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Thrombin treatment induces rapid changes in tyrosine phosphorylation in platelets

(pp60$^{c\text{-}src}$/tyrosine protein kinase)

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ABSTRACT **We previously demonstrated that platelets express high levels of the tyrosine protein kinase pp60$^{c\text{-}src}$. By a quantitative immunoblot assay, it is shown in this report that pp60$^{c\text{-}src}$ represents 0.2–0.4% of total platelet protein. The expression of high levels of pp60$^{c\text{-}src}$ in platelets correlated with high levels of total cell phosphotyrosine. Unstimulated platelets were shown to possess numerous phosphotyrosine-containing proteins by immunoblot analysis using antibodies that specifically recognize phosphotyrosine residues. To examine whether the pattern of phosphotyrosine-containing proteins changes upon platelet activation, lysates from thrombin- and phorbol ester-treated platelets were subjected to immunoblot analysis. Novel phosphotyrosine-containing proteins were detected within seconds following platelet stimulation. These results suggest that tyrosine phosphorylation, perhaps mediated by pp60$^{c\text{-}src}$, may be involved in events associated with platelet activation.**

Over the past decade, platelets have become a useful system to study the mechanisms that couple extracellular stimuli to intracellular responses. Platelets can be stimulated by a variety of agonists to undergo shape change, aggregation, and release of their granule components. Many of the biochemical events that accompany this activation have been well described (reviewed in ref. 1). The initial response to many agonists involves a rapid turnover of inositolphospholipids leading to the generation of second messengers, diacylglycerol and inositol 1,4,5-trisphosphate. This event is followed by changes in arachidonic acid metabolism, protease activation, proton and calcium fluxes, and cytoskeletal rearrangements. It is likely that these changes in cell physiology are mediated by changes in protein phosphorylation, since multiple cellular proteins are phosphorylated within seconds after treatment of platelets with various agonists (reviewed in ref. 1). Most of these phosphoproteins have been identified only by their molecular weight on SDS/polyacrylamide gels; however, the functions of only a few of these phosphoproteins have been determined, e.g., myosin light chain (M_r 20,000) (2) and actin-binding protein (M_r 250,000) (3). All of the known phosphorylation events that have been shown to accompany platelet activation are mediated by serine/threonine protein kinases. The observations that platelets possess high levels of tyrosine-specific protein kinase activity (4–7) raise the question of whether tyrosine kinases may represent another component of the signal-transducing systems in platelets.

Platelets have been shown to possess abundant levels of one tyrosine protein kinase, pp60$^{c\text{-}src}$ (6, 7), the cellular homolog of the transforming protein of Rous sarcoma virus (RSV) (reviewed in refs. 8–10). This gene product is an enzyme of M_r 60,000 that is localized to the cytoplasmic face of the plasma membrane and other cellular membranes (11, 12). The functional role of this tyrosine protein kinase in normal cells has not been identified. In this study, we have examined whether the expression of high levels of the c-*src* gene product, pp60$^{c\text{-}src}$, correlates with high levels of tyrosine phosphorylation in platelets *in vivo*. In addition, we have examined the profile of phosphotyrosine-containing proteins in unstimulated and agonist-stimulated platelets.

MATERIALS AND METHODS

Platelet Isolation and Agonist Treatment. Blood was collected from healthy human volunteers into 0.01 volume of 40% sodium citrate. The entire platelet isolation procedure was performed at room temperature. The blood was centrifuged at 800 × *g* for 3.5 min and the platelet-rich plasma was collected. Platelets were either washed three times in 150 mM NaCl/10 mM Tris·HCl, pH 7.5/5 mM EDTA or gel-filtered as described (13). Gel-filtered platelets were used in all experiments in which platelet agonists were employed. This isolation procedure is less harsh than the washing procedure and minimizes the potential for altering platelet functions. The gel-filtered platelets were treated with bovine thrombin (1 unit/ml of platelets) from Calbiochem or phorbol 12-myristate 13-acetate (PMA; 100 ng/ml of platelets) from Sigma. These concentrations of thrombin and PMA were sufficient to cause maximal platelet shape change and aggregation, as determined by microscopic examination. The platelets were spun out of suspension in a microcentrifuge and solubilized as described below.

