Role of Platelet Membrane Glycoprotein IIb-IIIa in Agonist-induced Tyrosine Phosphorylation of Platelet Proteins

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Abstract. Treatment of platelets with thrombin was shown previously to induce rapid changes in tyrosine phosphorylation of several platelet proteins. In this report, we demonstrate that a variety of agonists which induce platelet aggregation also stimulate tyrosine phosphorylation of three proteins with apparent molecular masses of 84, 95, and 97 kD. Since platelet aggregation requires the agonist-induced activation of an integrin receptor (GP IIb-IIIa) as well as the binding of fibrinogen to this receptor, we examined the relationship between tyrosine phosphorylation and the function of GP IIb-IIIa. When platelets were examined under conditions that either precluded the activation of GP IIb-IIIa (prior disruption of the complex by EGTA at 37°C) or the binding of fibrinogen (addition of RGDS or an inhibitory mAb), tyrosine phosphorylation of the 84-, 95-, and 97-kD proteins was not observed. However, although both GP IIb-IIIa activation and fibrinogen binding were necessary for tyrosine phosphorylation, they were not sufficient since phosphorylation was observed only under conditions in which the activated platelets were stirred and allowed to aggregate. In contrast, tyrosine phosphorylation was not dependent on another major platelet response, dense granule secretion. Furthermore, granule secretion did not require tyrosine phosphorylation of this set of proteins. These experiments demonstrate that agonist-induced tyrosine phosphorylation is linked to the process of GP IIb-IIIa-mediated platelet aggregation. Thus, tyrosine phosphorylation may be required for events associated with platelet aggregation or for events that follow aggregation.

PLATELETS are an excellent model system in which to study the mechanisms of signal transduction. Physiological agonists elicit a variety of cellular responses including shape change, aggregation, granule secretion, and generation of membrane procoagulant activity. The transduction mechanisms that lead to these platelet responses include activation of guanine nucleotide-binding proteins, hydrolysis of membrane phospholipids, release and metabolism of arachidonic acid, influx and mobilization of calcium, and stimulation of protein phosphorylation (Siess, 1989; Brass et al., 1990).

The role of protein phosphorylation in platelet responses has been the subject of intensive study (Kroll and Schafer, 1989; Siess, 1989). Many of the phosphorylation events observed upon platelet stimulation have been attributed to the actions of protein kinase C and myosin light chain kinase, both of which are serine/threonine-specific protein kinases. Recently, several tyrosine-specific protein kinases have been detected in platelets. These include the cellular *src* gene product (Golden et al., 1986; Varshney et al., 1986; Presek et al., 1988; Rendu et al., 1989), the cellular *fyn* gene product (Horak et al., 1989), and several unidentified kinases (Nakamura et al., 1985, 1988). Moreover, a number of proteins are phosphorylated on tyrosine residues after platelet activation by thrombin and other platelet agonists (Ishihara et al., 1985; Ferrell and Martin, 1988, 1989; Golden and Brugge, 1989; Nakamura and Yamamura, 1989). The identity of the protein-tyrosine kinases and their substrates and the precise role of tyrosine phosphorylation during platelet activation remain to be determined. Recently, Ferrell and Martin have shown that thrombin-induced tyrosine phosphorylation of several platelet proteins appears to require ligand binding to the platelet membrane glycoprotein (GP)[1] IIb–IIIa complex (Ferrell and Martin, 1989), a member of the integrin family of adhesion receptors (Pytela et al., 1986; Hynes, 1987).

GP IIb-IIIa plays a critical role in platelet aggregation, a process that requires the agonist-induced binding of fibrinogen to GP IIb-IIIa (Bennett and Vilaire, 1979; Plow and Ginsberg, 1989). Agonists activate GP IIb-IIIa, presumably by inducing a conformational change that exposes a binding site for fibrinogen, thus enabling fibrinogen to bind in a calcium-dependent manner (Shattil et al., 1985*a*, 1986).

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1. *Abbreviations used in this paper*: GP, glycoprotein.

Once fibrinogen is bound, platelets can aggregate, provided that the cells physically interact. Several lines of evidence suggest that fibrinogen-mediated aggregation is coupled to other platelet response pathways. First, the activated form of GP IIb-IIIa has been implicated in platelet Na^+/H^+ exchange and in activation of phospholipase A_2 (Sweatt et al., 1985, 1986; Banga et al., 1986). Second, when platelets are stimulated by so-called "weak" agonists such as ADP or epinephrine, platelet aggregation is required for arachidonic acid metabolism, thromboxane A_2 synthesis, and granule secretion (Huang and Detwiler, 1986). In contrast, "strong" agonists, such as thrombin or collagen can induce thromboxane A_2 synthesis and granule secretion independent of platelet aggregation. However, even in the case of thrombin or collagen, aggregation is required to promote association of GP IIb-IIIa with the Triton-insoluble membrane cytoskeleton (Phillips et al., 1980; Fox, 1987) and to activate calcium-dependent proteases (calpains) within the cell (Fox, 1987).

Given the presence of multiple-protein tyrosine kinases in platelets, the rapid induction of tyrosine phosphorylation after platelet activation, and the central role of GP IIb-IIIa in platelet function, we sought to further define the relationship between GP IIb-IIIa and tyrosine phosphorylation. GP IIb-IIIa activation was monitored both by aggregometry and by a sensitive flow cytometric technique that measures the binding of an mAb specific for the activated conformation of GP IIb-IIIa. Tyrosine phosphorylation was studied using immunoblots probed with an affinity-purified polyclonal antiphosphotyrosine antibody. We demonstrate here that activation of GP IIb-IIIa and fibrinogen binding to GP IIb-IIIa are required, but not sufficient, for tyrosine phosphorylation of three specific platelet proteins with apparent molecular masses of 84, 95, and 97 kD. The phosphorylation of these proteins also requires platelet aggregation.

Materials and Methods

Isolation of Platelets

Venous blood was collected from healthy drug-free volunteers and anticoagulated with 0.01 vol 40% sodium citrate or 0.15 vol NIH Formula A acid-citrate-dextrose solution. Platelet isolation was carried out at room temperature. Platelet-rich plasma was obtained by centrifugation of whole blood at 180 *g* for 20 min. After addition of 1 μM prostaglandin E_1 (PGE_1), platelets were separated from plasma by gel-filtration (Tangen et al., 1971) over a Sepharose-2B column equilibrated with an incubation buffer containing 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 (Sigma Chemical Co., St. Louis, MO), pH 7.4. Gel-filtered platelets were counted in an electronic particle counter (Coulter Electronics, Hialeah, FL) and adjusted to a concentration of 1–3 $\times$ 10^8 platelets/ml with incubation buffer.

Activation of Gel-filtered Platelets

Immediately after gel filtration, 200 μM $CaCl_2$ was added to the platelet suspension and platelets were stimulated for 1 min at 37°C with bovine α-thrombin (1 U/ml; Chrono-Log, Havertown, PA), collagen (5 μg/ml; Chrono-Log), ADP or a combination of ADP and epinephrine (10 μM each; Chrono-Log), phorbol 12-myristate 13-acetate (100 nM; Sigma Chemical Co.), A23187 (1 μM; Sigma Chemical Co.), or mastoparan (50 μM; Sigma Chemical Co. or an HPLC-purified preparation; gift from Elliott Ross [University of Texas Southwestern Medical Center, Dallas, TX]; Higashijima et al., 1988). In the case of ADP and epinephrine, purified human fibrinogen (50 μg/ml) was also added to support platelet aggregation when the suspensions were stirred (Bennett and Vilaire, 1979). In the case of the other agonists, sufficient fibrinogen was released from platelet alpha-granules to support the aggregation of stirred platelets. As specified in each experiment, incubations were carried out either without stirring (to prevent aggregation) or in the presence of a magnetic stir bar rotated at 1,000 rpm.

In some experiments, the binding of fibrinogen to GP IIb-IIIa was prevented by incubating the gel-filtered platelets with either the tetrapeptide RGDS (500 μM; Sigma Chemical Co. or Peninsula Laboratories, Belmont, CA), 10 mM EDTA, or the anti-GP IIb-IIIa mAb; A2A9 (50–100 μg/ml) (Bennett et al., 1983) for 2 min before the addition of an agonist. In other experiments, platelets in plasma were preincubated with 10 mM EGTA for 90 min at either 20° or 37°C, and then equimolar $CaCl_2$ was added back just before gel filtration. EGTA treatment at 37°C disrupts the integrity of the GP IIb-IIIa complex and renders it incompetent to bind fibrinogen or support aggregation even after the addition of equimolar $CaCl_2$ (Brass et al., 1985; Fitzgerald and Phillips, 1985; Shattil et al., 1985*b*; Pidard et al., 1986). As a control, some platelets were preincubated with EGTA at 20°C, a condition that does not disrupt GP IIb-IIIa (Shattil et al., 1985*b*; Pidard et al., 1986). In some experiments, the blood donor ingested 650 mg of aspirin 24 and 2 h before venepuncture to inhibit arachidonic acid metabolism through the cyclooxygenase pathway (Burch et al., 1978). To insure virtually complete inhibition of cyclooxygenase, experiments with these gel-filtered platelets were carried out in the presence of 10 μM indomethacin (Sigma Chemical Co.). In some experiments, the gel-filtered platelets were incubated with the cell-permeable calpain inhibitor E64d (100 mg/ml) (McGowen et al., 1989) for 5 min at 25°C before thrombin stimulation. Calpain inhibition was assayed by visualization of the total protein profile after staining the gels with Coomassie blue.

