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Platelet Tyrosine-Specific Protein Phosphorylation Is Regulated by Thrombin

JAMES E. FERRELL, JR.,* AND G. STEVEN MARTIN
Department of Zoology, University of California, Berkeley, California 94720

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Intact human platelets, terminally differentiated cells with no growth potential, were found to possess unusually high levels of tyrosine-specific protein phosphorylation. The physiological platelet activator thrombin transiently elevated platelet phosphotyrosine content, apparently through stimulation of one or more tyrosine-specific protein kinases. Immunoblotting with antiphosphotyrosine antiserum showed that thrombin caused dramatic changes in the tyrosine phosphorylation of a number of individual protein bands and that these changes occurred in three distinct temporal waves. Most but not all of the protein bands phosphorylated at tyrosine in response to thrombin were also tyrosine phosphorylated in response to chilling or the combination of ionophore A23187 and tetradecanoylphorbol acetate. Thrombin stimulated the phosphorylation of the tyrosine kinase pp60$^{c\text{-}src}$, primarily at Ser-12 and Tyr-527, although the effects of these phosphorylations on platelet pp60$^{c\text{-}src}$ function were not apparent. Together, these results suggest that tyrosine-specific protein kinases of uncertain identity are involved in signal transduction in platelets.

The phosphorylation of proteins at tyrosine residues is implicated in normal and abnormal cell growth: several growth factor receptors are tyrosine-specific protein kinases, as are the transforming proteins of one class of acutely oncogenic retroviruses (13). The best-studied such retrovirus is Rous sarcoma virus, whose transforming gene v-*src* was derived from a cellular tyrosine kinase gene, c-*src* (22, 23). The protein-tyrosine kinase activity of the v-*src* gene product pp60$^{v\text{-}src}$ has been activated by a C-terminal substitution and scattered point mutations (12). The transforming capacity of pp60$^{v\text{-}src}$ results, at least in part, from its increased kinase activity.

Through alternative splicing, the c-*src* gene gives rise to two distinct proteins, both of about 60 kilodaltons (kDa) (16, 18). Little is known about the physiological function of either c-*src* protein. A number of other proto-oncogene products are involved in the transduction of normal mitogenic signals (e.g., c-*erbB* [epidermal growth factor receptor], c-*fms* [colony-stimulating factor 1 receptor], c-*sis* [platelet-derived growth factor]). However, the tissue distribution of pp60$^{c\text{-}src}$ expression suggests that this is not its function; those cells which express the highest levels of pp60$^{c\text{-}src}$ do not divide. For example, postmitotic differentiated or differentiating neurons express high levels of both c-*src* proteins (1, 3, 7, 8, 17, 21). Mammalian platelets, terminally differentiated cells with no growth potential, express still higher levels of the smaller c-*src* protein (9). These observations suggest that tyrosine-specific protein kinases in general, and pp60$^{c\text{-}src}$ in particular, may regulate cellular processes that are distinct from growth control.

We have chosen human platelets as a system for investigating the role of protein-tyrosine phosphorylation in differentiated cells. Platelets are relatively simple cell fragments (they do not synthesize nucleic acids or proteins) and undergo a number of very rapid changes in response to activating agents (shape changes, secretion, eicosanoid release, and integrin- and fibrinogen-mediated aggregation). In this report we show that intact human platelets possess unusually high levels of protein-tyrosine phosphorylation and that thrombin, a physiological platelet activator, causes a transient elevation of platelet phosphotyrosine levels by stimulating one or more tyrosine-specific protein kinases.

* Corresponding author.

MATERIALS AND METHODS

Platelet isolation. After informed consent was obtained, human blood was drawn from adult volunteers who denied having taken aspirin or other drugs for 2 weeks prior to the donation. Washed platelets were isolated as described (25) and suspended at a concentration of 5×10^8 to 10×10^8 cells per ml in incubation buffer (137 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5.6 mM glucose, 5 mM NaHEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid sodium, pH 7.4], 0.1% bovine serum albumin [Sigma, essentially fatty acid free], and 1 U of apyrase [Sigma, grade V] per ml). Apyrase was omitted when platelets were to be treated with ADP.

Platelet incubation and radiolabeling. Suspensions of washed platelets were incubated at 37°C in a water bath or incubator with constant shaking. Cells were treated with various activating agents within 2 h of isolation or radiolabeled with carrier-free $^{32}P_i$ (1 to 10 mCi/ml; ICN) for 5 h prior to activation. When the responses of radiolabeled cells were to be directly compared with the responses of nonlabeled cells (see Fig. 6; data not shown), both the labeled and nonlabeled cells were incubated for 5 h before treatment with various agents. Cells remained responsive to thrombin throughout the radiolabeling, as assessed by thrombin-induced shape change, aggregation, phosphorylation of myosin light chain-1 (a 20-kDa protein whose phosphorylation by a calmodulin-dependent kinase is stimulated by thrombin [11]), and phosphorylation of the 47-kDa protein kinase C substrate (identified as inositol 1,4,5-trisphosphate phosphomonoesterase [4], although doubts have been raised about this identification [24]). In some experiments (see Fig. 6), prolonged incubation appeared to diminish thrombin-stimulated tyrosine phosphorylation, particularly of the protein band(s) around 100 to 108 kDa (cf. Fig. 4 and 5), although this diminution was not always apparent.