Anti-src Immunoblot Assay. Cell and platelet pellets were solubilized in 100°C lysis buffer (2% SDS/0.3% 2-mercaptoethanol/66 mM Tris·HCl, pH 7.5/10 mM EDTA) and immediately heated at 100°C for 10 min. Immunoblot assays were performed as described (6), with the following modifications. The filters were incubated in TN buffer (10 mM Tris·HCl, pH 7.5/170 mM NaCl) containing 1% Carnation instant low-fat dry milk to block nonspecific binding. Antibody incubations were performed in TN buffer/0.5% Carnation low-fat dry milk/0.05% Nonidet P-40/0.25% gelatin. Filters were washed in TN buffer/0.05% Nonidet P-40/0.5% gelatin. The bands were excised from the nitrocellulose filter paper and quantitated by γ counting (LKB 1275 Minigamma). The bacterial src protein used in these immunoblots was isolated from *Escherichia coli* expressing the chicken c-*src* gene engineered into a pT7 vector (ref. 14; S. Schuh and J.S.B., unpublished data). The amount of *E. coli* pp60$^{c\text{-}src}$ was quantitated by comparison to known amounts of Coomassie blue-stained protein standards.

Anti-phosphotyrosine Immunoblot Assays. Cells were solubilized in lysis buffer as described above. Immunoblot assays were performed as described (15). These immunoblots

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Abbreviations: PMA, phorbol 12-myristate 13-acetate; RSV, Rous sarcoma virus.

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were incubated with affinity-purified rabbit antibodies to phosphotyrosine [lots 3231 and 903 (derived from different rabbits), gifts from Jean Wang, University of California, San Diego]. These antibodies have been shown to have a strict specificity for phosphotyrosine residues (16, 17). In control immunoblots incubated with antibodies in the presence of phosphotyrosine, the immunoreactivity of all the platelet proteins recognized by the antibodies 903 and 3231 was eliminated.

Phospho Amino Acid Analysis. Two-dimensional phospho amino acid analysis was performed as described (15). The cell lines were labeled for 2 hr in phosphate-free Dulbecco's modified Eagle's medium with [^{32}P]orthophosphate at 1 mCi/ml (1 mCi = 37 MBq). Platelets were gel-filtered (as described above) and labeled in modified Tyrode's buffer for 2 hr.

Cell Lines. The vsrc-3T3ClA and csrc-3T3ClC cell lines were derived from G418-resistant clones resulting from transfection of NIH 3T3 cells with plasmid pLNSL7v-src or pLNSL7c-src, containing the v-*src* gene of the Schmidt-Ruppin A strain of RSV (18) or the intron-free chicken c-*src* gene [provided by H. Hanafusa, Rockefeller University (19)], respectively. The pLNSL7 plasmid is a derivative of the pLNL6 vector [provided by D. Miller, Fred Hutchinson Cancer Research Center (20)]. The vsrc-3T3ClA and csrc-3T3ClC cell lines were generated by S. Thomas and J.S.B. (unpublished data).

RESULTS

High Levels of pp60$^{c\text{-}src}$ and Phosphotyrosine in Platelets. We previously reported (6) that the tyrosine kinase pp60$^{c\text{-}src}$ is expressed at high levels in platelets compared to a variety of tissues including muscle, spleen, thymus, and bone marrow. To determine the abundance of pp60$^{c\text{-}src}$ relative to the total content of platelet protein, a sensitive immunoblot assay was performed using 25 ng of pp60$^{c\text{-}src}$ produced in *E. coli* as a reference standard to quantitate the c-*src* or v-*src* gene product. Fig. 1 shows a comparison of the levels of the c-*src* gene product in human platelets, NIH 3T3 cells, and two cell lines (derived from NIH 3T3 cells, S. Thomas and J.S.B., unpublished work) that express high levels of the chicken c-*src* gene product (csrc-3T3ClC) or the v-*src* gene product (vsrc-3T3ClA) encoded by RSV. These cell lines were included as reference standards for later studies in this report. The endogenous murine c-*src* gene product is expressed at low levels in NIH 3T3 cells, representing only 0.003% of total cell protein. In the csrc-3T3ClC and vsrc-3T3ClA cells, the avian c-*src* and v-*src* gene products were expressed at levels 15- to 20-fold higher than the endogenous pp60$^{c\text{-}src}$. Platelets expressed pp60$^{c\text{-}src}$ at levels 100- to 150-fold higher than in NIH 3T3 cells. By comparison to the bacterial pp60$^{c\text{-}src}$ marker, it was determined that pp60$^{c\text{-}src}$ represented 0.2–0.4% of the total platelet protein.