Measurement of Platelet Aggregation and Dense Granule Secretion

Platelet aggregation and secretion of dense granule ATP were monitored using a single channel lumi-aggregometer (model 400-VS; Chrono-Log) (Charo et al., 1977; Feinman et al., 1977). All samples were stirred for one minute unless otherwise stated. The extent of platelet aggregation in each sample was arbitrarily expressed relative to the aggregation of platelets simultaneously treated with 1 U/ml thrombin. For platelet samples in which no aggregation was apparent in the lumi-aggregometer, a small aliquot was examined by phase-contrast microscopy to determine the extent of formation of very small platelet aggregates (ranging from doublets to aggregates of 25–50 platelets). Platelet ATP secretion was quantitated by arbitrarily setting the luminescence of untreated platelet suspensions at 0% and thrombin-treated platelet suspensions at 100%.

Measurement of GP IIb-IIIa Activation and Platelet Alpha-Granule Secretion

Five murine mAbs were used in these studies. The antibody PAC1 is specific for the activated form of GP IIb-IIIa and binds to a fibrinogen binding site on GP IIb-IIIa (Shattil et al., 1985*a*; Taub et al., 1989). Antibody A2A9 binds to both the non-activated and activated forms of GP IIb-IIIa and therefore was used to quantitate total surface-expressed GP IIb-IIIa (Bennett et al., 1983). BIB5 is an antibody specific for GP IIb and SSA6 an antibody specific for GP IIIa (Silver et al., 1987). Antibody S12, specific for the platelet alpha-granule membrane protein, GMP-140, was a gift from Dr. Rodger P. McEver (Oklahoma Medical Research Foundation, Oklahoma City, OK). This antibody binds selectively to platelets that have undergone alpha-granule secretion (McEver and Martin, 1984). All antibodies were directly conjugated with FITC to a final fluorescein/protein ratio of 2.5–4.0 (Shattil et al., 1987).

Antibody binding to platelets was assessed by flow cytometry. Unstirred gel-filtered platelets were first incubated for 1 min at 37°C in the presence or absence of a platelet agonist. Then, aliquots (1 $\times$ 10^6 cells) were incubated an additional 10 min at 22°C in a total volume of 50 μl with a saturating concentration of each of the FITC-labeled antibodies (Abrams et al., 1990). After dilution of each sample to 500 μl with PBS, platelets were analyzed in a FACStar (Becton-Dickinson Co., Oxnard, CA) flow cytometer as described previously (Shattil et al., 1987; Abrams et al., 1990). Antibody binding was expressed in arbitrary fluorescence units as the mean fluorescence intensity of 10,000 platelets. This value for antibody binding is directly proportional to the number of antibody molecules bound per platelet (Shattil et al., 1987).

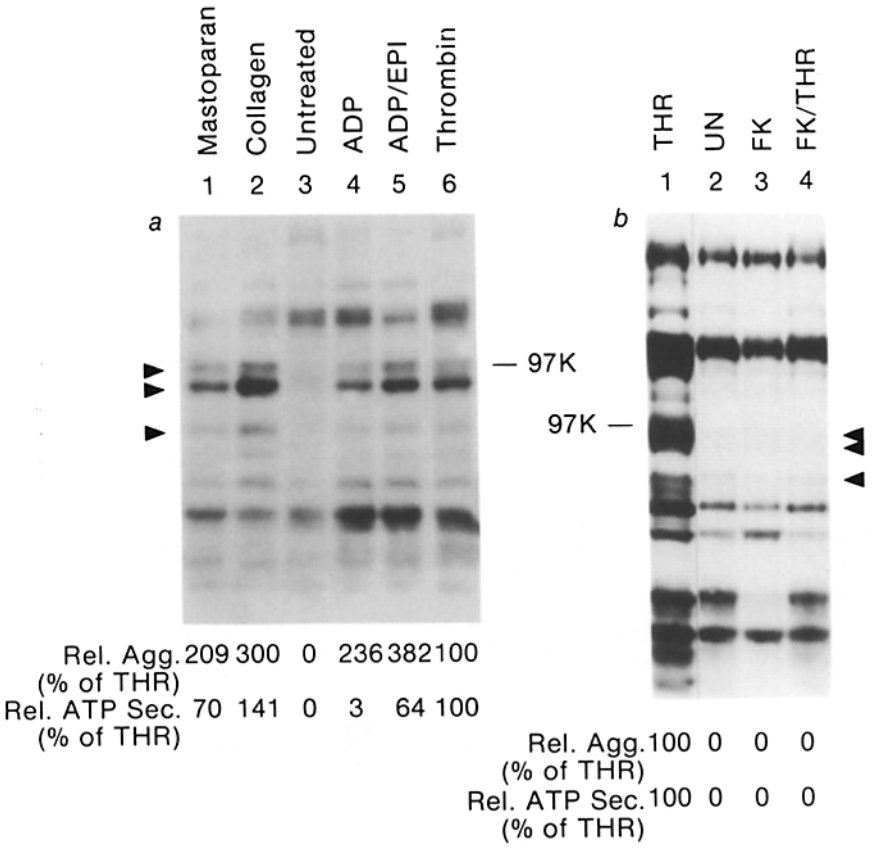


Figure 1. Agonist-induced tyrosine phosphorylation of platelet proteins. (*a*) As described in Materials and Methods, stirred gel-filtered platelets were treated with mastoparan (50 μM; lane *1*), collagen (5 μg/ml; lane *2*), no agonist (lane *3*), ADP (10 μM; lane *4*), ADP plus epinephrine (10 μM each; lane *5*), and thrombin (1 U/ml; lane *6*). Platelets were then solubilized with SDS, subjected to SDS-PAGE, and transferred to nitrocellulose filter paper. The immunoblots were incubated with anti-phosphotyrosine antibodies. The values below the immunoblot represent percent aggregation and ATP secretion relative to the thrombin-treated sample. Thus, values >100% indicate that a particular sample aggregated or secreted to a greater extent than the thrombin-treated sample. The value of 0% indicates that there was no aggregation or ATP secretion detected as assessed by the lumi-aggregometer. The aggregation and secretion data are expressed relative to the thrombin-treated sample, which was arbitrarily used as a reference index for the induction of tyrosine phosphorylation. (*b*) Gel-filtered platelets treated with thrombin (1 U/ml; lane *1*), no agonist (lane *2*), forskolin (50 μg/ml; lane *3*), and forskolin and thrombin (lane *4*) were prepared and immunoblotted as above. The numbers below the immunoblot represent aggregation and ATP secretion relative to the thrombin-treated sample.

Identification of Phosphotyrosine-containing Platelet Proteins by Immunoblotting

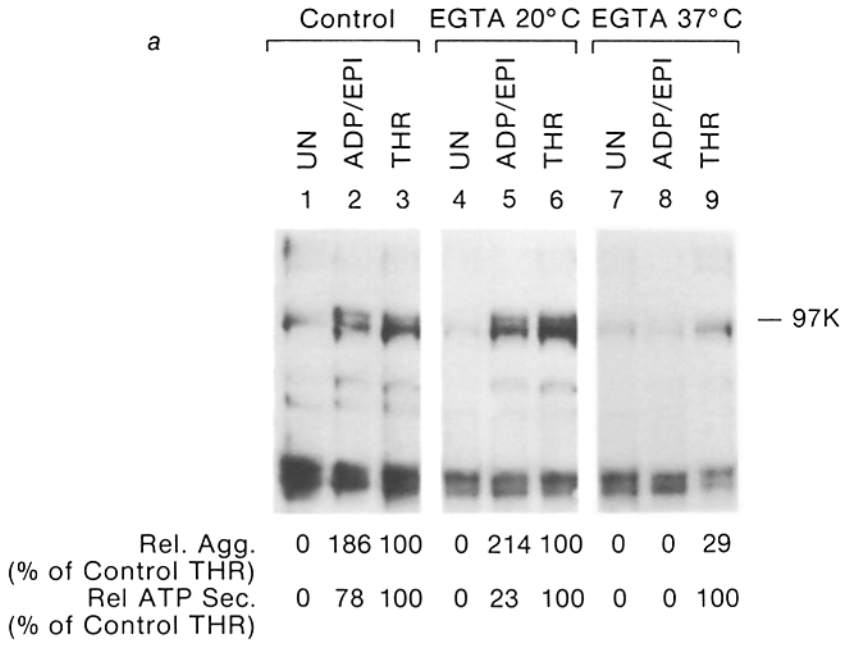
After activation of gel-filtered platelets for 1 min at 37°C as described above, the cells were sedimented for 4 s at 12,000 *g* in a microcentrifuge, solubilized in lysis buffer (2% SDS, 0.3% 2-mercaptoethanol, 66 mM Tris-HCl, pH 7.5, 10 mM EDTA) and heated at 95°C for 10 min. Immunoblot assays were performed as described (Yonemoto et al., 1987). The immunoblots were incubated with an affinity-purified rabbit antibody to phosphotyrosine (lot 903; a gift from Dr. Jean Wang [University of California, San Diego]). This antibody has been shown to be specific for phosphotyrosine residues (Wang, 1985; Morla and Wang, 1986). The binding of this antibody was detected using ^{125}I-labeled donkey anti-rabbit immunoglobulins (Amersham Corp., Arlington Heights, IL). ^{125}I-labeled protein bands were quantitated by densitometric scanning of the autoradiograms with a computing densitometer (Molecular Dynamics, Sunnyvale, CA).