Activators and antagonists. Platelet activators and antagonists were purchased from Sigma (unless specified otherwise) and added to washed platelet suspensions as 10- to

1,000-fold concentrates in either 138 mM NaCl (bovine thrombin, bovine collagen [type I], ADP, epinephrine, platelet-activating factor [PAF], sodium orthovanadate [pH 10.0, Aldrich], and aspirin), dimethylsulfoxide (DMSO) (12-*O*-tetradecanoylphorbol 13-acetate [TPA]), or ethanol (arachidonate, ionophore A23187, and 1,2-dioctanoyl-*rac*-glycerol [DG]). Final ethanol and DMSO concentrations were 1% or less. Ethanol and DMSO alone had no effect on the various quantities measured as described below.

Platelet morphology. Portions of platelets were fixed in 1% glutaraldehyde and examined by phase-contrast microscopy (100× objective) to ensure that the cells had not become activated during the isolation procedure and to monitor response to activators. Typical isolates showed 70 to 90% smooth discs and 10 to 30% discs with one or two filopodia and no spiculate spheres or aggregated cells. Cells activated with thrombin rapidly became spiculate spheres and then aggregated. Cells treated with collagen, ADP, PAF, A23187, or arachidonate or chilled to 4°C were spiculate but not aggregated. Cells treated with TPA or DG became lumpy spheres without spicules (although at concentrations higher than those needed to effect maximal phosphorylation of the 47-kDa kinase C substrate). Cells treated with TPA plus A23187 were spiculate and aggregated.

Phosphoamino acid analysis. Radiolabeled platelet suspensions were lysed in 0.1 volume of 10% (wt/vol) sodium dodecyl sulfate (SDS)–1 mM sodium orthovanadate, followed promptly by 0.25 volume of 100% (wt/vol) trichloroacetic acid. Precipitated proteins were washed once with 2:1 (vol/vol) chloroform-methanol and twice with cold ethanol. The precipitates were hydrolyzed for 2 h at 110°C in 6 M HCl, and the hydrolysates were lyophilized and analyzed by two-dimensional electrophoresis (pH 1.9 followed by pH 3.5) on cellulose thin-layer plates (100 μm; E. M. Laboratories) as described (5). The amounts of phosphoserine, phosphothreonine, and phosphotyrosine in each sample were determined by scintillation counting. The phosphoamino acid content of individual protein bands was analyzed by excising the bands from polyacrylamide gels and hydrolyzing the proteins in situ under the conditions described above. The hydrolysates were desalted by adsorption to AG1-X8 anion-exchange resin (Bio-Rad Laboratories, chloride form [5]) and analyzed by two-dimensional electrophoresis.

Immunoblotting. Platelet suspensions were lysed in SDS gel sample buffer supplemented with 10 mM EDTA (15) and promptly boiled for 3 min. Platelet proteins (30 to 60 μg per lane) were separated by SDS–7.5% polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride (PVDF) membranes (Millipore), and probed with antiphosphotyrosine antibodies followed by ^{125}I-protein A (ICN). Transfer, blocking, antibody incubation, and ^{125}I-protein A incubation were performed as described (14) except that transfer was carried out at 60 V instead of 40 V. Transfer efficiency was assessed by staining immunoblots with Coomassie blue. Transfer appeared to be complete for proteins below 190 kDa; above that, transfer efficiency was variable. Retention on the PVDF membrane of proteins above 29 kDa appeared to be nearly complete.

Immunoprecipitation. For immunoprecipitation with anti-pp60^{src} antibodies, platelet suspensions were lysed in 0.25 volume of immunoprecipitation buffer added as a fivefold concentrate (150 mM NaCl, 50 mM Tris hydrochloride [pH 7.0], 10 mM EDTA [pH 8.0], 5 mM sodium orthovanadate [pH 10.0], 5% [wt/vol] sodium deoxycholate, 5% [wt/vol] Nonidet P-40, 0.5% [wt/vol] SDS, 5% [vol/vol] aprotinin solution [Sigma], 500 μg of leupeptin per ml). Lysates were precleared with fixed *Staphylococcus aureus* (45 min, 4°C) and incubated with monoclonal antibody GD11 or EB8 (19) (45 min, 4°C). Immune complexes were adsorbed onto *S. aureus* saturated with rabbit anti-mouse immunoglobulin G (IgG) (45 min, 4°C), washed, eluted with SDS gel sample buffer, and analyzed by SDS–7.5% polyacrylamide gel electrophoresis.

For immunoprecipitation with antiphosphotyrosine antibodies, samples were lysed in SDS gel sample buffer, heated for 2 min at 100°C, and diluted 1:20 in immunoprecipitation buffer without SDS (yielding detergent concentrations of 1% sodium deoxycholate, 1% Nonidet P-40, and 0.1% SDS). Samples were then processed as described above, except that the immune complexes were adsorbed onto fixed *S. aureus* saturated with nonlabeled platelet lysate.

Kinase assays. The tyrosine-specific protein kinase activity of whole-platelet lysates was assessed by using angiotensin II as an exogenous substrate as described elsewhere (26) with the following modifications. Washed platelets were suspended in incubation buffer lacking apyrase and incubated for 3 min with or without thrombin. Cells were lysed in 0.25 volume of 5× immunoprecipitation buffer (see above). A portion of lysate (5 μl, containing approximately 5 μg of platelet protein) was mixed with 45 μl of kinase buffer (50 mM NaPIPES [sodium piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), pH 7.0], 10 mM $MnCl_2$, 40 μM ATP, 200 μCi of [γ-^{32}P]ATP per ml, and 2 mM angiotensin II [Sigma]) and incubated for 15 min at 37°C. Reactions were stopped by addition of excess EDTA, and phosphorylated angiotensin II was separated from other radiolabeled species by electrophoresis toward the cathode on cellulose thin-layer plates at pH 3.5. Angiotensin II spots were detected by ninhydrin staining. Phosphorylated angiotensin II was retarded by a factor of about 0.5 with respect to the stained spots.