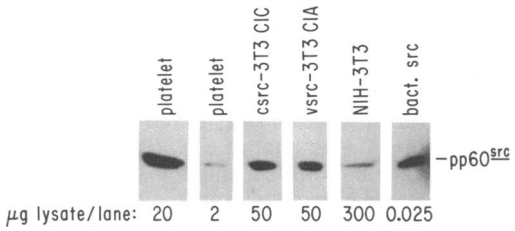

FIG. 1. Anti-src immunoblot. Cell lysates were prepared and immunoblotted as stated in *Materials and Methods*. pp60^{src} was detected with monoclonal antibody 327 (21). Cell type is indicated above each lane, and amount of lysate protein loaded into each lane is designated below. pp60$^{c\text{-}src}$ expressed in *E. coli* (bact. src, 25 ng) was included for quantitation.

To determine whether the high levels of the c-*src* gene product in platelets correlated with high levels of tyrosine phosphorylation, the total cell phosphotyrosine content was examined in platelets incubated with [^{32}P]orthophosphate for 2 hr (Fig. 2). Total cell proteins were extracted from platelets and the three NIH 3T3-derived cell lines, and the phospho amino acid content of radiolabeled proteins was determined following partial acid hydrolysis. This analysis revealed that platelets possessed high levels of total cell phosphotyrosine, with phosphotyrosine accounting for ≈0.9% of the total radiolabeled phospho amino acids. The NIH 3T3 cells had very low levels of total cell phosphotyrosine (≈0.13%). The csrc-3T3ClC cell line contained slightly higher levels (0.2%), consistent with the observations that the mere overexpression of the c-*src* gene product does not cause a dramatic elevation in total cell phosphotyrosine (22, 23). The vsrc-3T3ClA cells contained the highest percentage of total cell phosphotyrosine (1.9%). These results suggested that unstimulated platelets possess high levels of total cell phosphotyrosine.

The high levels of [^{32}P]phosphotyrosine in platelets labeled for 2 hr reflect the turnover of phosphate groups on tyrosine residues rather than the steady-state levels of phosphotyrosine. Immunoblot assays using antibodies that recognize phosphotyrosine residues were performed to provide a more accurate representation of the steady-state levels of phosphotyrosine-containing proteins and to examine the profile of phosphotyrosine-containing proteins in platelets. These antibodies react specifically with proteins containing phosphotyrosine (16, 17). The immunoblot in Fig. 3 shows a comparison of phosphotyrosine-containing proteins in unstimulated washed platelets and the three NIH 3T3-derived cell lines. The highest level of immunoreactivity was detected in the v-*src*-transformed 3T3 cells; however, platelets contained

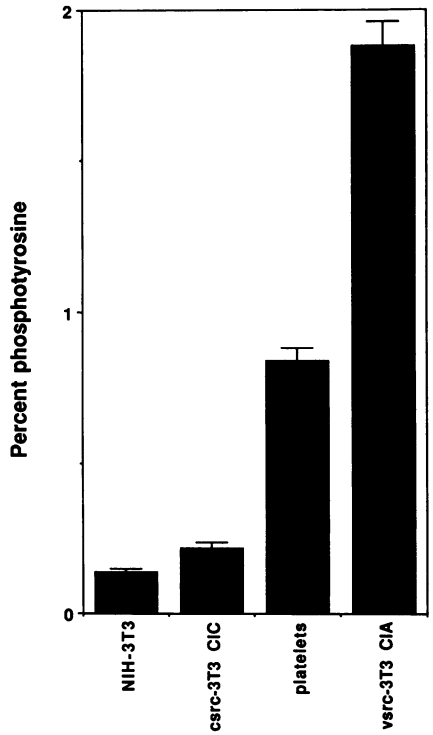

FIG. 2. Total cell phospho amino acid analysis of NIH 3T3, csrc-3T3ClC, and vsrc-3T3ClA cells and platelets labeled for 2 hr with [^{32}P]orthophosphate. The amount of phosphotyrosine, as a percentage of total [^{32}P]phospho amino acids, detected from each cell type is plotted. Actual values (mean ± SEM) were 0.13 ± 0.01% for the NIH 3T3 cells, 0.22 ± 0.02% for the csrc-3T3ClC cells, 0.84 ± 0.04% for the platelets, and 1.88 ± 0.04% for the vsrc-3T3ClA cells.