Results

Effect of Platelet Activation on Tyrosine Phosphorylation of Platelet Proteins

Fig. 1 *a* shows an autoradiographic profile of platelet proteins phosphorylated on tyrosine residues 1 min after the addition of agonists to a suspension of gel-filtered platelets stirred at 37°C. Strong physiological agonists such as thrombin or collagen reproducibly induced the phosphorylation of three proteins with apparent molecular masses of 84, 95, and 97 kD. Weak agonists such as ADP, or ADP in combination with epinephrine, also evoked this response in stirred platelets provided that fibrinogen (50 μg/ml) had been added to the incubation medium. This pattern of phosphorylation was also observed when stirred platelets were incubated with agonists that circumvent the normal receptor-mediated pathways of cell activation: mastoparan, a peptide from wasp venom that directly activates G proteins, (Hirai et al., 1979); (Fig. 1 *a*) and phorbol 12-myristate 13-acetate, which directly activates protein kinase C (Nishizuka, 1986); data previously shown in Golden and Brugge, 1989. Forskolin, an inhibitor of platelet activation that acts by raising the intracellular concentration of cAMP (Feinstein et al., 1983), prevented the changes in tyrosine phosphorylation induced by thrombin (Fig. 1 *b*) and collagen (data not shown). Thus, it appears that an identical set of proteins is phosphorylated on tyrosine residues in stirred platelets within a minute of stimulation by a variety of agonists. This observation suggests that these phosphorylation events are linked to some common platelet activation process and not coupled solely to a single agonist receptor.

In the experiments shown in Fig. 1, the platelets were activated under conditions in which both aggregation and secretion occurred. Specifically, the platelets were stirred in the presence of fibrinogen and calcium. For ADP and epinephrine treatment, fibrinogen had been added to the platelet suspension. For the other agonists, sufficient fibrinogen was released from platelet alpha-granules to support aggregation. Indeed, platelet aggregation, ATP secretion from platelet dense granules (Fig. 1), and alpha-granule secretion (as measured by platelet binding of the anti-GMP-140 antibody, FITC-S12; data not shown) were observed in response to all



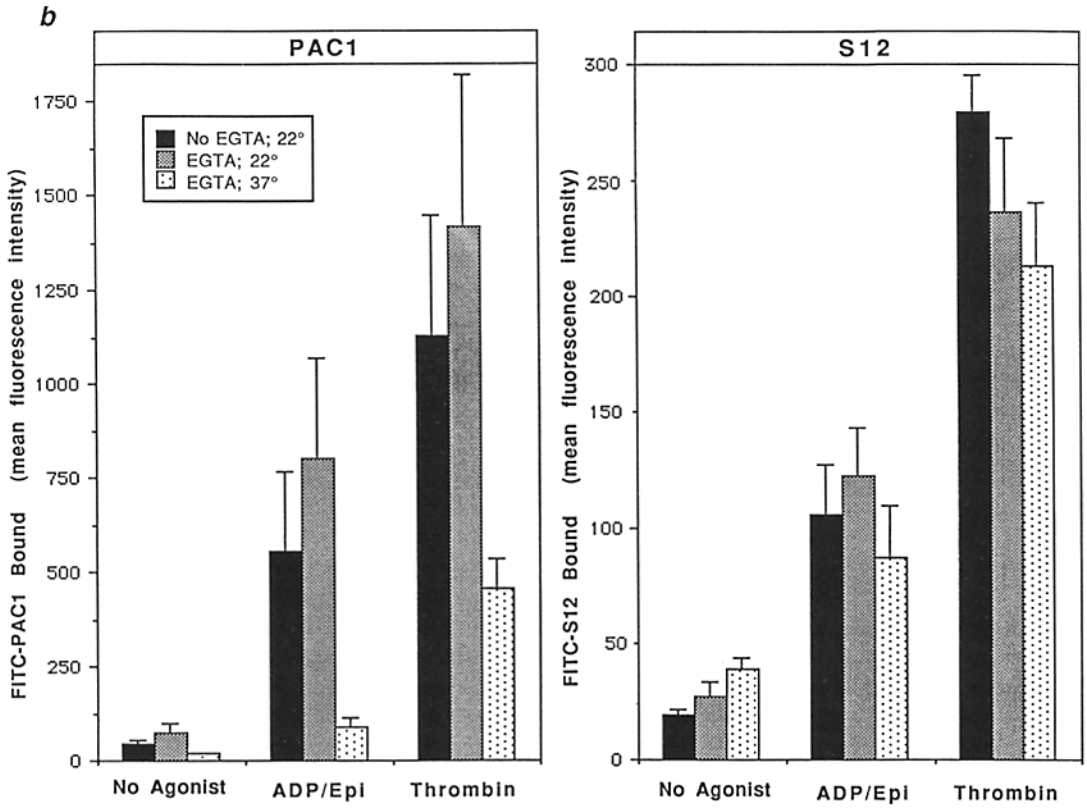
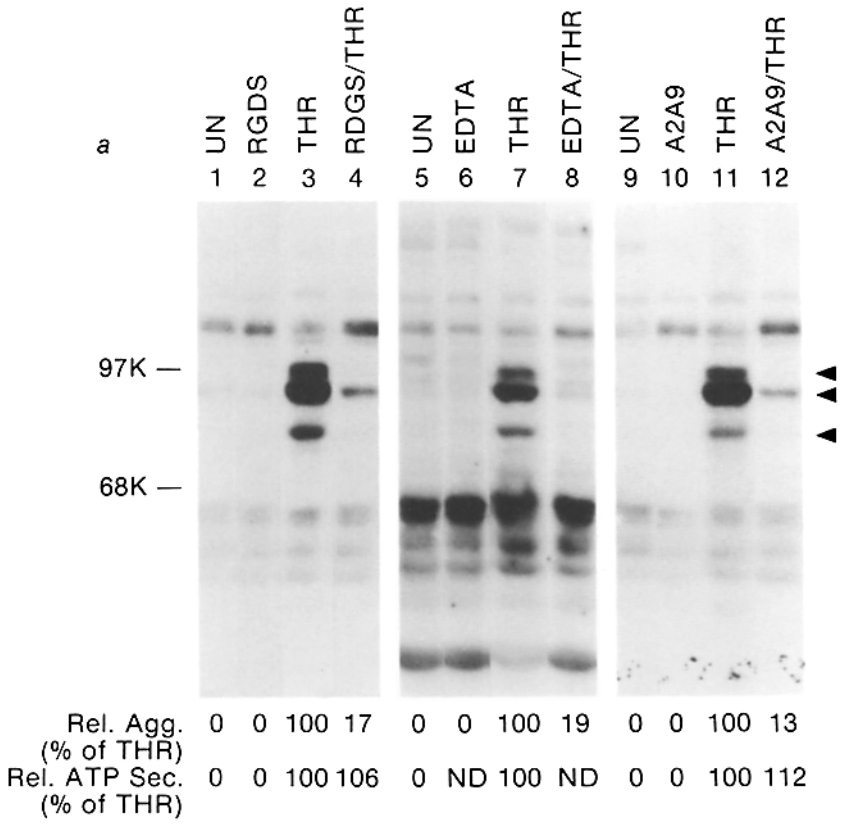


Figure 2. Disruption of the GP IIb-IIIa complex inhibits agonist-induced tyrosine phosphorylation. (*a*) Platelet-rich plasma was treated without EGTA (Control; lanes *1–3*), treated with 10 mM EGTA at 20°C for 90 minutes (lanes *4–6*), or treated with 10 mM EGTA at 37°C for 90 min (lanes *7–9*) before the addition of equimolar calcium and gel filtration. The gel-filtered platelets were then incubated without agonist (lanes *1*, *4*, and *7*), or with ADP and epinephrine (10 μM each; lanes *2*, *5*, and *8*), or thrombin (1 U/ml; lanes *3*, *6*, and *9*). Lysates were prepared and immunoblotted as described in Fig. 1. The values below the immunoblot represent aggregation and ATP secretion relative to the thrombin-treated sample for the control platelets incubated without EGTA. (*b*) Effect of EGTA and temperature on agonist-induced PAC1 and S12 binding to platelets. Antibody binding to platelets was assessed by flow cytometry as described in Materials and Methods and expressed in arbitrary fluorescence units as the mean fluorescence intensity per platelet. The data represent the mean +/− SEM of six experiments.