To assess the kinase activity of platelet pp60$^{c\text{-}src}$ in vitro, the protein was isolated by immunoprecipitation with monoclonal antibody EB8 as described above and suspended in 100 mM NaCl–10 mM NaPIPES (pH 7.0). The initial rate of phosphorylation of IgG heavy-chain, acid-treated rabbit muscle enolase (Sigma) or pp60$^{c\text{-}src}$ itself was assayed essentially as described (6).

Tryptic peptide mapping. Platelet pp60$^{c\text{-}src}$ was immunoprecipitated with GD11 or EB8, eluted from gel slices, digested with tolylsulfonyl phenylalanyl chloromethyl ketone-inactivated trypsin (Sigma), and analyzed by electrophoresis on cellulose thin-layer plates at pH 1.9 followed by chromatography in *n*-butanol–pyridine–acetic acid–water (75:50:15:60) as described (3).

RESULTS

Platelet phosphoamino acid analysis. Washed human platelets were labeled with $^{32}P_i$ and subjected to phosphoamino acid analysis. Phosphotyrosine was found to constitute 0.89 ± 0.16% (standard deviation [SD], $n = 9$) of the recovered phosphoamino acids (Fig. 1). The proportion of phosphotyrosine found in platelets is unusually high; under somewhat different labeling conditions, fibroblasts typically yield 0.02 to 0.06% phosphotyrosine, and transformed and growth factor-stimulated fibroblasts yield 0.1 to 0.5% (5).

Thrombin-stimulated tyrosine phosphorylation. Radiolabeled platelets were incubated with thrombin at a concentration sufficient to cause maximal shape change and aggregation (1 U/ml), and the platelet [^{32}P]phosphotyrosine content was measured as a function of time. As shown in Fig. 2A, thrombin effected a modest increase in the absolute

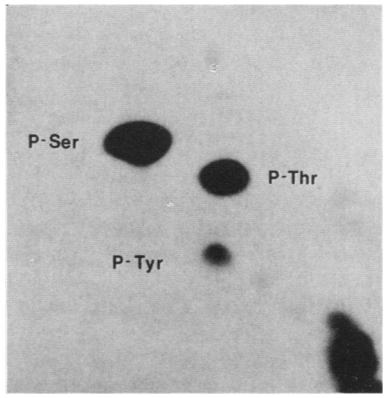

FIG. 1. Phosphoamino acid analysis of intact human platelets. Washed platelets were labeled with $^{32}P_i$, lysed, and analyzed by two-dimensional electrophoresis on thin-layer cellulose plates followed by autoradiography. First dimension, pH 1.9, anode at left; second dimension, pH 3.5, anode at top.

amount of platelet [^{32}P]phosphotyrosine. The increase was detectable within 20 s of thrombin stimulation and was maximal by 3 min. Tyrosine phosphorylation lagged behind platelet shape change, which was near maximal after 5 s and maximal by 20 s and occurred concomitantly with aggregation (and, by inference, with secretion). Phosphotyrosine levels peaked at 132 to 176% of basal levels (in four experiments), compared with maximal phosphoserine and phosphothreonine levels of 156 to 183% and 184 to 236%, respectively. The rate of increase in [^{32}P]phosphotyrosine following thrombin stimulation was 30- to 100-fold higher than the maximum rate of labeling observed in resting cells (Fig. 3).

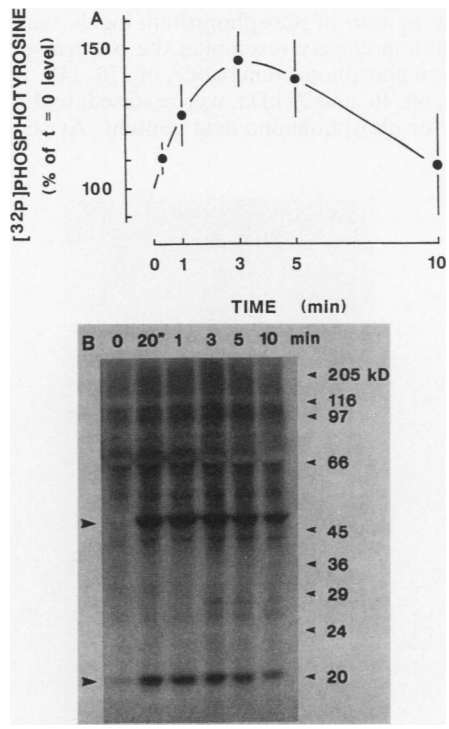

FIG. 2. (A) Platelet [^{32}P]phosphotyrosine content at various times following stimulation by thrombin (1 U/ml). Data are averages ± standard error from four independent experiments. (B) Overall protein phosphorylation at various times following thrombin stimulation. Platelets were labeled with ^{32}P, lysed, and subjected to SDS–10% polyacrylamide gel electrophoresis followed by autoradiography. The prominent 47- and 20-kDa phosphoproteins are substrates of protein kinase C and calmodulin-dependent myosin light-chain-1 kinase, respectively (4, 11).