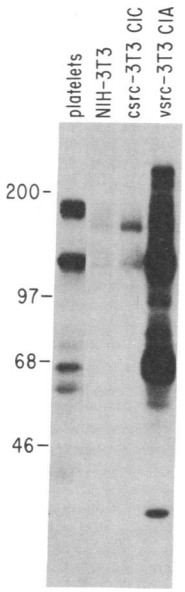

FIG. 3. Anti-phosphotyrosine immunoblot of platelets and NIH 3T3, csrc-3T3ClC, and vsrc-3T3ClA cells. Phosphotyrosine-containing proteins were detected using affinity-purified rabbit antibodies (lot 3231). Each lane contained 100 μg of lysate protein. Migration of prestained molecular weight markers ($M_r \times 10^{-3}$) is indicated at left.

high levels of immunoreactivity compared to the NIH 3T3 and csrc-3T3ClC cells. Multiple protein species were detected in platelets, the most immunoreactive proteins having molecular weights of approximately 170,000–180,000, 122,000, 66,000, and 59,000. (The M_r 59,000 protein is not the c-*src* gene product: it does not comigrate with radiolabeled pp60$^{c\text{-}src}$ markers, and pp60$^{c\text{-}src}$ immunoprecipitated with anti-src monoclonal antibodies is not recognized by these anti-phosphotyrosine antibodies.) These results indicate that unstimulated washed platelets possess several phosphotyrosine-containing proteins.

Agonist-Induced Tyrosine Phosphorylation in Platelets. Treatment of platelets with a variety of agonists that stimulate aggregation and secretion has been shown to be accompanied by the rapid phosphorylation of multiple proteins on serine and threonine residues (reviewed in ref. 1). To determine whether changes in tyrosine phosphorylation also accompany platelet activation, anti-phosphotyrosine immunoblot analysis was performed with lysates of thrombin- and PMA-treated platelets (Figs. 4 and 5). Thrombin, a strong physiological agonist of platelets, induces multiple changes in platelet physiology including shape changes, aggregation, and secretion of intracellular vesicles (reviewed in ref. 24). The phorbol ester PMA also causes platelet aggregation and secretion (25), presumably by mimicking diacylglycerol activation of protein kinase C (reviewed in ref. 26).

Fig. 4 shows two representative profiles of phosphotyrosine-containing proteins from platelets treated with thrombin for various times. The two blots were probed with two different antibodies that specifically recognize phosphotyrosine residues. The most prominent thrombin-induced change in tyrosine phosphorylation detected with antibody 903 (Fig. 4*A*) involved a protein doublet at M_r 95,000–97,000. The induction of phosphorylation of these two proteins occurred within 5 sec after thrombin treatment, and the level of phosphorylation of these proteins did not change appreciably during 15 min. Two less-immunoreactive protein species of M_r 77,000 and 83,000 were also detected with similar kinetics as observed with the M_r 95,000–97,000 protein doublet. In addition, the tyrosine phosphorylation of a protein of M_r 66,000 was diminished within 5 sec of thrombin treatment. The immunoreactivity of this protein was restored during the continued incubation with thrombin. The profile with antibody 3231 (Fig. 4*B*), a different antiphosphotyrosine antibody, reveals similar changes in tyrosine phosphorylation of the proteins of M_r 95,000–97,000, 83,000, 77,000, and 66,000; however, several additional immunoreactive proteins were

