelets were stirred at 1,000 rpm during the incubation. At the end of the activation period the platelets were lysed by the addition of an equal volume of 2× modified RIPA buffer (1× is 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 158 mM NaCl, 10 mM Tris-HCl, pH 7.2, 1 mM sodium EGTA, 1 mM phenylmethylsulfonylfluoride [PMSF], 1 mM Na_3VO_4 [Fisher Scientific Co., Pittsburgh, PA], and 100 kallikrein inactivator units/ml of Trasylol [FBA Pharmaceuticals, West Haven, CT]) at 4°C for 30 min. In some experiments, platelets were pretreated with 10 μM cytochalasin D (Aldrich Chemical Co., Milwaukee, WI) for 20 min before their stimulation with thrombin or collagen. In other experiments, the gel-filtered platelets were preincubated for 20 min with the anti-GP IIb-IIIa mAb 7E3 (10 μg/ml) or mAb 10E5 (20 μg/ml) generously provided by Robert Jordon (Centocor, Inc., Malvern, PA) and Barry Coller (State University of New York of Stony Brook), respectively. Collagen binding to GP Ia-IIa was prevented by preincubating the gel-filtered platelets for 20 min with the anti-GP Ia-IIa mAb 6F1 (20 μg/ml) (8), generously provided by Barry Coller (State University of New York at Stony Brook).

Identification of Phosphotyrosine-Containing Proteins by Immunoblotting

Platelet lysates or immunoprecipitates (from 8 to 10 × 10^8 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 1:1,500 dilution of horseradish peroxidase-conjugated goat-anti-mouse IgG (Bio-Rad Laboratories, Richmond, CA) in buffer A. Immunoreactivity was determined using the ECL chemiluminescence reaction (Amersham 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 pp125^{FAK}, 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 pp125^{FAK} (28). Immune complexes were incubated for 30 min with 30 μl/ml 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 pp125^{FAK}, immunoprecipitates were eluted from Pansorbin, separated by SDS-PAGE, and immunoblotted onto nitrocellulose filters which where then probed with anti-phosphotyrosine mAbs, 4G10 or PY20 as described above. To examine the autophosphorylating activity of pp125^{FAK}, immunoprecipitates were washed twice in RIPA buffer, once with low salt buffer (100 mM NaCl, 10 mM Tris, pH 7.5, 5 mM $MnCl_2$) and incubated in 30 μl of kinase reaction mixture containing 10 mM Tris, pH 7.4, 5 mM $MnCl_2$, 1 μM ATP, and 10 μCi[γ^{32}P]ATP [1 Ci=37 GB_q, 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 ^{32}P-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).

1. *Abbreviations used in this paper*: CEF, chicken embryo fibroblast; CD, cytochalasin D; FAK, focal adhesion kinase; PGE_1, prostaglandin E_1.