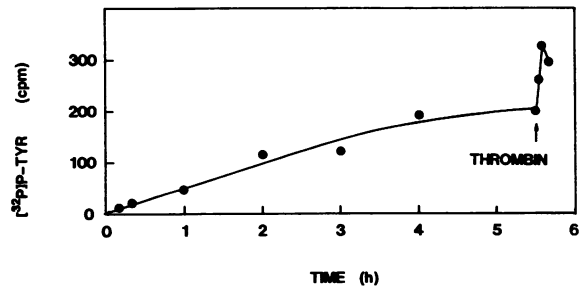

FIG. 3. Time course of [^{32}P]phosphotyrosine ([^{32}P]P-Tyr) labeling before and after treatment with thrombin (1 U/ml). Phosphoamino acid analyses were performed on samples of platelet suspension containing equal numbers of cells.

The thrombin-stimulated increase in phosphotyrosine radiolabeling was followed by a decrease in labeling to roughly basal levels (Fig. 2A). The extent of the decrease varied greatly from experiment to experiment. The decrease was paralleled by similar decreases in the labeling of phosphoserine, phosphothreonine, and nearly all of the phosphoprotein bands detectable by autoradiography following SDS-polyacrylamide slab gel electrophoresis (Fig. 2B). These decreases may reflect activation of a phosphoprotein phosphatase(s) (2).

Immunological detection of phosphotyrosine-containing proteins. To examine individual phosphotyrosine-containing proteins, we made use of an affinity-purified antiserum raised against polymerized phosphotyrosine, alanine, glycine, and keyhole limpet hemocyanin (14). This approach offers two important advantages: the phosphotyrosine content of many protein bands can be assessed individually, and there is no potential for interference from changes in specific activity of radiolabeled proteins.

As shown in Fig. 4, lysates from resting platelets analyzed by immunoblotting with antiphosphotyrosine antiserum revealed a number of candidate phosphotyrosine-containing protein bands, including prominent protein bands of 130 and 60 kDa. Authentic pp60$^{c\text{-}src}$ was obtained by immunoprecipitation with monoclonal antibody EB8, radiolabeled by autophosphorylation in vitro, and included as an internal standard on an antiphosphotyrosine immunoblot. The 60-kDa immunoblotted band was found to comigrate exactly with pp60$^{c\text{-}src}$ (not shown).

Upon stimulation with thrombin, many of the immunoblotted bands became more intense, and several novel bands appeared (Fig. 4). Some of the increases were marked, such as the four- to fivefold increase in the 130-kDa band(s) (estimated by densitometry of pre-flashed autoradiograms), and some were less dramatic, for example, the 25 to 75% increase in pp60$^{c\text{-}src}$ tyrosine phosphorylation.

The proteins phosphorylated at tyrosine in response to thrombin can be divided into three groups by the kinetics of their phosphorylation. The first wave included four protein bands of 70, 68, 34, and 27 kDa. Their phosphorylation was rapid and transient, peaking within 5 to 20 s of thrombin treatment (Fig. 4). In some experiments, the phosphorylations of the 70- and 68-kDa bands appeared to be more sustained (not shown).

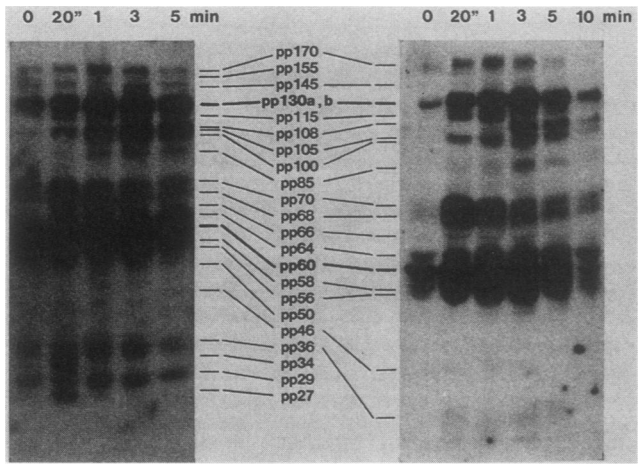

FIG. 4. Time course of thrombin-induced tyrosine phosphorylation by immunoblotting. Platelets were stimulated with thrombin (1 U/ml) for various lengths of time from 20 s (20") to 10 min. Samples were subjected to SDS–7.5% polyacrylamide gel electrophoresis, transferred to PVDF membranes, probed with antiphosphotyrosine antiserum and ^{125}I-protein A, and autoradiographed. Two blots are shown, from similar experiments with different washed platelet preparations. The blot on the left shows a wide range of phosphotyrosine-containing proteins. For the blot on the right, the time of electrophoresis was increased to effect better resolution of the 100- to 130-kDa proteins. Masses (in kilodaltons) were assigned by comparison with standards (not shown).

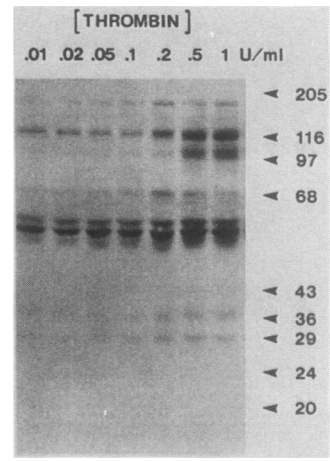

FIG. 5. Dose dependence of thrombin-induced tyrosine phosphorylation by immunoblotting. Platelets were stimulated for 5 min with various concentrations of thrombin. Samples were subjected to SDS–7.5% polyacrylamide gel electrophoresis, transferred to PVDF membranes, probed with antiphosphotyrosine antiserum and ^{125}I-protein A, and autoradiographed.