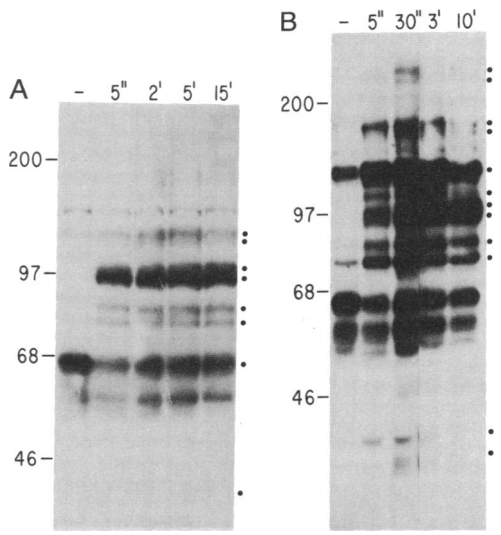

FIG. 4. Anti-phosphotyrosine immunoblots of gel-filtered platelets stimulated with bovine thrombin (1 unit/ml) and solubilized after various times of treatment (5 sec to 15 min). Lane at left represents unstimulated platelets. Immunoblotting was performed with affinity-purified antibodies 903 (*A*) and 3231 (*B*). Dots at right designate phosphotyrosine-containing proteins that appear to change in intensity upon thrombin stimulation. Migration of prestained molecular weight markers ($M_r \times 10^{-3}$) is indicated at left.

detected. Most notably, the immunoreactivity of a protein of M_r 122,000 was enhanced significantly upon thrombin treatment. This protein represented the most immunoreactive protein in platelets. In addition, thrombin induced an increase in the levels of phosphotyrosine in proteins of M_r 250,000, 170,000, 116,000, 38,000, and 34,000. In this blot, the thrombin-induced loss of immunoreactivity of the M_r 66,000 protein was readily detectable. These results indicate that thrombin treatment induces rapid changes in the phosphorylation of cellular proteins on tyrosine residues. These changes involve predominantly an increase in tyrosine phosphorylation of a specific set of cellular proteins; however, a single protein species of M_r 66,000 displays a reproducible loss of phosphotyrosine. While the differences between the profiles shown in these two immunoblots appear to represent differences in the immunoreactivity of the two different antibody preparations, we have also noted minor variations in profiles from individual subjects and observed differences in the basal levels of phosphotyrosine-containing proteins in platelets that were isolated by different methods. Note that the pattern of tyrosine phosphorylation in washed platelets (Fig. 3) varies slightly from the pattern observed in untreated gel-filtered platelets (Fig. 4): the M_r 170,000–180,000 phosphotyrosine-containing protein in washed platelets is not detected in gel-filtered platelets.

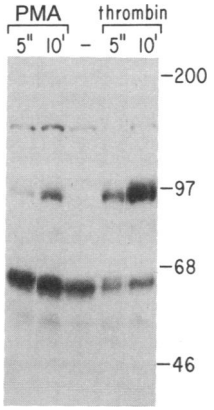

FIG. 5. Anti-phosphotyrosine immunoblot of gel-filtered platelets stimulated with PMA (100 ng/ml) or bovine thrombin (1 unit/ml) and solubilized in SDS lysis buffer after 5 sec or 10 min. Center lane represents an untreated platelet lysate. Immunoblotting was performed with affinity-purified anti-phosphotyrosine antibodies (lot 903). Migration of prestained molecular weight markers ($M_r \times 10^{-3}$) is indicated at right.

PMA treatment of gel-filtered platelets also caused an increase in the immunoreactivity of a protein doublet of M_r 95,000–97,000 (Fig. 5) that comigrated with the thrombin-induced phosphotyrosine-containing protein doublet. Although these proteins represented the most prominent and consistent changes in tyrosine phosphorylation, the intensity of the signal was not as high as that detected following thrombin treatment and the kinetics of induction with PMA were slightly different than with thrombin. In addition, we have not observed any reduction in phosphorylation of the M_r 66,000 protein after PMA treatment.

DISCUSSION

We have demonstrated that human platelets contain very high levels of the tyrosine protein kinase pp60$^{c\text{-}src}$. This protein was found to represent 0.2–0.4% of the total protein of platelets. It was unexpected to find an enzyme expressed at levels comparable to those of structural proteins such as myosin, tropomyosin, and fodrin (reviewed in refs. 27 and 28).