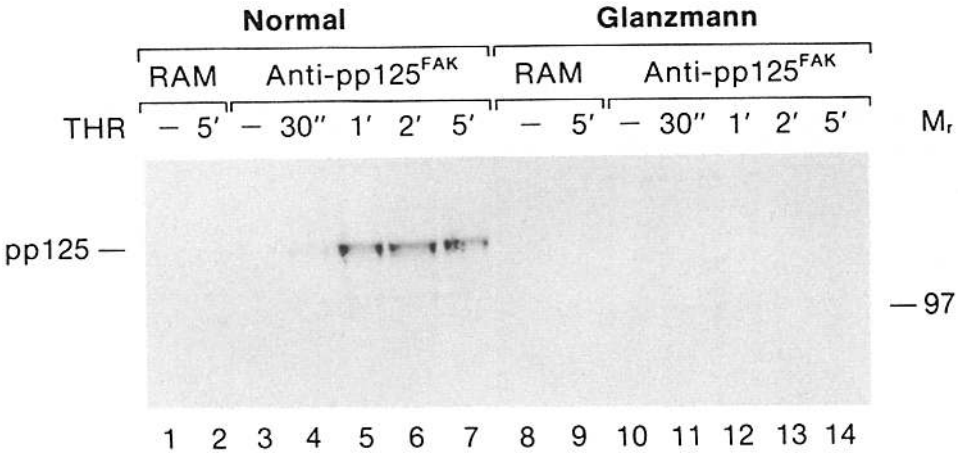

Figure 1. Time course of pp125^{FAK} tyrosine phosphorylation in thrombin-stimulated platelets. Gel-filtered platelets from a normal donor (lanes *1–7*) or from a donor with Glanzmann's thrombasthenia (lanes *8–14*) were stirred in the absence of agonist (lanes *1*, *3*, *8*, and *10*), or with thrombin (THR; 1 U/ml) for 30 s (lanes *4* and *11*), 1 min (lanes *5* and *12*), 2 min (lanes *6* and *13*), or 5 min (lanes *7* and *14*), and then lysed in RIPA buffer. Lysates were incubated with anti-pp125^{FAK} mAb 2A7 plus RAM (pp125^{FAK}; lanes *3–7* and *4–10*), or with RAM alone (RAM; lanes *1*, *2*, *8*, and *9*). Immunoprecipitated proteins were analyzed on immunoblots and probed with mAbs specific for phosphotyrosine residues.

Results

Tyrosine Phosphorylation of pp125^{FAK} 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 pp125^{FAK} is phosphorylated on tyrosine in activated platelets, pp125^{FAK} was immunoprecipitated from lysates of resting or thrombin-treated platelets, transferred to nitrocellulose, and probed with mAbs specific for phosphotyrosine residues (Fig. 1). The pp125^{FAK} immunoprecipitates from untreated platelets did not show any reactivity with the mAbs to phosphotyrosine. However, a 125,000 M_r phosphotyrosine-containing protein was immunoprecipitated from all thrombin-treated platelet lysates (lanes *5–7*). This protein displayed the predicted electrophoretic mobility of pp125^{FAK}, 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 pp125^{FAK} 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 pp125^{FAK} phosphorylation suggested that tyrosine phosphorylation of pp125^{FAK} 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 pp125^{FAK} was dependent on fibrinogen binding to GP IIb-IIIa by analyzing tyrosine phosphorylation of pp125^{FAK} 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 pp125^{FAK} in Glanzmann's platelets (lanes *10–14*), however, we detected normal levels of pp125^{FAK} in these platelets in immunoblot assays (data not shown). These results indicate that tyrosine phosphorylation of pp125^{FAK} is dependent on fibrinogen binding to GP IIb-IIIa.

pp125^{FAK} Phosphorylation in Collagen-Treated Platelets

Tyrosine phosphorylation of pp125^{FAK} was also examined in platelets treated with collagen, which interacts with another integrin receptor, GP Ia-IIa ($\alpha_2\beta_1$) (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 pp125^{FAK} at levels similar to those induced by thrombin (lanes *2* and *3*). As in thrombin-treated platelets, the induction of tyrosine phosphorylation of pp125^{FAK} by collagen was dependent on GP IIb-IIIa because pp125^{FAK} 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 pp125^{FAK} 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 pp125^{FAK} phosphorylation.

Stimulation of pp125^{FAK} Protein Tyrosine Kinase Activity

pp125^{FAK} 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 pp125^{FAK} is stimulated by thrombin, pp125^{FAK} immunoprecipitates from untreated or thrombin-treated platelets were

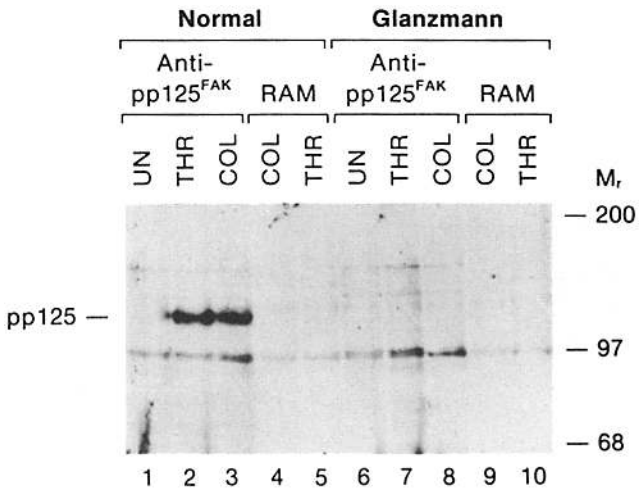

Figure 2. Induction of pp125^{FAK} tyrosine phosphorylation in thrombin- or collagen-treated platelets. Gel-filtered platelets from a normal donor (lanes *1–5*) or from a donor with Glanzmann's thrombasthenia (lanes *6–10*) were stirred for 2 min in the absence of agonist (UN; lanes *1* and *6*), with 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-pp125^{FAK} mAb 2A7 plus RAM (pp125^{FAK}; 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.