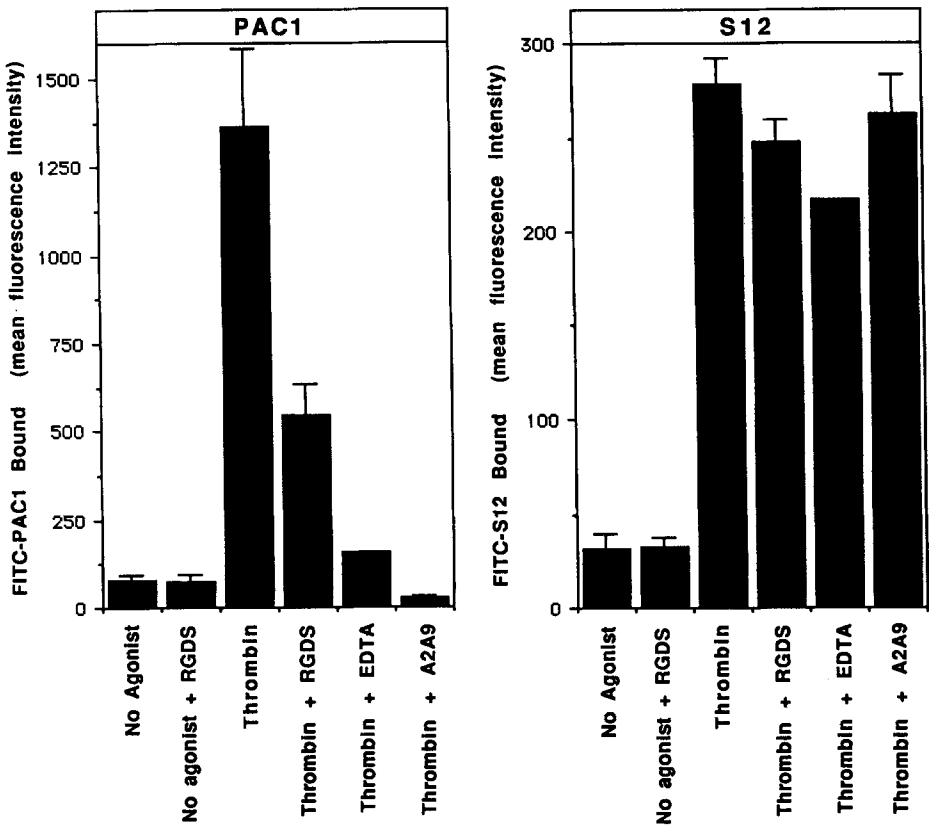


Figure 3. Inhibition of fibrinogen binding and platelet aggregation reduces agonist-induced tyrosine phosphorylation. (*a*) Gel-filtered platelets were incubated without agonist (lanes *1*, *5*, and *9*), or with thrombin (1 U/ml; lanes *3*, *7*, and *11*), RGDS (500 μM; lane 2), RGDS and thrombin (lane *4*), 10 mM EDTA (lane *6*), 10 mM EDTA and thrombin (lane *8*), antibody A2A9 (50 μg/ml; lane *10*), or mAb A2A9 and thrombin (lane *12*), and prepared and immunoblotted as described in Fig. 1. The values below the immunoblot represent aggregation and ATP secretion relative to the thrombin-treated sample. The ATP secretion values in lanes *6* and *8* were not determined (*ND*) since EDTA interferes with the luciferase reaction in the lumi-aggregometer. (*b*) Effect of RGDS, EDTA, and A2A9 on thrombin-induced PAC1 and S12 binding to platelets. The data represent the mean +/− SEM of two to six experiments.

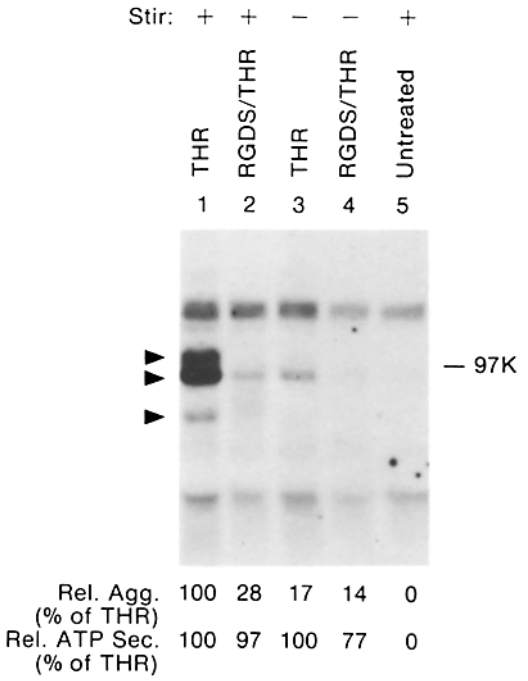


Figure 4. Agonist-stimulated platelets display minimal changes in tyrosine phosphorylation unless they are stirred and aggregate. Gel-filtered platelets were treated with thrombin (1 U/ml; lanes *1* and *3*), 500 μM RGDS and thrombin (lanes *2* and *4*), or no agonist (lane *5*). The platelets in lanes *3* and *4* were not stirred upon addition of agonists. All these samples were prepared and immunoblotted as described in Fig. 1. The values below the immunoblot represent aggregation and ATP secretion relative to the thrombin-treated sample.

agonists tested. These results suggest that tyrosine phosphorylation of the 84-, 95-, and 97-kD proteins is induced by some component of stimulus-response coupling that is linked to platelet aggregation and/or granule secretion. The following experiments were performed to explore the relationship between tyrosine phosphorylation and events leading to platelet aggregation.

Relationship of Tyrosine Phosphorylation to Platelet Responses Mediated by GP IIb-IIIa

The process of platelet aggregation requires the activation of GP IIb-IIIa, resulting in exposure of binding sites for fibrinogen. This event is followed by fibrinogen binding and, provided that the cell suspension is stirred, platelet aggregation. To assess whether GP IIb-IIIa activation was necessary for the agonist-induced tyrosine phosphorylation pattern shown in Fig. 1, platelets in plasma were initially incubated with 10 mM EGTA at 37°C for 90 min, $CaCl_2$ (10 mM) was then added, and the platelets were gel filtered. This treatment results in the irreversible disruption of surface GP IIb-IIIa and the failure of GP IIb-IIIa activation by ADP and epinephrine (Brass et al., 1985; Fitzgerald and Phillips, 1985; Shattil et al., 1985*b*; Pidard et al., 1986). Incubation with EGTA at 37°C prevented ADP and epinephrine-induced aggregation (Fig. 2 *a*, lane *8*), and inhibited the binding to platelets of FITC-PAC1, an mAb specific for the activated form of GP IIb-IIIa (Fig. 2 *b*). It also caused a significant reduction in ADP and epinephrine-induced tyrosine phosphorylation of the 84-, 95-, and 97-kD proteins (Fig. 2 *a*, lane *8*). In contrast, pretreatment of platelets with EGTA at 20°C, which does not disrupt the integrity of GP IIb-IIIa, did not affect the subsequent binding of FITC-PAC1, platelet aggregation, or tyrosine phosphorylation induced by ADP and epinephrine (Fig. 2 *a*, lane *5*, and Fig. 2 *b*).

Pretreatment of platelets with EGTA at 37°C disrupts surface GP IIb-IIIa, but does not affect internal pools of GP IIb-IIIa, which become surface-expressed after thrombin-stimulation and which support aggregation (Woods et al., 1986). Consistent with this observation, we found that such thrombin-stimulated platelets bound FITC-PAC1, aggregated, and underwent agonist-induced tyrosine phosphorylation, although each of these responses was only about 30% the level of control platelets (Fig. 2, *a* and *b*).

In contrast, pretreatment with EGTA at 37°C did not impair thrombin-induced dense granule secretion (ATP release) or alpha-granule secretion (FITC-S12 binding; Fig. 2 *b*). Together, these data suggest that tyrosine phosphorylation requires a structurally intact form of GP IIb-IIIa that is capable of activation by agonists.

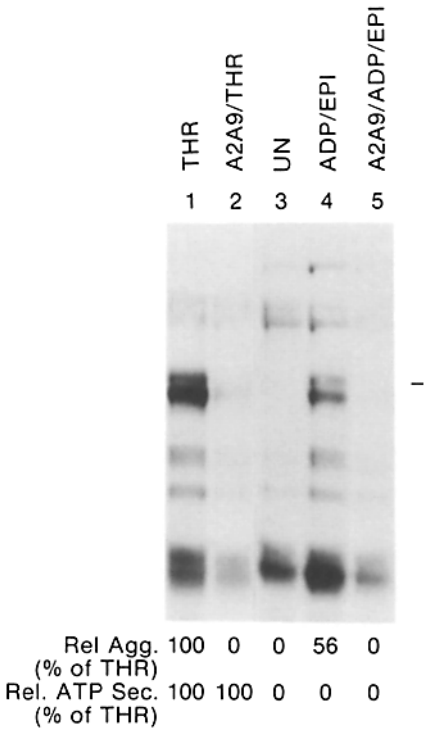


Figure 5. Inhibition of platelet cyclooxygenase does not inhibit agonist-induced tyrosine phosphorylation. Gel-filtered platelets isolated from donors who had ingested aspirin were incubated with 10 μM indomethacin to further insure complete inhibition of arachidonic acid metabolism. The platelets were then treated with thrombin (1 U/ml; lane *1*), 100 μg/ml antibody A2A9 and thrombin (lane *2*), no agonist (lane *3*), ADP and epinephrine (10 μM each; lane *4*), or A2A9, ADP, and epinephrine (lane *5*), and prepared and immunoblotted as described in Fig. 1. The values below the immunoblot represent aggregation and ATP secretion relative to the thrombin-treated sample. In platelets from donors not treated with aspirin, indomethacin did not cause a reduction in thrombin-induced tyrosine phosphorylation of the 84-, 95-, and 97-kD proteins (data not shown).