The expression of high levels of the c-*src* gene product in platelets correlated with high levels of total cell phosphotyrosine; indeed, the amount of phosphotyrosine in platelets was only 50% lower than the levels found in cells transformed by the oncogenic *src* gene product encoded by RSV. Other cell types that express high levels of pp60$^{c\text{-}src}$, such as chicken embryo or mouse 3T3 fibroblasts containing plasmids engineered to express the wild-type c-*src* gene product at 10- to 20-fold higher levels than the endogenous c-*src* gene product, display only a slight increase in total cellular phosphotyrosine (refs. 22 and 23; Fig. 2). In addition, cultured neurons and astrocytes, which express pp60$^{c\text{-}src}$ at levels comparable to those in the 3T3 or chicken embryo fibroblast c-*src* overexpressor cells, also display a low level of total cell phosphotyrosine (S. A. Lynch and J.S.B., unpublished data). Thus, platelets are unique in that they possess high levels of both pp60$^{c\text{-}src}$ and phosphotyrosine.

The concentration of pp60$^{c\text{-}src}$ in all of the above-mentioned cells, although higher than that in fibroblasts, is only 1/5th to 1/10th that of pp60$^{c\text{-}src}$ in platelets. The specific activity of pp60$^{c\text{-}src}$ in platelets (which is similar to that of fibroblast pp60$^{c\text{-}src}$, unpublished data) is 1/10th to 1/20th that of pp60$^{v\text{-}src}$ (22, 23). If both the specific activity and the levels of pp60^{src} are taken into consideration, the total units of *src* tyrosine kinase activity are comparable in platelets and the v-*src*-transformed 3T3 cells employed in this study. Thus, one might predict that platelets would contain elevated levels of phosphotyrosine-containing proteins compared to fibroblasts and the c-*src* overexpressor cells employed in this study.

Tyrosine phosphorylation in normal cells is generally associated with events that are transiently induced in response to extracellular signals such as growth factors (reviewed in refs. 29–31). The presence of a high, constitutive signal of tyrosine phosphorylation in unstimulated platelets suggests the possibility that tyrosine phosphorylation may play a "housekeeping" (maintenance) function in the platelets. However, given the extreme sensitivity of platelets to spontaneous activation, we cannot rule out the possibility that the signal of phosphorylation that we detect in unstimulated platelets reflects a low level of activation generated by the experimental manipulations used to isolate the platelets from peripheral blood. We have noted some variation in the phosphotyrosine-containing protein profiles among different platelet preparations, which could reflect different degrees of activation.

Following thrombin treatment of platelets, novel phosphotyrosine-containing proteins were observed, most notably a protein doublet of M_r 95,000–97,000. The appearance of these phosphotyrosine-containing proteins was very rapid, detectable within seconds after stimulation, and the phosphorylation of these proteins was maintained throughout the 15-min assay. Several other phosphotyrosine-containing proteins were also detected. The M_r 95,000–97,000 proteins were consistently observed with different preparations of antibodies that recognize phosphotyrosine and under a variety of conditions of platelet isolation.

The increase in the immunoreactivity of protein species in the anti-phosphotyrosine immunoblot assays of thrombin-treated platelets could reflect either increased phosphorylation or decreased dephosphorylation of these proteins. Alternatively, since thrombin itself is a protease and also activates platelet proteases, it is possible that thrombin-induced immunoreactive proteins could represent proteolytic cleavage products of phosphotyrosine-containing proteins present in unstimulated platelets. Since the only protein that displayed a detectable loss of immunoreactivity after thrombin treatment was a M_r 66,000 protein, the only observed proteins that could be candidate proteolytic cleavage products are the M_r 38,000 and 34,000 proteins (Fig. 4*B*). Although we cannot exclude the possibility that thrombin inhibits cellular phosphotyrosine phosphatases, the impressive increase in the immunoreactivity of several proteins within seconds following thrombin treatment is more consistent with the possibility that thrombin causes an increase in the phosphorylation of several platelet proteins, possibly by activating a tyrosine kinase(s) or by altering substrate accessibility to a tyrosine kinase(s).