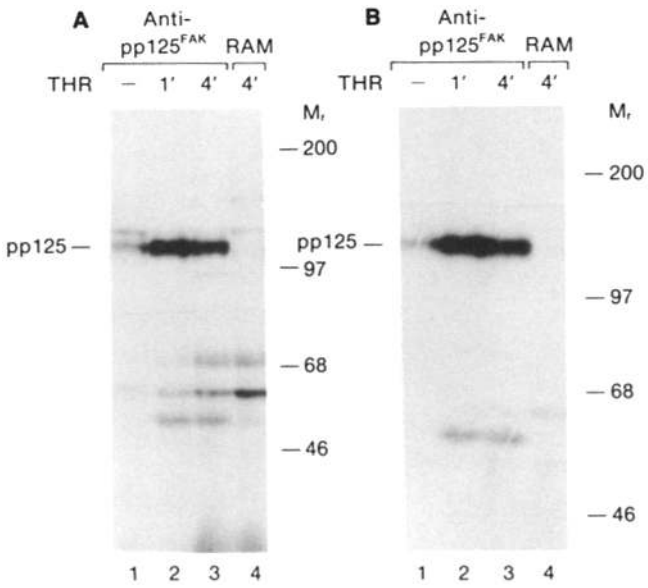

Figure 3. Stimulation of pp125^{FAK} in vitro tyrosine kinase activity. (*A*) Gel-filtered platelets were stirred in the absence of agonist (lane *1*), or in the presence of thrombin (THR; 1 U/ml) for 1 min (lane *2*) or 4 min (lanes *3* and *4*) and lysed in RIPA buffer. Lysates were incubated with anti-pp125^{FAK} mAb 2A7 plus RAM (pp125^{FAK}; lanes *1–3*) or with RAM alone (RAM; lane *4*). The immunocomplexes were incubated with [γ^{32}P]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.

incubated with [γ^{32}P]ATP (Fig. 3 *A*). Under these conditions, only a low level of phosphate was incorporated into a 125-kD protein in anti-pp125^{FAK} immunoprecipitates from untreated platelets (lane *1*). However, platelet activation by thrombin resulted in enhanced ^{32}P-incorporation into this protein (lanes *2* and *3*). Control immunoprecipitates did not contain a similar phosphorylated protein (lane *4*). Several other ^{32}P-labeled proteins of M_r 56,000, 62,000, and 73,000 were detected in these assays. Since the proteins of M_r 73,000 and 62,000 were also observed in the control immunoprecipitates (lane *4*), they appear to represent nonspecific phosphorylated proteins. The 56-kD protein detected in anti-pp125^{FAK} immunoprecipitates from thrombin-treated platelets could represent a coprecipitating substrate of pp125^{FAK}, a coprecipitating protein kinase, or a protein that nonspecifically associated with the immune complexes. The phosphate label on the M_r 125,000 and 56,000 proteins was stable to alkali treatment strongly suggesting that these proteins may be phosphorylated on tyrosine (Fig. 3 *B*). Phosphoamino acid analysis of pp125^{FAK} labeled in vitro revealed phosphotyrosine only (M. D. Schaller and J. T. Parsons, data not shown). These results suggest that thrombin stimulates the autophosphorylating activity of pp125^{FAK}, though we cannot absolutely rule out the involvement of another kinase which is activated by thrombin.