Two slower, more sustained, and more prominent waves of phosphorylation followed (Fig. 4). The second wave included bands of 130, 115, and 105 kDa and the putative pp60$^{c\text{-}src}$ band. Their phosphorylation peaked 1 to 3 min after thrombin treatment. The third and last wave of phosphorylation peaked 3 to 5 min after thrombin treatment and included proteins of 108 to 100 kDa (distinguishable in some experiments from the more rapidly phosphorylated 105-kDa band), and an 85-kDa protein. In some experiments, the prominent 130-kDa band seen in the second phosphorylation wave appeared to become a closely spaced doublet during the third wave.

The subsequent decreases in the most prominent immunoblotted bands, like the decrease noted above in whole-cell [^{32}P]phosphotyrosine content, varied greatly from experiment to experiment.

Dose-response studies. Platelets were incubated for 5 min with various concentrations of thrombin, and cell shape, aggregation, and tyrosine phosphorylation were assessed. Tyrosine phosphorylation was maximal at 0.5 U of thrombin per ml (Fig. 5). This coincided with the concentration needed to effect maximal aggregation. By contrast, thrombin-induced shape change was maximal at 0.02 U/ml. The individual waves of tyrosine phosphorylation may have slightly different dose dependences. In one study (Fig. 5), the first and second temporal waves of tyrosine were maximal at 0.2 U/ml and the third wave at 0.5 U/ml. In a similar experiment, the second wave was maximal at 0.1 U of thrombin per ml, and the first and third waves (and aggregation) were maximal at 0.2 U/ml (not shown).

Antibody specificity. The specificity of the antiphosphotyrosine antibody was addressed by competition with phenylphosphate (40 mM), phosphotyrosine (1 mM), phosphoserine (1 mM), or phosphothreonine (1 mM). Phenylphosphate and phosphotyrosine decreased the intensity of all the immunoblotted bands to undetectable levels, whereas phosphoserine and phosphothreonine had no noticeable effect (not shown). This suggests that the immunoblotted bands represent bona fide phosphotyrosine-containing proteins.

To address the issue of antibody specificity further, platelets were radiolabeled, stimulated with thrombin for 3 min, lysed, and subjected to immunoprecipitation. As shown in Fig. 6, the pattern of phosphoprotein bands seen by immunoprecipitation closely resembled the pattern seen by blotting. Eleven phosphoprotein bands, of 170, 145, 130, 105, 85, 70, 68, 64, 60, 46, and 29 kDa, were excised, hydrolyzed, and analyzed for phosphoamino acid content. As shown in Fig.

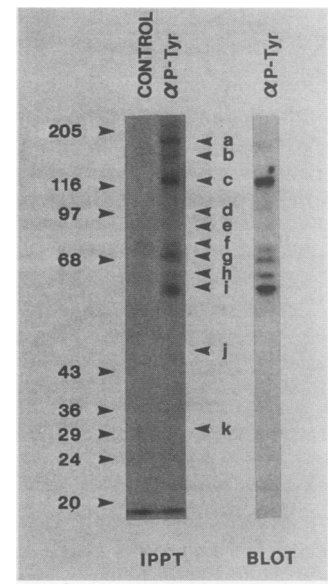

FIG. 6. Immunoprecipitation of phosphotyrosine-containing proteins. Platelets were labeled with ^{32}P, incubated with thrombin (1 U/ml) for 3 min, lysed, and immunoprecipitated (IPPT) with antiphosphotyrosine antiserum (αP-Tyr) or normal rabbit serum. Eleven candidate phosphotyrosine-containing protein bands (labeled a through k) were detected. On the right is an immunoblot of unlabeled lysates from thrombin-stimulated platelets for comparison.

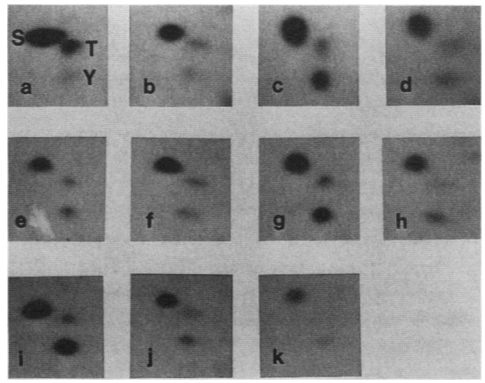

FIG. 7. Phosphoamino acid analysis of platelet protein bands immunoprecipitated with antiphosphotyrosine antiserum. The 11 bands marked a through k in Fig. 6 were excised, hydrolyzed, and subjected to phosphoamino acid analysis by two-dimensional electrophoresis. S, T, Y, Phosphoserine, phosphothreonine, and phosphotyrosine, respectively.