The stimulation of tyrosine phosphorylation of the M_r 95,000–97,000 proteins suggests that tyrosine phosphorylation may mediate some early events induced by thrombin. Thrombin has been shown to rapidly induce inositolphospholipid hydrolysis, generating diacylglycerol, which directly activates protein kinase C. To determine whether the activation of tyrosine phosphorylation is an event that takes place after the initial hydrolysis of inositolphospholipids, we investigated the effect of PMA, an agonist that acts downstream of inositolphospholipid hydrolysis to activate protein kinase C. As shown in this report, treatment of platelets with PMA generated the phosphorylation of the M_r 95,000–97,000 protein doublet. Since this phosphorylation event occurred after treatment of platelets with PMA as well as thrombin, it is possible that the phosphorylation of these proteins is dependent on the generation of diacylglycerol and the subsequent activation of protein kinase C.

In our platelet studies, the PMA-induced signal of phosphorylation of the M_r 95,000–97,000 protein doublet was not as strong as the signal induced by thrombin treatment. Therefore, it is difficult to conclude from this preliminary report that protein kinase C is an intermediate in the induction of phosphorylation of these proteins by thrombin.

PMA has been reported to induce the phosphorylation of proteins on tyrosine residues in fibroblasts (32–34). This evidence suggests that PMA stimulation of protein kinase C may lead to the activation of cellular tyrosine kinases. PMA and other agonists that cause activation of protein kinase C have been shown to induce serine/threonine phosphorylation of pp60$^{c\text{-}src}$ and other tyrosine kinases (35–41). We have found that thrombin or PMA treatment of platelets causes a dramatic increase in the phosphorylation of the pp60$^{c\text{-}src}$ tryptic peptide that contains serine-12 (data not shown), the major site of phosphorylation by protein kinase C (37, 38). However, the phosphorylation of serine-12 does not result in an activation of the kinase activity of pp60$^{c\text{-}src}$ as detected in *in vitro* kinase assays (ref. 37 and unpublished observations). It is possible that the effects of this phosphorylation involve more subtle changes in other properties such as the localization of pp60$^{c\text{-}src}$ or substrate accessibility.

The tyrosine kinase(s) responsible for phosphorylating the many phosphotyrosine-containing proteins in unstimulated and stimulated platelets remains to be identified. We (unpublished data) and others (7) have found that the c-*src* gene product represents the major tyrosine kinase activity in fractionated platelet lysates. Although this evidence makes $pp60^{c\text{-}src}$ a likely candidate for the tyrosine kinase responsible for the high levels of tyrosine phosphorylation in platelets, we cannot rule out the possibility that another tyrosine kinase is responsible for the observed tyrosine phosphorylation. We have not detected any alteration in the kinase activity of $pp60^{c\text{-}src}$ extracted from untreated, thrombin-treated, or PMA-treated platelets by *in vitro* kinase assays (data not shown). However, the activation of $pp60^{c\text{-}src}$ kinase activity following agonist treatment may not be preserved during detergent solubilization and immunoprecipitation. It is possible that the platelet thrombin receptor possesses an intracellular tyrosine kinase domain or, alternatively, that the thrombin receptor directly interacts with an intracellular tyrosine kinase, such as the c-*src* gene product, to transmit its signal into the platelet interior. It has recently been demonstrated that $p56^{lck}$, which is closely related to $pp60^{c\text{-}src}$, is associated with the CD4 and CD8 transmembrane proteins in T cells (42). This evidence supports the possibility that the *src* family of tyrosine kinases may be functionally coupled to transmembrane receptors. However, since the thrombin receptor in platelets has not been identified, the mechanism involved in triggering the most immediate events that follow thrombin treatment cannot be determined.

Further studies are needed to determine whether tyrosine phosphorylation of the M_r 95,000–97,000 proteins, or the other thrombin-induced phosphotyrosine-containing proteins, is necessary to mediate a particular platelet response (i.e., platelet aggregation or secretion) and thus establish whether a cause–effect relationship exists. The availability of reagents for the immunopurification of phosphotyrosine-containing proteins should allow isolation and characterization of the functional activities of these phosphoproteins.

Note. While this manuscript was under review, Ferrell and Martin (43) reported showing similar changes in tyrosine phosphorylation following thrombin treatment of platelets.

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