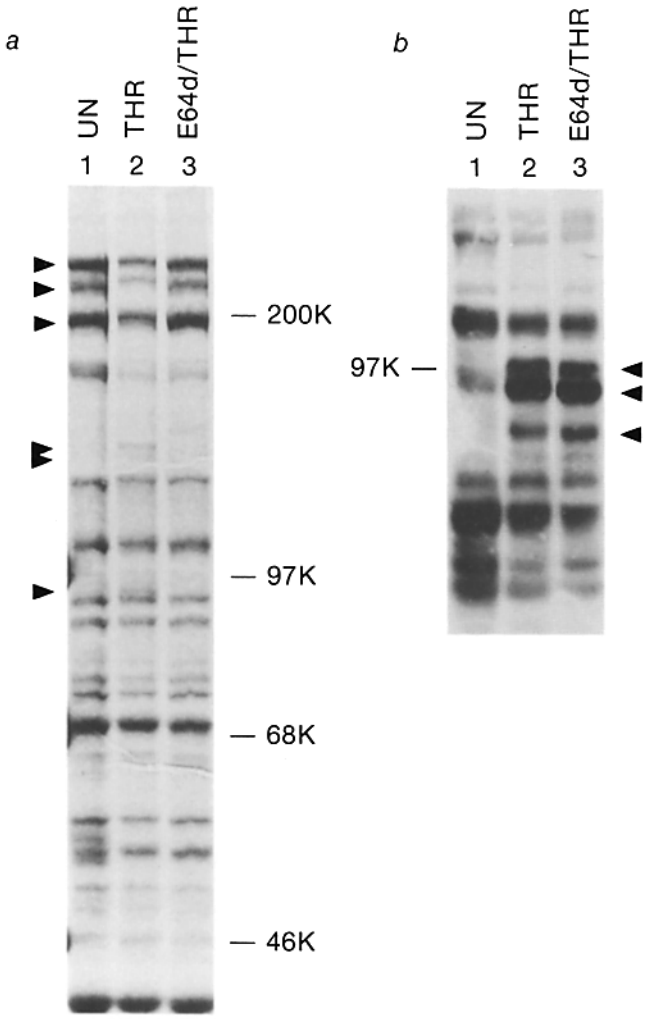


Figure 6. Inhibition of platelet calpain does not inhibit agonist-induced tyrosine phosphorylation. (*a*) Coomassie blue–stained gel of lysates from gel-filtered platelets that had been incubated without agonist (lane *1*), or with thrombin (1 U/ml; lane *2*), or with E64d (100 mg/ml) for 5 min at 25°C before thrombin addition (lane *3*). The three arrowheads at top point to talin, actin-binding protein, and myosin heavy chain. The lower three arrowheads point to the degradation products of myosin heavy chain (135 kD) and of actin-binding protein (93 kD) which are only apparent in lane *2*. (*b*) Gel-filtered platelets were incubated as described in *a* and prepared and immunoblotted as described in Fig. 1.

Since activation of GP IIb-IIIa and fibrinogen binding to GP IIb-IIIa are closely related, but sequential events, we then asked whether tyrosine phosphorylation was dependent on fibrinogen binding to GP IIb-IIIa. To address this question, platelets were stimulated with thrombin in the presence of either the tetrapeptide, RGDS, the divalent cation chelator, EDTA, or the mAb A2A9. Each of these agents is known to prevent fibrinogen and PAC1 binding to activated GP IIb-IIIa (Bennett and Vilaire, 1979; Bennett et al., 1983; Gartner and Bennett, 1985). None of these inhibitors affected the basal pattern of tyrosine phosphorylation when incubated in the absence of agonists (Fig. 3 *a*, lanes *2*, *6*, and *10*). However, each agent inhibited thrombin-induced FITC-PAC1 binding by 70-95% (Fig. 3 *b*), aggregation by >80%, and thrombin-induced tyrosine phosphorylation of the 84-, 95-, and 97-kD proteins by ~90% (Fig. 3 *a*). Similar results were obtained when platelets were stimulated with ADP and epinephrine in the presence of these inhibitors (data not shown). Control monoclonal antibodies specific for GP IIb (B1B5) or GP IIIa (SSA6), which do not inhibit fibrinogen binding, had no effect on thrombin-induced PAC1 binding, platelet aggregation, or tyrosine phosphorylation (data not shown).

Although RGDS, EDTA, and A2A9 severely reduced platelet aggregation and agonist-induced tyrosine phosphorylation, these agents had no effect on thrombin-induced alpha-granule secretion (platelet binding of FITC-S12; Fig. 3 *b*) or dense granule secretion (ATP release; Fig. 3 *a*). These data indicate that the induction of tyrosine phosphorylation requires the agonist-induced binding of fibrinogen to GP IIb-IIIa. They also suggest that tyrosine phosphorylation of this set of proteins is not required for platelet secretion in response to thrombin.

In separate experiments, induction of tyrosine phosphorylation was not observed when platelets were stimulated to agglutinate in the presence of von Willebrand factor, ristocetin, and EDTA (data not shown). Under these conditions, platelets clump together in a process that requires the binding of von Willebrand factor to GP Ib and is independent of GP IIb-IIIa (Zucker et al., 1977).

To examine whether platelet aggregation was required for tyrosine phosphorylation, platelets were activated by thrombin or ADP plus epinephrine in the absence of stirring. Under these conditions, GP IIb-IIIa is activated and is capable of binding PAC1 (see Fig. 3, *a* and *b*) and fibrinogen (Bennett et al., 1979), and aggregation is prevented. In the absence of stirring, platelets exhibited only a minimal level of agonist-induced tyrosine phosphorylation of the 84-, 95-, and 97-kD proteins when compared with stirred thrombin-treated platelets (Fig. 4; compare lanes *1* and *3*). Collectively, these studies demonstrate that the induction of tyrosine phosphorylation requires GP IIb-IIIa activation, fibrinogen binding, and fibrinogen-dependent platelet aggregation.

Relationship of Tyrosine Phosphorylation to Platelet Events Subsequent to Aggregation

Since phosphorylation of the 84-, 95-, and 97-kD proteins was inhibited under conditions that blocked the process of platelet aggregation, we considered the possibility that activation of tyrosine phosphorylation was induced by some platelet response that is dependent upon platelet aggregation. Although the events that follow platelet aggregation are not completely understood, three such responses have been clearly identified: (*a*) conversion of arachidonic acid to thromboxane A_2, (*b*) granule secretion (when platelets are stimulated with ADP or epinephrine), and (*c*) activation of platelet calcium-dependent proteases (calpains). To examine whether tyrosine phosphorylation required arachidonic acid metabolism and granule secretion, platelets were pretreated with aspirin and indomethacin to inhibit cyclooxygenase. Since platelet secretion by ADP and epinephrine is mediated by arachidonic acid metabolites, blockade of cyclooxygenase prevents granule secretion. Platelets treated with aspirin and indomethacin underwent primary wave aggregation in response to ADP and epinephrine when stirred in the presence of fibrinogen. Under these conditions, dense granule secretion (ATP release) was completely inhibited (Fig. 5). Aspirin and indomethacin did not prevent the tyrosine phosphoryla-

Table I. Tyrosine Phosphorylation of the 84-, 95-, and 97-kD Proteins Is Dependent on Platelet Aggregation Mediated by GP IIb-IIIa

Platelet treatment			Cellular response			
Agonist	Inhibitor	Stir	Ligand binding to GP IIb-IIIa*	Aggregation	Secretion	84-, 95-, 97-kD phosphorylation
None	None	Yes or No	−‡	−	−	−
Thrombin	None	No	+	−	+	−
Thrombin	None	Yes	+	+	+	+
Thrombin	RGDS or A2A9 or EDTA	Yes	−	−	+	−
Thrombin	Aspirin	Yes	+	+	+	+
Thrombin	Forskolin	Yes	−	−	−	−
ADP/Epi/Fibrinogen	None	No	+	−	−	−
ADP/Epi/Fibrinogen	None	Yes	+	+	+	+
ADP/Epi/Fibrinogen	RGDS or A2A9 or EDTA	Yes	−	−	−	−
ADP/Epi/Fibrinogen	Aspirin	Yes	+	+	−	+
Collagen	None	Yes	+	+	+	+
Phorbol ester	None	Yes	+	+	+	+
Mastoparan	None	Yes	+	+	+	+
Ristocetin/vWf	EDTA	Yes	−	Agglutination	−	−

* Ligand binding refers to PAC1 or fibrinogen binding.
‡ The plus symbol refers to a cellular response ≥75% of maximal; the minus symbol refers to a cellular response ≤30% of maximal.

tion of the 84-, 95-, and 97-kD proteins induced by ADP and epinephrine (Fig. 5). Thus, neither arachidonic acid metabolism nor platelet dense granule secretion appear to be absolutely necessary for the induction of tyrosine phosphorylation. The reduced level of tyrosine phosphorylation in ADP/epinephrine treated platelets (lane *4*) could be a consequence of the inhibited secretion of factors that potentiate the aggregation response.