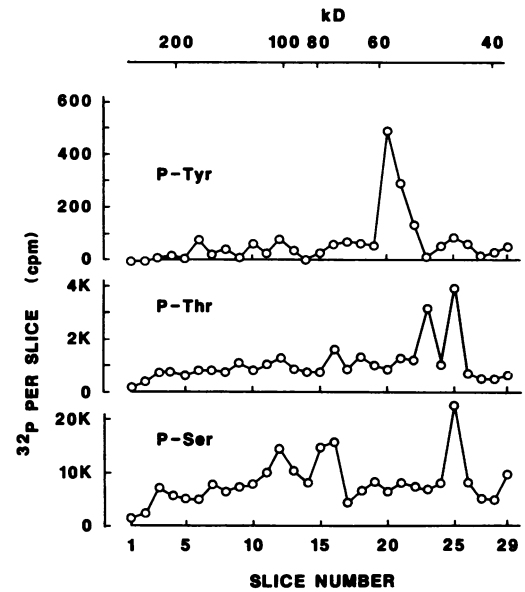

FIG. 8. Molecular mass distribution of platelet phosphotyrosine-containing proteins. Platelets were radiolabeled, stimulated with thrombin (1 U/ml) for 3 min, and subjected to SDS–6% polyacrylamide gel electrophoresis. The gel was sliced, and the phosphoamino acid composition of each slice was determined by two-dimensional electrophoresis.

7, each immunoprecipitated band was found to contain substantial amounts of phosphotyrosine, with percentages ranging from 4 to 31% of the recovered phosphoamino acids.

Nonspecific precipitation with normal rabbit serum used in place of the antiphosphotyrosine antiserum yielded several minor phosphoprotein bands, which probably contributed a background of additional phosphoserine and phosphothreonine to the specific immunoprecipitation (Fig. 6). The phosphoamino acid content of two such bands, of 130 and 29 kDa, was determined. Neither band contained detectable phosphotyrosine (not shown).

To determine whether the immunoprecipitates were enriched in phosphotyrosine relative to whole-platelet lysates, we analyzed the size distribution of phosphotyrosine-containing proteins. Platelets were radiolabeled, stimulated with thrombin for 3 min, lysed, and subjected to SDS-polyacrylamide slab gel electrophoresis followed by phosphoamino acid analysis of proteins present in individual gel slices (Fig. 8). About 40% of the phosphotyrosine was derived from a broad peak centered at 60 kDa, encompassing three prominent bands found by immunoblotting (Fig. 4). Slices within this peak contained up to 7% phosphotyrosine. This is significantly less than the 31% phosphotyrosine found at 60 kDa in the antiphosphotyrosine immunoprecipitate (Fig. 6 and 7, band i). Elsewhere in the gel, phosphotyrosine constituted less than 2% of the recovered phosphoamino acids, compared with 4 to 30% in the immunoprecipitates.

By gel slicing, no [^{32}P]phosphotyrosine was detected in proteins above 170 kDa and little was detected in those below 50 kDa (Fig. 8), in agreement with the immunoblotting results. Each gel slice that contained proteins phosphorylated at tyrosine corresponded to one or more immunoblotted protein bands. These results suggest that the antiphosphotyrosine antiserum recognized a large percentage, if not all, of the major phosphotyrosine-containing proteins in platelets.

Other agonists and antagonists. Platelets activated by incubation with ADP (50 μM, in the absence of apyrase), collagen (10 μg/ml), PAF (1 μM), arachidonate (1 or 10 μM), epinephrine (50 μM, with no added Ca^{2+}), or ionophore A23187 (100 nM) showed no increase in protein tyrosine phosphorylation, as judged by immunoblotting (Fig. 9 and data not shown). Incubation at 4°C for 60 min, a treatment which activates platelets by an unknown mechanism, caused a modest increase in the intensity of several immunoblotted bands (Fig. 9). The platelet antagonist prostaglandin E_1, which activates platelet adenylate cyclase, stimulated the phosphorylation of proteins at 27 and 22 kDa (not shown) but caused no change in tyrosine phosphorylation (Fig. 9), platelet [^{32}P]phosphotyrosine content (not shown), or pp60$^{c\text{-}src}$ phosphorylation (not shown).

In some experiments, TPA caused a small increase in the intensity of the 130- and 60-kDa bands, but this increase was not always detectable and, when present, required higher levels of TPA than those needed to effect maximal phosphorylation of the 47-kDa protein kinase C substrate (approximately 100 ng/ml versus 10 ng/ml; data not shown). DG (10 μM to 1 mM) had no effect on tyrosine phosphorylation, as judged by immunoblotting (not shown). The combination of A23187 and TPA caused a modest increase in the intensity of several immunoblotted bands (Fig. 9).

Pretreating platelets with aspirin (100 μM, 5 min), an inhibitor of thromboxane A_2 synthesis, did not affect thrombin-induced aggregation or tyrosine phosphorylation (Fig. 9 and data not shown), while under the same conditions, aspirin inhibited ADP-induced aggregation of platelet-rich plasma (not shown). Thrombin-induced tyrosine phosphorylation was similar in the presence of 1 mM Ca^{2+} and no added Ca^{2+}. The third wave of phosphorylation, but not the first or second, was largely inhibited by 10 mM EDTA, which also inhibited aggregation (not shown). The phosphotyrosine phosphatase inhibitor sodium orthovanadate (10 and 100 μM, 1- to 10-min incubations) had no effect on platelet tyrosine phosphorylation, as assessed by immunoblotting (not shown).

