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 can be 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 (Pytel 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., 1985a, 1986).

\(^{1}\) 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 Ib-IIIa has been implicated in platelet Na+/H+ exchange and in activation of phospholipase A2 (Sweat 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 A2 synthesis, and granule secretion (Huang and Detwiler, 1986). In contrast, "strong" agonists, such as thrombin or collagen can induce thromboxane A2 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 Ib-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).

The presence of multiple-protein tyrosine kinases in platelets, the rapid induction of tyrosine phosphorylation after platelet activation, and the central role of GP Ib-IIIa in platelet function, we sought to further define the relationship between GP Ib-IIIa and tyrosine phosphorylation. GP Ib-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 Ib-IIIa. Tyrosine phosphorylation was studied using immunoblotting probed with an affinity-purified polyclonal antiphosphotyrosine antibody. We demonstrate here that activation of GP Ib-IIIa and fibrinogen binding to GP Ib-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 Et (PGE), platelets were separated from plasma by gel-filtration (Tang et al., 1971) over a Sepharose-2B column equilibrated with an incubation buffer containing 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl2, 56 mM glucose, 1 mM CaCl2, 3 mM NaH2PO4, 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 × 108 platelets/ml with incubation buffer.

**Activation of Gel-filtered Platelets**

Immediately after gel filtration, 200 μM CaCl2 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 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 Ib-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 Ib-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 CaCl2 was added back just before gel filtration. EGTA treatment at 37°C disrupts the integrity of the GP Ib-IIIa complex and renders it incompetent to bind fibrinogen or support aggregation even after the addition of equimolar CaCl2 (Brass et al., 1985; Fitzgerald and Phillips, 1985; Shattil et al., 1985b; Pidard et al., 1986). As a control, some platelets were preincubated with EGTA at 20°C, a condition that does not disrupt GP Ib-IIIa (Shattil et al., 1985b; 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 μg/ml) (McCown 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 Ib-IIIa Activation and Platelet Alpha-granule Secretion**

Five murine mAbs were used in these studies. The antibody PACI is specific for the activated form of GP Ib-IIIa and binds to a fibrinogen binding site on GP Ib-IIIa (Shattil et al., 1985a, 1986; Taub et al., 1989). Antibody A2A9 binds to both the non-activated and activated forms of GP Ib-IIIa and therefore was used to quantitate total surface-expressed GP Ib-IIIa (Bennett et al., 1983). BIB5 is an antibody specific for GP Ib and SSA6 an antibody specific for GP IIa (Silver et al., 1987). Antibody S12, specific for the platelet alpha-granule membrane protein, GMP-140, was a gift from Dr. Roderic 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. Unstained gel-filtered platelets were first incubated for 1 min at 37°C in the presence or absence of a platelet agonist. Then, aliquots (1 × 108 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 fluorescent 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).
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 125I-labeled donkey anti-rabbit immunoglobulins (Amersham Corp., Arlington Heights, IL). 125I-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 (Nishiznka, 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.
sine 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 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.

### 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, CaCl2 (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., 1985b; 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).

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### 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).
binding protein (93 kD) which are only apparent in lane 2. (b) Gel-degradation products of myosin heavy chain (135 kD) and of actin- 

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 (BIB5) 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 A2, (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

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<th>Secretion</th>
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* 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.

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-
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 aggregation by ristocetin and von Willebrand factor, a process that involves GPIb and not that platelet aggregation was necessary for these phosphorylation events, occurred. Although unstirred platelets aggregated in response to thrombin or 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, 126,000, 108,000, and 100,000, and that these proteins are not phosphorylated in thrombathemic platelets which lack GP IIb-IIIa. The anti-phosphotyrosine 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 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....

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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 Ib-IIa 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 Ib-IIa 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\(^{\text{ck}}\) (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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