Activation of platelet calpains requires platelet aggregation (Fox, 1987; Weidmer et al., 1990). Calpain activation can be monitored indirectly on Coomassie blue-stained SDS gels as a decrease in the amount of the calpain substrates, actin-binding protein (270 kD), talin (235 kD), and myosin heavy chain (200 kD) and as a concomitant increase in the degradation products of actin-binding protein (190 and 93 kD), talin (190 kD), and myosin heavy chain (135 kD) (Fig. 6 *a*). However, when stirred platelets were stimulated with thrombin in the presence of E64d, a cell permeable inhibitor of calpains (McGowen et al., 1989), no evidence of calcium-dependent protease activity could be detected (Fig. 6 *a*). Furthermore, E64d treatment had no effect on platelet aggregation or the induction of tyrosine phosphorylation of the 84-, 95-, and 97-kD proteins (Fig. 6 *b*).

Discussion

These studies demonstrate that activation of platelets by a variety of agonists induces tyrosine phosphorylation of three proteins with apparent molecular masses of 84, 95, and 97 kD. Since two-dimensional immunoblot analysis has not been performed, the exact number of phosphoproteins represented by these three bands is unknown. This pattern of phosphorylation was observed whether platelets were stimulated in a receptor-mediated fashion by thrombin, collagen, or ADP and epinephrine, or in a receptor-independent fashion by an activator of G proteins (mastoparan) or protein kinase C (phorbol ester). Using a variety of experimental systems and monitoring techniques, we established that this pattern of tyrosine phosphorylation is dependent on agonist-induced activation of GP IIb-IIIa, fibrinogen binding to GP IIb-IIIa, and platelet aggregation. In contrast, platelet arachidonic acid metabolism, dense granule secretion, and calpain activity are not necessary for tyrosine phosphorylation of these proteins. Table I summarizes the experimental support for these conclusions.

Upon platelet activation, the surface membrane GP IIb-IIIa complex undergoes a conformational change that permits it to function as a receptor for adhesive ligands, including fibrinogen, von Willebrand factor, and under some circumstances, fibronectin (Plow and Ginsberg, 1989). In plasma, fibrinogen is the dominant ligand (Pietu et al., 1984; Gralnick et al., 1984; Schullek et al., 1984). The binding of fibrinogen and RGD-containing peptides to GP IIb-IIIa has been shown to induce clustering of GP IIb-IIIa complexes within the plane of the plasma membrane (Isenberg et al., 1989). In the present study, RGDS binding per se did not induce tyrosine phosphorylation in platelets, suggesting that clustering of GP IIb-IIIa complexes is not sufficient to induce tyrosine phosphorylation. However, the experiments illustrated in Fig. 2 in which agonist-induced tyrosine phosphorylation was inhibited by preincubating platelets with EGTA at 37°C indicated that the functional integrity of GP IIb-IIIa is required for these tyrosine phosphorylation events.

Fibrinogen binding to activated GP IIb-IIIa and platelet aggregation can be inhibited by RGD-containing peptides, by chelation of divalent cations, and by certain anti-GP IIb-IIIa mAbs (Gartner and Bennett, 1985; Plow et al., 1985; Ben-

nett et al., 1983; Shattil et al., 1986). The experiments in which fibrinogen binding and aggregation were inhibited by RGDS, EDTA, or mAb A2A9 (Fig. 3) suggested that agonist-induced tyrosine phosphorylation required the binding of fibrinogen to GP IIb-IIIa. Although unstirred platelets stimulated by agonists displayed evidence of GP IIb-IIIa activation (FITC-PAC1 binding), they did not display the inducible pattern of tyrosine phosphorylation (Fig. 4), implying that platelet aggregation was necessary for these phosphorylation events to occur. Platelet agglutination by ristocetin and von Willebrand factor, a process that involves GP Ib and not GP IIb-IIIa, did not induce tyrosine phosphorylation. Thus, these studies clearly indicate that GP IIb-IIIa activation and fibrinogen binding are necessary for tyrosine phosphorylation, but these events are not sufficient. Rather, once fibrinogen has become bound to activated platelets, the platelets must aggregate in order for the induction of tyrosine phosphorylation to occur.

We obtained substantial evidence that dense granule secretion is not required for tyrosine phosphorylation of the 84-, 95-, and 97-kD proteins and that secretion of dense and alpha granules is not dependent on tyrosine phosphorylation of these proteins. Pretreatment with aspirin and indomethacin, which blocked arachidonic acid metabolism and granule secretion, did not inhibit the induction of tyrosine phosphorylation by ADP and epinephrine (Fig. 5). In addition, stirred platelets underwent full thrombin-induced granule secretion under conditions where platelet aggregation and tyrosine phosphorylation were blocked by RGDS, EDTA, or the mAb A2A9 (Fig. 3). Finally, unstirred platelets exhibited normal granule secretion when stimulated with thrombin, but they did not aggregate or undergo agonist-induced tyrosine phosphorylation (Fig. 4). It is also likely that tyrosine phosphorylation is unrelated to agonist-induced platelet shape change, the process whereby the cell undergoes spheration and pseudopodial extension. Unstirred platelets or platelets in EDTA can undergo shape change in response to thrombin or ADP (Siess, 1989); however, agonist-induced tyrosine phosphorylation was barely detectable under these conditions.

The observation that fibrinogen binding to GP IIb-IIIa is necessary for the induction of tyrosine phosphorylation is consistent with the recent findings of Ferrell and Martin (1989) who demonstrated that RGDS inhibited thrombin-induced tyrosine phosphorylation of proteins of M_r 126,000, 108,000 and 100,000, and that these proteins are not phosphorylated in thrombasthenic platelets which lack GP IIb-IIIa. The antiphosphotyrosine antibodies used in this study detected several additional thrombin-induced proteins that were not sensitive to RGDS, suggesting that all thrombin-induced tyrosine phosphorylation events are not dependent on fibrinogen binding. Our results and conclusions differ in two respects to those of Ferrell and Martin. First, although Ferrell and Martin observed an induction of tyrosine phosphorylation in response to thrombin, they did not detect such phosphorylation when platelets were stimulated with ADP in the presence of fibrinogen. They concluded that ADP-induced activation of GP IIb-IIIa and aggregation were insufficient to induce the tyrosine phosphorylation of proteins in the 100-kD range. However, we observed that ADP or ADP and epinephrine could activate GP IIb-IIIa and induce both aggregation and tyrosine phosphorylation. Technical considerations may explain this apparent discrepancy. In the study of Ferrell and Martin, platelets were washed by repeated centrifugation rather than gel filtration. It is difficult to avoid inadvertent platelet activation when platelets are washed by centrifugation. Platelets partially activated during the washing process may become relatively refractory to subsequent stimulation by agonists (Peerschke, 1985). Indeed, Ferrell and Martin did not observe tyrosine phosphorylation of proteins in the 100-kD range until 3–5 min after thrombin stimulation, while we have observed this pattern within 30 s of stimulation (Golden and Brugge, 1989).

The second difference between our findings and those of Ferrell and Martin is that we found that induction of tyrosine phosphorylation was dependent on stirring the suspension of activated platelets. We concluded that GP IIb-IIIa, fibrinogen binding to GP IIb-IIIa, and platelet aggregation are necessary for induction of tyrosine phosphorylation. In contrast, Ferrell and Martin concluded that "GP IIb-IIIa-ligand interaction per se, and not aggregation", was required for the induction of tyrosine phosphorylation. Again, technical considerations may explain this discrepancy. It is difficult to avoid platelet microaggregation when platelets are centrifuged, washed, and then incubated with thrombin. Indeed, we have found that when some degree of tyrosine phosphorylation is observed in a platelet sample believed to contain no aggregates, as determined in a conventional platelet aggregometer, microscopic platelet aggregates are invariably present.

What is the exact relationship between tyrosine phosphorylation and platelet aggregation? It is possible that agonist-induced tyrosine phosphorylation is necessary for an event that follows fibrinogen binding to GP IIb-IIIa and that is required for the process of aggregation. These "postoccupancy" events remain to be characterized, however, the ability of desensitized platelets to bind fibrinogen normally (in the absence of aggregating) suggests that fibrinogen binding is not sufficient for full platelet aggregation (Peerschke, 1985; Shattil et al., 1986). Alternatively, tyrosine phosphorylation may be involved in some cellular process that is dependent on platelet aggregation (i.e., activation of the Na^+/H^+ pump). Although further studies will be required to establish more precise cause-effect relationships, the present studies clearly establish that induction of tyrosine phosphorylation does not require platelet arachidonic acid metabolism or dense granule secretion. In addition, experiments using the cell permeable calpain inhibitor, E64d, imply that tyrosine phosphorylation does not require calpain activation.