Response of pp60$^{c\text{-}src}$ to thrombin. ^{32}P-labeled platelets were stimulated with thrombin (1 U/ml), and samples were taken for immunoprecipitation of pp60$^{c\text{-}src}$ with monoclonal antibody GD11. The pp60$^{c\text{-}src}$ bands were excised and subjected to phosphoamino acid analysis. In resting platelets, the ratio of phosphoserine to phosphotyrosine in pp60$^{c\text{-}src}$

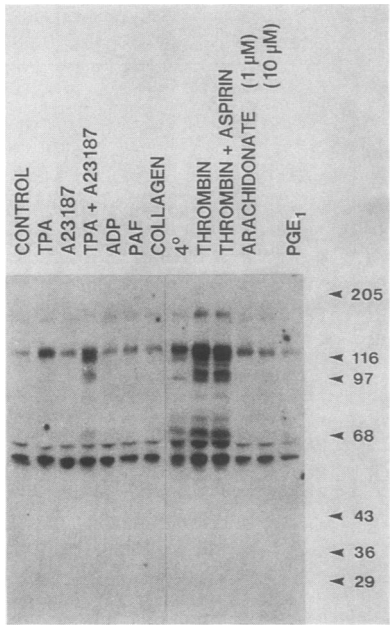

FIG. 9. Platelet tyrosine phosphorylation in response to other agents. Cells were incubated with either no additive, TPA (50 ng/ml), A23187 (100 nM), TPA plus A23187 (50 ng/ml and 100 nM, respectively), ADP (50 μM), PAF (1 μM), collagen (10 μg/ml), chilling to 4°C, thrombin (1 U/ml), aspirin (100 μM) followed by thrombin (1 U/ml), arachidonic acid (1 and 10 μM), and prostaglandin E_1 (PGE_1, 250 nM). All incubations were carried out in incubation buffer supplemented with 1 mM $CaCl_2$. Apyrase was omitted for the ADP incubation. Incubations were for 3 min; the 4°C sample was chilled for 1 h. Samples were subjected to SDS–7.5% polyacrylamide gel electrophoresis and processed for immunoblotting with antiphosphotyrosine antiserum.

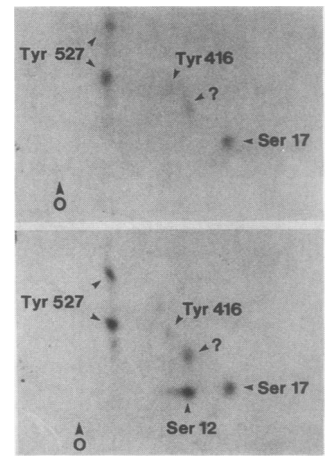

FIG. 10. Two-dimensional tryptic mapping of $pp60^{c\text{-}src}$ from resting (top) and thrombin-stimulated (bottom, 1 U/ml for 3 min) platelets. Tryptic ^{32}P-phosphopeptides were identified by comparison with published patterns (3, 10). The identity of the Tyr-416-containing phosphopeptide was confirmed by mixing experiments with in vitro-labeled $pp60^{c\text{-}src}$, which is labeled almost exclusively at Tyr-416. O, Origin.

was found to be 1.5 ± 0.1 (SD, $n = 4$). No phosphothreonine was detectable. Within 20 s of thrombin treatment, the amount of phosphoserine in $pp60^{c\text{-}src}$ roughly doubled (not shown). Phosphotyrosine levels rose by a smaller amount.

To examine these changes in greater detail, the sites of phosphorylation were analyzed by two-dimensional tryptic mapping. In resting platelets, $pp60^{c\text{-}src}$ yielded four prominent tryptic phosphopeptides. The phosphorylation sites within these peptides have been determined to be Ser-17, Tyr-527, and an unidentified N-terminal serine residue (3), as shown in Fig. 10 and Table 1. A small amount of Tyr-416 phosphorylation (about 7% of the total phosphotyrosine) was detected. No Ser-12 phosphorylation was detected.

(The phosphorylation of platelet $pp60^{c\text{-}src}$ has been analyzed previously by two-dimensional tryptic mapping [10]. In contrast to the present findings, essentially no phosphotyrosine-containing phosphopeptides were found. This discrepancy has been attributed to poor solubility of such peptides in pH 8.9 electrophoresis buffer in the previous study [K. Gould, personal communication].)

In thrombin-stimulated platelets, $pp60^{c\text{-}src}$ became prominently phosphorylated at Ser-12 (Fig. 10), presumably through activation of protein kinase C (10). Thrombin also increased the labeling of the Tyr-416 and Tyr-527 phosphopeptides.

The thrombin-induced changes in $pp60^{c\text{-}src}$ phosphorylation had no effect on its in vitro protein kinase activity, as judged by autophosphorylation, enolase phosphorylation, or IgG heavy-chain phosphorylation (not shown). Likewise, lysates from thrombin-stimulated platelets showed no enhancement in their angiotensin II kinase activity relative to control platelets (not shown).

DISCUSSION

The present work shows that in intact human platelets, an unusually high proportion of protein phosphorylation occurs at tyrosine residues. Platelet tyrosine phosphorylation is regulated by the physiological activator thrombin, which transiently increases both the rate of radiolabeling of phosphotyrosine (Fig. 3) and the absolute amount of phosphotyrosine (Fig. 4).