The activation of pp125^{FAK} phosphorylation in vivo and in vitro was examined under several additional experimental conditions. To determine whether tyrosine phosphorylation of pp125^{FAK} was dependent on fibrinogen binding to GP IIb-IIIa, platelets were preincubated with a GP IIb-IIIa–specific mAb (7E3) that prevents fibrinogen binding to this receptor (8). 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 pp125^{FAK} in thrombin-treated platelets (Fig. 4 *A*) and prevented thrombin-induced pp125^{FAK} 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 pp125^{FAK} in vivo (Fig. 4 *A*, lane *5*) or in vitro (Fig. 4 *B*, lane *5*). However, this antibody prevented collagen stimulation of pp125^{FAK} phosphorylation in vivo. There were no detectable differences in the levels of pp125^{FAK} in these immunoprecipitations (as assayed by reprobing with the polyclonal anti-pp125^{FAK} serum, data not shown). A 62-kD protein was also detected in the in vitro kinase assays of anti-pp125^{FAK} immunoprecipitates from thrombin- and collagen-treated cells; however, the phosphorylation of this protein did not correlate with pp125^{FAK} phosphorylation. These results indicate the stimulation of the phosphorylation activity of pp125^{FAK} is dependent on fibrinogen-binding to GPIIb-IIIa in both thrombin- and collagen-treated platelets, and demonstrate a correlation between tyrosine phosphorylation of pp125^{FAK} in vivo and activation of the in vitro phosphorylation activity of pp125^{FAK}.

To examine whether fibrinogen binding to GP IIb-IIIa is sufficient to stimulate pp125^{FAK} 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-

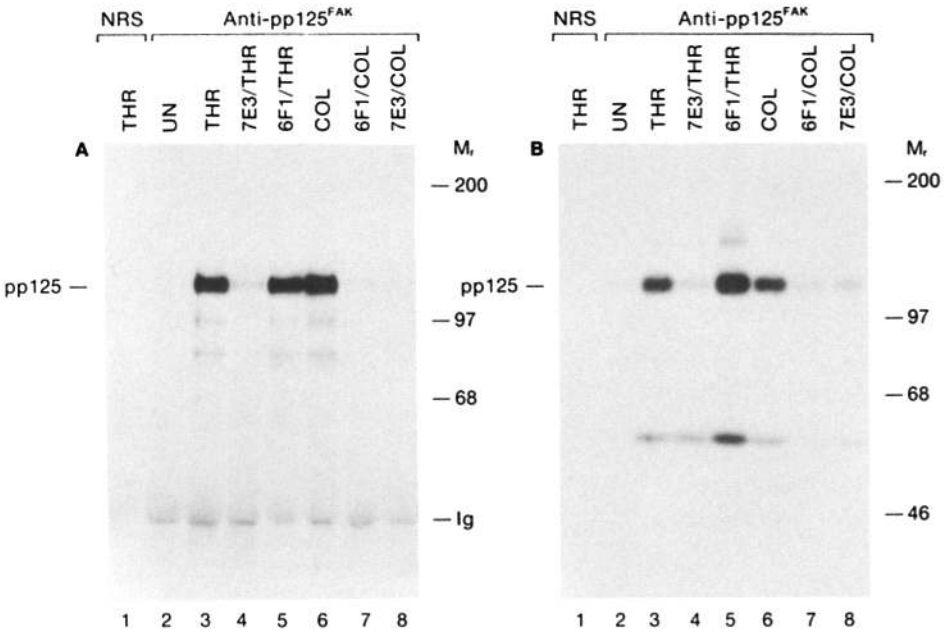

Figure 4. Activation of the in vitro tyrosine kinase activity associated with pp125^{FAK} is dependent on the integrin GP IIb-IIIa. Gel-filtered platelets unstimulated (UN; lane *2*), or stimulated with thrombin (THR; lanes *1*, *3*, *4*, and *5*) or collagen (COL; lanes *6*, *7*, and *8*) were lysed 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-pp125^{FAK} 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 μC [γ^{32}P]ATP as described in Materials and Methods (*B*) to allow for in vitro phosphorylation. The gel shown in *B* was treated with alkali (*9*).

tions, 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). 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 pp125^{FAK} in platelets treated with the strong agonist, thrombin, or the combination of relatively