Platelet aggregation is associated with a structural reorganization of the platelet cytoskeleton that is correlated with the physical association of GP IIb-IIIa with one or more Triton-insoluble cytoskeletal elements (Phillips et al., 1980). Recent studies suggest that activated GP IIb-IIIa may become associated with a specific cytoskeletal protein, talin Beckerle et al., 1989). Although the precise role of such interactions is speculative, they might be involved in GP IIb-IIIa-mediated adhesion of platelets to the subendothelial matrix (Weiss et al., 1989) or in clot retraction (Cohen et al., 1989). A direct or indirect association of GP IIb-IIIa with a protein-tyrosine kinase could be one means by which such functions are regulated. In other cells, protein-tyrosine kinases can induce alterations in the organization of the membrane cytoskeleton. These kinases include the epidermal growth factor receptor (Schlessinger and Geiger, 1981), the

platelet-derived growth factor receptor (Herman and Pledger, 1985), and the viral *src* (David-Pfeuty and Singer, 1980), *yes* (Gentry and Rohrschneider, 1984), *abl* (Rohrschneider and Najita, 1984), and *fps* (Tarone et al., 1985) gene products.

These speculations raise the question as to whether the GP IIb-IIIa complex might communicate with a protein-tyrosine kinase. Despite the high levels of the *c-src* gene product in platelets (0.2% of total cell protein [Golden and Brugge, 1989]), there is as yet no evidence that this protein associates with GP IIb-IIIa or is the kinase responsible for the agonist-induced tyrosine phosphorylation of the 84-, 95-, and 97-kD proteins (Ferrell and Martin, 1988; Golden and Brugge, 1989). Recently, the CD4 and CD8 receptors of helper and cytotoxic T-lymphocytes have been shown to associate with and regulate a *src*-related protein-tyrosine kinase, $p56^{lck}$ (Veillette et al., 1988). Thus, the coupling of cell surface receptors with protein-tyrosine kinases could represent a common mechanism for mediating signal transduction events triggered by cell–cell, and perhaps cell–matrix interactions.

The identities of the 84-, 95-, and 97-kD tyrosine-phosphorylated proteins in agonist-stimulated platelets are unknown. Although the 95- and 97-kD proteins are similar in apparent molecular weight to nonreduced GP IIIa, preliminary immunoblotting and immunoprecipitation studies suggest that neither of these proteins represents GP IIIa (Ferrell, J., and G. S. Martin, personal communication; S. Nemeth, L. Lipfert, A. Golden, and J. S. Brugge, personal observation). Identification of these proteins and their functions will be facilitated by their isolation and by analysis of their primary structure.

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References

Abrams, C. S., N. Ellison, A. Z. Budzynski, and S. J. Shattil. 1990. The direct detection of activated platelets and platelet-derived microparticles in man. *Blood.* 75:128–138.

Banga, H., 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.

Beckerle, M. C., D. E. Miller, M. E. Bertagnolli, and S. J. Locke. 1989. Activation-dependent redistribution of the adhesion plaque protein, talin, in intact human platelets. *J. Cell Biol.* 109:3333–3346.

Bennett, J. S., and G. Vilaire. 1979. Exposure of platelet fibrinogen receptors by ADP and epinephrine. *J. Clin. Invest.* 64:1393–1401.

Bennett, J. S., J. A. Hoxie, S. F. Leitman, G. Vilaire, and D. B. Cines. 1983. Inhibition of fibrinogen binding to stimulated human platelets by a monoclonal antibody. *Proc. Natl. Acad. Sci. USA.* 80:2417–2421.

Brass, L. F., S. J. Shattil, T. J. Kunicki, and J. S. Bennett. 1985. Effect of calcium on the stability of the platelet membrane glycoprotein IIb-IIIa complex. *J. Biol. Chem.* 260:7875–7881.

Brass, L. F., D. Manning, and S. J. Shattil. 1990. GTP-binding proteins and platelet activation. *Prog. Hem. Thromb.* In press.

Burch, J. W., N. L. Baenziger, N. Stanford, and P. W. Majerus. 1978. Sensitivity of fatty acid cyclooxygenase from aorta to acetylation by aspirin. *Proc. Natl. Acad. Sci. USA.* 75:5181–5184.

Charo, I. F., R. D. Feinman, and T. C. Detwiler. 1977. Interrelations of platelet aggregation and secretion. *J. Clin. Invest.* 60:866–873.

Cohen, I., D. L. Burk, and J. G. White. 1989. The effect of peptides and monoclonal antibodies that bind to platelet glycoprotein IIb-IIIa complex on the development of clot tension. *Blood.* 73:1880–1887.

David-Pfeuty, T., and S. J. Singer. 1980. Altered distributions of the cytoskeletal proteins vinculin and alpha-actinin in cultured fibroblasts transformed by Rous sarcoma virus. *Proc. Natl. Acad. Sci. USA.* 77:6687–6691.

Feinman, R. D., J. Lubowsky, I. Charo, and M. P. Zabinski. 1977. The lumi-aggregometer: a new instrument for simultaneous measurement of secretion and aggregation by platelets. *J. Lab. Clin. Med.* 90:125–129.

Feinstein, M. B., J. J. Egan, R. I. Sha'afi, and J. White. 1983. The cytoplasmic concentration of free calcium in platelets is controlled by stimulators of cyclic AMP production (PGD_2, PGE_1, forskolin). *Biochem. Biophys. Res. Commun.* 113:598–604.

Ferrell, J. E., and G. S. Martin. 1988. Platelet tyrosine-specific protein phosphorylation is regulated by thrombin. *Mol. Cell Biol.* 8:3603–3610.

Ferrell, J. E., and G. S. Martin. 1989. Tyrosine-specific protein phosphorylation is regulated by glycoprotein IIb-IIIa in platelets. *Proc. Natl. Acad. Sci. USA.* 86:2234–2238.

Fitzgerald, L. A., and D. R. Phillips. 1985. Calcium regulation of the platelet membrane glycoprotein IIb-IIIa complex. *J. Biol. Chem.* 260:11366–11374.

Fox, J. E. B. 1987. The platelet cytoskeleton. *In* Thrombosis and Hemostasis. M. Verstraete, J. Vermylen, R. Lijnen, and J. Arnaout, editors. Leuven University Press, Leuven, Belgium. 175–225.

Gartner, T. K., and J. S. Bennett. 1985. The tetrapeptide analogue of the cell attachment site of fibronectin is an inhibitor of platelet aggregation and fibrinogen binding. *J. Biol. Chem.* 260:11891–11894.

Gentry, L. E., and L. R. Rohrschneider. 1984. Common features of the *yes* and *src* gene products defined by peptide-specific antibodies. *J. Virol.* 51:539–546.

Golden, A., S. P. Nemeth, and J. S. Brugge. 1986. Blood platelets express high levels of the $pp60^{c\text{-}src}$-specific tyrosine kinase activity. *Proc. Natl. Acad. Sci. USA.* 83:852–856.

Golden, A., and J. S. Brugge. 1989. Thrombin treatment induces rapid changes in tyrosine phosphorylation in platelets. *Proc. Natl. Acad. Sci. USA.* 86: 901–905.

Gralnick, R. H., S. B. Williams, and B. S. Coller. 1984. Fibrinogen competes with von Willebrand factor for binding to the glycoprotein IIb/IIIa complex when platelets are stimulated with thrombin. *Blood.* 64:797–800.

Herman, B., and W. J. Pledger. 1985. Platelet-derived growth factor-induced alterations in vinculin and actin distribution in BALB/c-3T3 cells. *J. Cell Biol.* 100:1031–1040.

Higashijima, T., S. Uzu, T. Nakajima, and E. M. Ross. 1988. Mastoparan, a peptide toxin from wasp venom, mimics receptors by activating GTP-binding regulatory proteins (G proteins). *J. Biol. Chem.* 263:6491–6494.

Hirai, Y., M. Kuwada, T. Yasuhara, H. Yoshida, and T. Nakajima. 1979. A new mast cell degranulating peptide homologous to mastoparan in the venom of Japanese hornet (*Vespa xanthoptera*). *Chem. Pharmacol. Bull.* 27:1945–1946.

Horak, I. D., M. L. Corcoran, P. A. Thompson, L. M. Wahl, and J. B. Bolen. 1990. Expression of $p60^{fyn}$ in human platelets. *Oncogene.* 5:597–602.

Huang, E. M., and T. C. Detwiler. 1986. Stimulus-response coupling mechanisms. *In* Biochemistry of Platelets. D. R. Phillips and M. A. Shuman, editors. Academic Press, Inc., Orlando, FL. 1–68.

Hynes, R. O. 1987. Integrins: A family of cell surface receptors. *Cell.* 48: 549–554.