The data in Fig. 3 suggest that the thrombin-stimulated increase in tyrosine phosphorylation arises largely from an increase in tyrosine kinase activity rather than from a decrease in phosphotyrosine phosphatase activity. The initial rate of protein-tyrosine phosphorylation shown in Fig. 3 approximates the activity of the kinases in vivo in the absence of phosphatases. The fact that thrombin accelerates the rate of phosphotyrosine labeling 30- to 100-fold above this basal kinase activity indicates that kinases are being stimulated and that thrombin-induced phosphatase inhibition could account for only a small fraction of the observed increase in labeling. The stimulation of platelet tyrosine kinases observed in vivo was not manifested in an increase in angiotensin II kinase activity in vitro. Perhaps platelet tyrosine kinases are regulated by protein or lipid cofactors whose interaction with the kinases is disrupted in vitro.

The proteins phosphorylated at tyrosine in response to thrombin appear in three temporal waves. This suggests

TABLE 1. Phosphorylation of individual sites in $pp60^{c\text{-}src}$ isolated from resting and thrombin-stimulated platelets

Platelets	Phosphorylation (mean cpm ± SD)[a] at site:				
	Tyr-527	Tyr-416	Ser-12	Ser-17	Uncertain[b]
Resting	12 ± 1	1 ± 0	0 ± 0	6 ± 2	5 ± 1
Thrombin stimulated	26 ± 6	4 ± 1	19 ± 2	8 ± 1	6 ± 1

[a] Data represent mean ± SD for two experiments.
[b] Unidentified N-terminal serine residue (3).

either that three distinct tyrosine-specific protein kinases are stimulated by thrombin; that one kinase is stimulated in three waves; or that distinct phosphatase activities come into play at different times.

The first wave may include two thrombin-specific tyrosine phosphorylations; thus far, tyrosine phosphorylation of the 34- and 27-kDa bands has only been observed after thrombin treatment. The phosphorylations of the second wave appear not to be thrombin specific. They also occur in response to chilling, an activation treatment that was accompanied by maximal shape change but no aggregation, and in response to TPA plus A23187. The third wave of tyrosine phosphorylation appears to correlate with aggregation. It occurs in response to thrombin or TPA plus A23187, requires relatively high concentrations of thrombin (approx. 0.5 U/ml) and long incubation times (3 min), and is inhibited by 10 mM EDTA.

The identity of the kinase(s) regulated by thrombin is unknown. Although the functional thrombin receptor is as yet unidentified, one can speculate that it might be a tyrosine-specific protein kinase: thrombin is a peptide mitogen, and several peptide-mitogen receptors are protein-tyrosine kinases (e.g., the receptors for insulin, epidermal growth factor, platelet-derived growth factor, and colony-stimulating factor 1). If so, the thrombin receptor may be responsible for the rapid, thrombin-specific phosphorylations.

By virtue of its abundance, pp60$^{c\text{-}src}$ is another plausible thrombin-regulated tyrosine kinase. Thrombin causes rapid, protein kinase C-mediated phosphorylation of pp60$^{c\text{-}src}$ at Ser-12; however, this phosphorylation does not appear to be sufficient to stimulate the kinase activity of pp60$^{c\text{-}src}$ either in vitro or in vivo. As reported previously (10), phosphorylation at Ser-12 does not alter the in vitro kinase activity of pp60$^{c\text{-}src}$. Furthermore, several agents that stimulate kinase C (including DG and low doses of TPA) fail to stimulate tyrosine phosphorylation, implying that kinase C activation is not sufficient to activate pp60$^{c\text{-}src}$ or other putative tyrosine kinases in vivo.

Thrombin also increased the tyrosine phosphorylation of pp60$^{c\text{-}src}$, mainly at Tyr-527. In other systems this phosphorylation decreases the kinase activity of pp60$^{c\text{-}src}$ (12 and references therein), although we detected no such decrease in platelet pp60$^{c\text{-}src}$ in vitro. It seems likely either that pp60$^{c\text{-}src}$ is not activated in thrombin-stimulated platelets or that it is activated by some factor over and above the changes in its phosphorylation state (for example, the binding of a regulatory cofactor, as mentioned above).

The DG-kinase C system is only one of several signal transduction pathways activated by thrombin. Thrombin also elevates intracellular Ca^{2+} levels, decreases the level of cyclic AMP, and mobilizes arachidonic acid for eicosanoid synthesis. None of these changes individually appears to be sufficient to activate platelet tyrosine phosphorylation. Epinephrine, which lowers cyclic AMP levels, does not stimulate tyrosine phosphorylation, and A23187 does so only in combination with TPA. Arachidonic acid does not stimulate tyrosine phosphorylation, and thrombin-stimulated phosphorylation is not inhibited by aspirin. Thus, we suspect that one or more thrombin-stimulated tyrosine kinases, possibly including pp60$^{c\text{-}src}$, are activated by a combination of second messengers or by events "downstream" from the second messengers.

What role does tyrosine phosphorylation play in platelet physiology? Parsons and Creutz have shown that pp60$^{c\text{-}src}$ colocalizes with chromaffin granules in adrenal medullary tissue and suggested that the protein is involved in granule secretion (19). The time course we observed for thrombin-stimulated tyrosine phosphorylation in platelets is consistent with a role for pp60$^{c\text{-}src}$ (or some other tyrosine-specific protein kinase) in secretion in this cell as well. Equally attractive is the possibility that tyrosine phosphorylation is involved in aggregation; for example, in rendering platelet integrin competent to bind fibrinogen, or in communicating to the cell that fibrinogen binding has occurred. Such a scheme, if true, would provide an intriguing link between the physiology of platelet tyrosine-specific protein kinases and the pathology of viral kinases such as pp60$^{v\text{-}src}$.

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