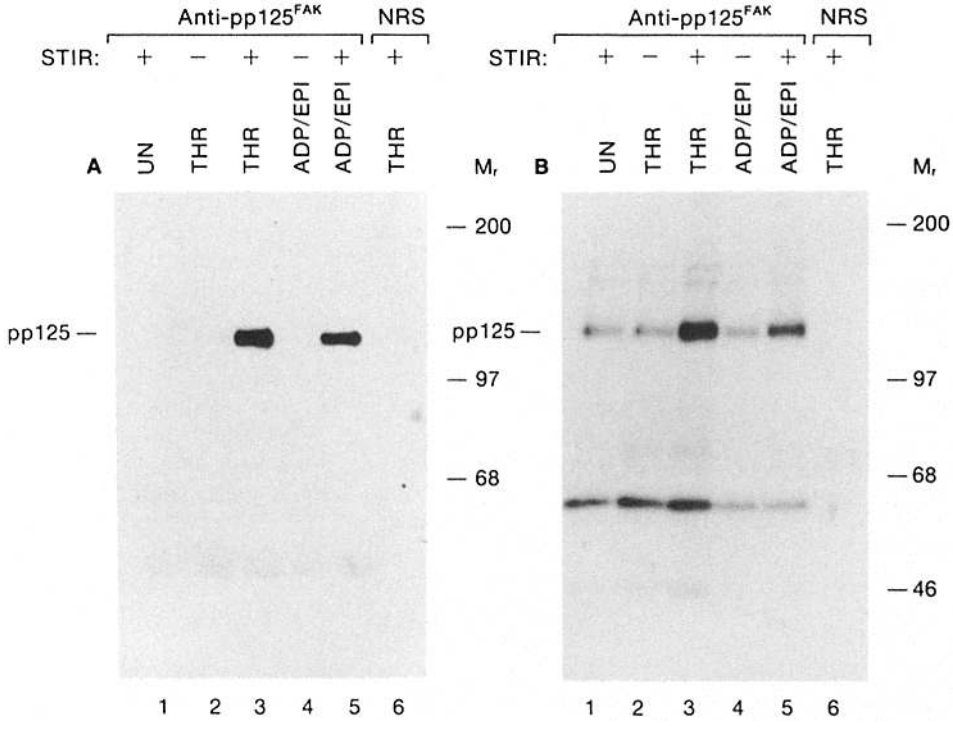

Figure 5. Platelet aggregation is required for stimulation of tyrosine phosphorylation of pp125^{FAK}. Gel-filtered platelets unstimulated (UN; lane *1*) or stimulated with thrombin (THR; lanes *2*, *3*, and *6*) or a combination of ADP, epinephrine, and fibrinogen (ADP/EPI; lanes *4* and *5*) were either left unstirred (lanes *2* and *4*) or stirred (lanes *1*, *3*, *5*, and *6*) for 1 min before lysing in RIPA buffer. Platelet aggregation and secretion of ATP were monitored by lumiaggregometry. Lysates were incubated with the polyclonal rabbit anti-pp125^{FAK} 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^{32}P]ATP (as described in Materials and Methods) to allow for in vitro phosphorylation. Phosphorylated proteins were separated on an SDS/7.5% polyacrylamide 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.

weak agonists, ADP and epinephrine (Fig. 5 *A*, lanes *2* and *4*). 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.