Isenberg, W. M., R. P. McEver, D. R. Phillips, M. A. Shuman, and D. F. Bainton. 1989. Immunogold-surface replica study of ADP-induced ligand binding and fibrinogen receptor clustering in human platelets. *Am. J. Anat.* 185:142–148.

Ishihara, N., K. Nagao, and B. Kobayashi. 1985. Tyrosine phosphorylation of platelet protein induced by phorbol ester. *Thromb. Haemostasis.* 54:579–585.

Kroll, M. H., and A. I. Schafer. 1989. Biochemical mechanisms of platelet activation. *Blood.* 74:1181–1195.

McEver, R. P., and M. N. Martin. 1984. A monoclonal antibody to a membrane glycoprotein binds only to activated platelets. *J. Biol. Chem.* 259: 9799–9804.

McGowen, E. B., E. Becker, and T. C. Detwiler. 1989. Inhibition of calpain in intact platelets by the thiol protease inhibitor E-64d. *Biochem. Biophys. Res. Commun.* 158:432–435.

Morla, A. O., and J. Y. J. Wang. 1986. Protein tyrosine phosphorylation in the cell cycle of Balb/c 3T3 fibroblasts. *Proc. Natl. Acad. Sci. USA.* 83:8191–8195.

Nakamura, S., Takeuchi, T. Tomizawa, N. Takasaki, H. Kondo, and H. Yamamura. 1985. Two separate tyrosine protein kinases in human platelets. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 184:56–59.

Nakamura, S., S. Yanagi, and H. Yamamura. 1988. Purification and characterization of cytosolic protein-tyrosine kinase from bovine platelets. *Eur. J. Biochem.* 174:471–477.

Nakamura, S., and H. Yakamura. 1989. Thrombin and collagen induce rapid phosphorylation of a common set of cellular proteins on tyrosine in human

platelets. *J. Biol. Chem.* 264:7089–7091.
Nishizuka, Y. 1986. Perspectives on the role of protein kinase C in stimulus-response coupling. *J. Natl. Cancer Inst.* 76:363–370.
Peerschke, E. I. B. 1985. Ca^{2+} mobilization and fibrinogen binding of platelets refractory to adenosine diphosphate stimulation. *J. Lab. Clin. Med.* 106: 111–122.
Phillips, D. R., L. K. Jennings, and H. H. Edwards. 1980. Identification of membrane proteins mediating the interaction of human platelets. *J. Cell Biol.* 86:77–86.
Pidard, D., D. Didry, T. J. Kunicki, and A. T. Nurden. 1986. Temperature-dependent effects of EDTA on the membrane glycoprotein IIb-IIIa complex and platelet aggregability. *Blood.* 67:604–611.
Pietu, G., G. Cherel, G. A. Marguerie, and D. Meyer. 1984. Inhibition of von Willebrand factor platelet interaction by fibrinogen. *Nature (Lond.).* 308:648–650.
Plow, E. F., and M. H. Ginsberg. 1989. Cellular Adhesion: GP IIb-IIIa as a prototypic adhesion receptor. *Prog. Hem. Thromb.* 9:117–156.
Plow, E. F., M. D. Pierschbacher, E. Ruoslahti, G. A. Marguerie, and M. H. Ginsberg. 1985. The effect of Arg-Gly-Asp-containing peptides on fibrinogen and von Willebrand factor binding to platelets. *Proc. Natl. Acad. Sci. USA.* 82:8057–8061.
Presek, P., C. Reuter, D. Findik, and P. Bette. 1988. High-yield purification of $pp60^{c\text{-}src}$ related protein-tyrosine kinase from human platelets. *Biochem. Biophys. Acta.* 969:271–290.
Pytela, R., M. D. Pierschbacher, M. H. Ginsberg, E. F. Plow, and E. Ruoslahti. 1986. Platelet membrane glycoprotein IIb/IIIa: membrane of a family of arg-gly-asp-specific adhesion receptors. *Science (Wash. DC).* 231:1559–1562.
Rendu, R., M. Lebret, S. Danielian, R. Fagard, S. Levy-Toledano, and S. Fischer. 1989. High $pp60^{c\text{-}src}$ level in human platelet dense bodies. *Blood.* 73:1545–1551.
Rohrschneider, L. R., and L. M. Najita. 1984. Detection of the *v-abl* gene product at cell-substratum contact sites in Abelson murine leukemia virus-transformed fibroblasts. *J. Virol.* 51:547–552.
Schlessinger, J., and B. Geiger. 1981. Epidermal growth factor induces redistribution of actin and alpha-actinin in human epidermal carcinoma cells. *Exp. Cell Res.* 134:273–279.
Schullek, J., J. Jordan, and R. R. Montgomery. 1984. Interaction of von Willebrand factor with human platelets in the plasma milieu. *J. Clin. Invest.* 73:421–428.
Shattil, S. J., J. A. Hoxie, M. Cunningham, and L. F. Brass. 1985*a*. Changes in the platelet membrane glycoprotein IIb-IIIa complex during platelet activation. *J. Biol. Chem.* 260:11107–11114.
Shattil, S. J., L. F. Brass, J. S. Bennett, and P. Pandhi. 1985*b*. Biochemical and functional consequences of dissociation of the platelet membrane glycoprotein IIb-IIIa complex. *Blood.* 66:92–98.
Shattil, S. J., H. Motulsky, P. A. Insel, L. Flaherty, and L. F. Brass. 1986. Expression of fibrinogen receptors during activation and subsequent desensitization of human platelets by epinephrine. *Blood.* 68:1224–1231.
Shattil, S. J., M. Cunningham, and J. A. Hoxie. 1987. Detection of activated platelets in whole blood using activation-dependent monoclonal antibodies and flow cytometry. *Blood.* 70:307–315.
Siess, W. 1989. Molecular mechanisms of platelet activation. *Physiol. Rev.* 69:58–178.
Silver, S. M., M. M. McDonough, G. Vilaire, and J. S. Bennett. 1987. The in vitro synthesis of polypeptides for the platelet membrane glycoprotein IIb and IIIa. *Blood.* 69:1031–1037.
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.
Sweatt, J. D., T. M. Connolly, E. J. Cragoe, and L. E. Limbird. 1986. Evidence that Na^+/H^+ exchange regulates receptor-mediated phospholipase A_2 activation in human platelets. *J. Biol. Chem.* 261:8667–8673.
Tangen, O., H. J. Berman, and P. Marfey. 1971. Gel filtration: a new technique for separation of blood platelets from plasma. *Thromb. Diath. Haemorrh.* 25:268–278.
Tarone, G., D. Cirillo, F. G. Giancotti, P. M. Comoglio, and P. C. Marchisio. 1985. Rous sarcoma virus-transformed fibroblasts adhere primarily at discrete protrusions of the ventral membrane called podosomes. *Exp. Cell Res.* 159:141–157.
Taub, R., R. J. Gould, V. M. Garsky, T. M. Ciccarone, J. Hoxie, P. A. Friedman, and S. J. Shattil. 1989. A monoclonal antibody against the platelet fibrinogen receptor contains a sequence that mimics a receptor recognition domain in fibrinogen. *J. Biol. Chem.* 264:259–265.
Varshney, G. C., J. Henry, A. Kahn, and F. Phan-Dinh-Tuy. 1986. Tyrosine kinases in normal human blood cells: platelet but not erythrocyte band 3 tyrosine kinase is $pp60^{c\text{-}src}$. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 205:97–103.
Veillette, A., M. A. Bookman, E. M. Horak, and J. B. Bolen. 1988. The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase $p56^{lck}$. *Cell.* 55:301–308.
Wang, J. Y. J. 1985. Isolation of antibodies for phosphotyrosine by immunization with a *y-abl* oncogene-encoded protein. *Mol. Cell. Biol.* 5:3640–3643.
Weidmer, T., S. J. Shattil, M. Cunningham, and P. J. Sims. 1990. The role of calcium and calpain in complement-induced vesiculation of the platelet plasma membrane and in the exposure of the platelet factor Va receptor. *Biochemistry.* 29:623–632.
Weiss, H. J., J. Hawiger, Z. M. Ruggeri, V. T. Turitto, P. Thiagarajan, and T. Hoffmann. 1989. Fibrinogen-independent platelet adhesion and thrombus formation on subendothelium mediated by glycoprotein IIb-IIIa complex at high shear rate. *J. Clin. Invest.* 83:288–297.
Woods, V. L., L. E. Wolff, and D. M. Keller. 1986. Resting platelets contain a substantial centrally located pool of glycoprotein IIb-IIIa complex which may be accessible to some but not other extracellular proteins. *J. Biol. Chem.* 261:15242–15251.
Yonemoto, W., A. J. Filson, A. E. Queral-Lustig, J. Y. J. Wang, and J. S. Brugge. 1987. Detection of phosphotyrosine-containing proteins in polyomavirus middle tumor antigen-transformed cells after treatment with a phosphotyrosine phosphatase inhibitor. *Mol. Cell. Biol.* 7:905–913.
Zucker, M. B., S.-J. Kim, J. McPherson, and R. A. Grant. 1977. Binding of factor VIII to platelets in the presence of ristocetin. *Br. J. Haematol.* 35:535–549.