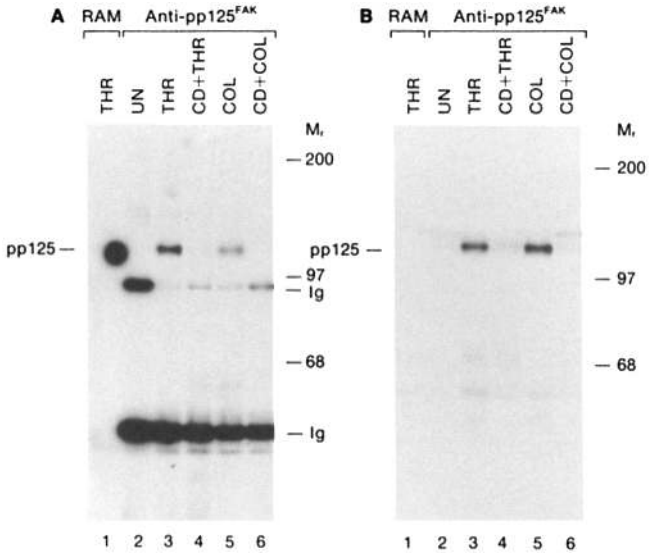

Figure 6. Treatment of platelets with cytochalasin D blocks activation of the in vitro tyrosine kinase activity associated with pp125^{FAK}. Gel-filtered platelets unstimulated (UN; lane *2*), or stimulated with thrombin (TH; lanes *1*, *3*, and *4*), or collagen (COL; lanes *5* and *6*), were lysed with RIPA buffer. Parallel samples were pretreated with 10 μM CD for 20 min before stimulation with thrombin (CD + THR; lane *4*) or collagen (CD and COL; lane *6*). Lysates were incubated with anti-pp125^{FAK} mAb 2A7 and RAM (lanes *2–6*) or with RAM alone (RAM; lane *1*). (*A*) Immunoprecipitated proteins were analyzed on immunoblots probed with mAbs specific for phosphotyrosine residues. The 97-K Ig band represents a single dimer of the heavy and light immunoglobulin chains. (*B*) The immunocomplexes were incubated with 10 μCi [γ^{32}P]ATP (as described in Materials and Methods) to allow for in vitro phosphorylation. Phosphorylated proteins were separated on SDS/7.5% polyacrylamide gels.

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 mAb-crossing 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-

tion; L. S. Kornberg and R. L. Juliano, personal communication; M. D. Schaller, B. S. Cobb, and J. T. Parsons, unpublished results). Taken together, all of these results suggest a link between integrin receptors, tyrosine phosphorylation of cellular proteins and the activation of tyrosine phosphorylation of pp125^{FAK}.

It is important to establish the mechanisms involved in coupling integrin receptors with protein tyrosine kinases. The induction of tyrosine phosphorylation of pp125^{FAK} by collagen in solution was dependent on fibrinogen binding to GP IIb-IIIa, suggesting that pp125^{FAK} 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 pp125^{FAK} 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 pp60^{src}, associate with a detergent-insoluble cell fraction following platelet activation (15, 35). Furthermore, we have found that the association of pp60^{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. S. Brugge, unpublished data).

The results presented in this report show a strong correlation between tyrosine phosphorylation of pp125^{FAK} in vivo and enhanced phosphorylation of pp125^{FAK} 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 pp125^{FAK} in vivo also inhibited the phosphorylation of pp125^{FAK} in vitro. These results suggest that tyrosine phosphorylation of pp125^{FAK} by another kinase or by autophosphorylation might stimulate the phosphorylating activity of this kinase. Alternatively, autophosphorylation of pp125^{FAK} in vivo may not affect its catalytic activity, but merely be a consequence of its "activated" state. Further studies to determine whether pp125^{FAK} is phosphorylated in vivo by autophosphorylation, and whether this phosphorylation is necessary for its in vitro phosphorylating activity are in progress.

Although we can not rule out the possibility that pp125^{FAK} is phosphorylated in immunocomplexes by an associated protein tyrosine kinase, our current data does not strongly support this possibility. In anti-pp125^{FAK} immunocomplexes formed with mAb 2A7, we detected a 56-kD alkali-stable phosphorylated protein whose phosphorylation correlated with pp125^{FAK} phosphorylation (Fig. 3). However, this protein was not detected in any of the in vitro kinase assays using the polyclonal rabbit anti-pp125^{FAK} serum. A 62-kD protein was detected in the rabbit anti-pp125^{FAK} immunoprecipitates; however, the phosphorylation of this protein did not correlate with pp125^{FAK} phosphorylation in vitro (Figs. 4 and 5) and, whereas pp125^{FAK} phosphorylating activity could be removed by preclearing with anti-pp125^{FAK} 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 pp125^{FAK} 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 pp125^{FAK} 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 M_r 115,000–130,000, and a few minor phosphotyrosine-containing proteins were detected in immunoblots probed with antibodies to phosphotyrosine. Although this diffuse M_r 115,000–130,000 immunoreactive band could contain other phosphotyrosine-containing proteins in addition to pp125^{FAK}, these results suggest that there are only a few candidate targets of pp125^{FAK} 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.

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References

1. Albelda, S. M., and C. A. Buck. 1990. Integrins and other cell adhesion molecules. *FASEB J.* 4:2868–2889.
2. Banga, H. E., E. Simons, L. Brass, and S. Rittenhouse. 1986. Activation of phospholipases A and C in human platelets exposed to epinephrine: the role of glycoproteins IIb-IIIa. *Proc. Natl. Acad. Sci. USA.* 83:9197–9201.
3. Baumgarter, H. R. 1977. Platelet interaction with collagen fibrils in flowing blood. I. Reaction of human platelets with alpha-chymotrypsin-digested subendothelium. *Thromb. Haemost.* 37:1–16.
4. Bennett, J. S., and G. Vilaire. 1979. Exposure of platelet fibrinogen receptors by ADP and epinephrine. *J. Clin. Invest.* 64:1393–1401.
5. Burridge, K., G. Nuckolls, C. Otey, F. Pavalko, K. Simon, and C. Turner. 1990. Actin–membrane interaction in focal adhesions. *Cell Differ. Dev.* 32:337–342.

6. Carlsson, L., F. Markey, I. Bilkstad, T. Persson, and U. Lindberg. 1979. Reorganization of actin in platelets stimulated by thrombin as measured by the DNase I inhibition assay. *Proc. Natl. Acad. Sci. USA.* 76:6376–6380.
7. Carroll, R. C., R. G. Butler, and P. A. Morris. 1982. Separable assembly of platelet pseudopodal and contractile cytoskeletons. *Cell.* 30:385–393.
8. Coller, B. S., J. H. Beer, L. E. Scudder, and M. H. Steinberg. 1989. Collagen-platelet interactions: evidence for direct interaction of collagen with platelet GPIa/IIa and an interaction with platelet GPIIb/IIIa medicated by adhesive proteins. *Blood.* 74:182–192.
9. Cooper, J. A., and T. Hunter. 1981. Changes in protein phosphorylation of Rous sarcoma virus-transformed chicken embryo cells. *Mol. Cell Biol.* 1:165–178.
10. Drucker, B., T. Mamon, and T. M. Roberts. 1989. Oncogenes, growth factors, and signal transduction. *N. Engl. J. Med.* 321:1383–1391.
11. Ferrell, J. E., and G. S. Martin. 1989. Tyrosine-specific protein phosphorylation is regulation by glycoprotein IIb-IIIa in platelets. *Proc. Natl. Acad. Sci. USA.* 86:2234–2238.
12. Fox, J. E. B. 1987. The Platelet Cytoskeleton. *In* Thrombosis and Haemostasis. M. Verstaete, J. Vermylen, R. Linjnen, and J. Arnout, editors. Leuven University Press. 175–225.
13. Fox, J. E. B., and D. R. Phillips. 1981. Inhibition of actin polymerization in blood platelets by cytochalasins. *Nature (Lond.).* 292:650–652.
14. Golden, A., J. S. Brugge, and S. Shattil. 1991. Role of platelet membrane glycoprotein IIb-IIIa in agonist-induced tyrosine phosphorylation of platelet proteins. *J. Cell Biol.* 111:3117–3127.
15. Grondin, P., M. Plantavid, C. Sultan, M. Breton, G. Mauco, and H. Chap. 1991. Interaction of $pp60^{c\text{-}src}$, phospholipase c, inositol-lipid, and diacylglycerol kinases with the cytoskeletons of thrombin-stimulated platelets. *J. Biol. Chem.* 266:15705–15709.
16. Guan, J.-L., J. E. Trevithick, and R. O. Hynes. 1991. Fibronectin/integrin interaction induces tyrosine phosphorylation of a 120-kDa protein. *Cell Regul.* 2:951–964.
17. Hemler, M. E. 1990. VLA proteins in the integrin family: structures, functions and their role on leukocytes. *Annu. Rev. Immunol.* 8:365–400.
18. Huang, E. M., and T. C. Detwiler. 1986. Stimulus-response coupling mechanisms. *In* Biochemistry of Platelets. D. R. Philips and M. A. Shuman, editors. Academic Press, Inc., Orlando. 1–68.
19. Hynes, R. O. 1992. Integrins: versatility, modulation, and signalling in cell adhesion. *Cell.* 69:11–25.
20. Hynes, R. O., and A. D. Lander. 1992. Contact and adhesive specificities in the associations, migrations, and targeting of cells and axons. *Cell.* 68:303–322.
21. Jennings, L. K., J. E. B. Fox, H. H. Edwards, and D. R. Phillips. 1981. Changes in the cytoskeletal structure of human platelets following thrombin activation. *J. Biol. Chem.* 256:6927–6932.
22. Kanner, S. B., A. B. Reynolds, R. R. Vines, and J. T. Parsons. 1990. Monoclonal antibodies to individual tyrosine-phosphorylated protein substrates of oncogene-encoded tyrosine kinases. *Proc. Natl. Acad. Sci. USA.* 87:3328–3332.
23. Kieffer, N., and D. R. Phillips. 1990. Platelet membrane glycoproteins: functions in cellular interactions. *Annu. Rev. Cell Biol.* 6:329–357.
24. Kornberg, L. J., H. S. Earp, C. E. Turner, C. Prockop, and R. L. Juliano. 1991. Signal transduction by integrins: increased protein tyrosine phosphorylation caused by clustering of β integrins. *Proc. Natl. Acad. Sci. USA.* 88:8392–8396.
25. Phillips, D. R., I. F. Choro, and R. M. Scarborough. 1991. GPIIb-IIIa: the responsive integrin. *Cell.* 65:359–362.
26. Plow, E. F., and M. H. Ginsberg. 1989. Cellular adhesion. GPIIb-IIIa as a prototypic adhesion receptor. *Prog. Hemost. Thromb.* 9:117–156.
27. Santoro, S. 1986. Identification of 160,000 dalton platelet membrane protein that mediates the initial divalent cation-dependent adhesion of platelets to collagen. *Cell.* 46:913–920.
28. Schaller, M. D., C. A. Borgman, B. S. Cobb, R. R. Vines, A. B. Reynolds, and J. T. Parsons. 1992. pp125FAK, a structurally distinctive protein tyrosine kinase associated with focal adhesions. *Proc. Natl. Acad. Sci. USA.* 89:5192–5196.
29. Sweatt, J. D., T. M. Connolly, E. J. Cragoe, and L. E. Limbird. 1986. Evidence that Na^+/H^+ exchange regulates receptor-medicated phospholipase A2 activation in human platelets. *J. Biol. Chem.* 261:8667–8673.
30. Sweatt, J. D., S. L. Johnson, E. J. Cragoe, and L. E. Limbird. 1985. Inhibitors of Na^+/H^+ exchange block stimulus-provoked arachidonic acid release in human platelets. Selective effects on platelet activation by epinephrine, ADP, and lower concentrations of thrombin. *J. Biol. Chem.* 260:12910–12919.
31. Tangen, O., H. J. Berman, and P. Marfey. 1971. Gel filtration: a new technique for separation of blood platelets from plasma. *Thromb. Haemost.* 25:268–278.
32. Wang, J. Y. J., and D. Baltimore. 1985. Localization of the tyrosine kinase coding region in v-abl oncogene by the expression of v-abl-encoded proteins in bacteria. *J. Biol. Chem.* 260:64–71.
33. Weiss, H. J., J. Hawiger, Z. M. Ruggeri, V. T. Turitta, P. Thiagarajen, and T. Hoffman. 1989. Fibrinogen-independent platelet adhesion and thrombus formation subendothelium mediated by glycoprotein IIb-IIIa. *J. Clin. Invest.* 83:288–297.
34. Weiss, H. J., V. T. Turitto, and H. R. Baumgartner. 1986. Platelet adhesion and thrombus formation on subendothelium in platelets deficient in glycoproteins IIb-IIIa, and storage granules. *Blood.* 67:322–330.
35. Zhang, J., M. J. Fry, M. D. Waterfield, S. Jaken, L. Liao, J. E. B. Fox, and S. E. Rittenhouse. 1992. Activated phosphoinositide 3-kinase associates with membrane skeleton in thrombin-exposed platelets. *J. Biol. Chem.* 267:4686–4692.