Thrombin Stimulates the Activities of Multiple Previously Unidentified Protein Kinases in Platelets*

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We have used a renaturation method to search for previously unidentified protein kinases in human platelets. The method involves subjecting lysates to denaturing gel electrophoresis, transferring the proteins to blotting membranes, and treating the blotted proteins with guanidine. The guanidine is then removed to allow the proteins to renature, and the blots are overlaid with [γ-32P]ATP. We have identified 14 electrophoretically distinct, serine/threonine-specific protein kinases. Eleven of the kinases clearly differ in molecular weight from all previously described platelet serine/threonine kinases. Ten of these novel kinases (PK220, PK200, PK170, PK150, PK64, PK60, PK56, PK52, PK48, and PK40) were found to possess markedly increased in vitro activity when isolated from thrombin-stimulated platelets, presumably as a result of thrombin-stimulated covalent modification. Treatment of intact platelets with the calcium ionophore ionomycin and phorbol 12-myristate 13-acetate also increased the in vitro activity of these kinases. The agonist-stimulated kinases could be divided into three classes: 1) one kinase whose activity was increased by in vivo phorbol ester treatment but not by ionomycin (PK150); 2) two kinases whose activity was increased by ionomycin but not phorbol ester (PK48 and PK40); 3) seven kinases whose activity was markedly increased by combinations of phorbol ester and ionomycin, but not by either agent alone (PK220, PK200, PK170, PK64, PK60, PK56, and PK52). This third mode of regulation is what would be expected of enzymes that mediate the biological effects of inositol-mobilizing stimuli.

The platelet activating agent thrombin causes rapid breakdown of inositides, to transient elevations of intracellular calcium and intramembrane diacylglycerol (1-3). Exogenous calcium ionophores and diacylglycerol (or phorbol esters) act synergistically to activate platelets, suggesting that the two signals mediate the biological effects of thrombin and other platelet activating agents (4).

The effects of calcium and diacylglycerol are exerted, at least in part, through the activation of protein kinases. Calcium activates calmodulin-dependent myosin light chain-1 kinase (5); diacylglycerol activates protein kinase(s) C (6, 7).

Tyrosine-specific protein kinases may also be involved in platelet activation. Platelets possess high levels of pp60src (8), and platelet tyrosine phosphorylation is transiently elevated after thrombin stimulation (9, 10). Some platelet tyrosine phosphorylations occur only when extracellular matrix proteins bind to an integrin, glycoprotein IIb-IIIa, suggesting that these phosphorylations may be involved in cell-matrix recognition (11). As yet it is unclear whether pp60src is responsible for any or all of the changes in phosphorylation seen in activated platelets.

Some protein kinases may act to oppose platelet activation. Cyclic AMP-dependent protein kinase may mediate the response of platelets to prostacyclin (reviewed in Ref. 12), a prostanooid that renders platelets resistant to activating agents.

We set out to determine whether platelets possess other protein kinases and, if so, whether the kinases might be involved in signal transduction. Celenza and Carlson (13) recently demonstrated that the yeast SNF1 gene product is a protein kinase by means of an in vitro kinase assay performed on renatured, blotted proteins. We reasoned that such an assay might allow the identification of kinases whose presence in platelets was not already established. In this report, we present a modified renaturation assay and establish conditions under which it can be used to assess kinase reaction rates. We then show that at least 14 platelet proteins possess serine/threonine protein kinase activity and that the in vitro activities of many of these kinases are increased after treatment of intact platelets with thrombin, apparently by covalent modification. We also demonstrate that the thrombin-regulated kinases can be activated by treating intact platelets with ionomycin/calcium, phorbol esters, or combinations of these agents.

MATERIALS AND METHODS

Platelet Isolation—Blood was drawn from drug-free volunteers into plastic syringes containing 0.1 volume of 100 mM sodium citrate, 1.5 units/ml apyrase (Sigma, grade V), and was centrifuged at 150 × g for 15 min at room temperature. The supernatant was centrifuged again at 150 × g for 15 min to pellet any contaminating erythrocytes and leukocytes. The resulting platelet-rich plasma was acidified to pH 6.5 with 150 mM citric acid and centrifuged at 500 × g for 15 min to pellet the platelets. The platelets were washed twice with 100 volumes of 137 mM NaCl, 11 mM glucose, 1 mM CaCl2, 0.4 mM Na2HPO4, 5.6 mM glucose, 5 mM HEPES (pH 7.4), 1 unit/ml apyrase, at a

1 The abbreviations used are: HEPES, N-4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Fura 2, 1-[2-(5-carboxyoxazo1-2-y1)-6-amino-2-ninobenzofuran-5-oxy]-2-(12-amin-5'-methylphenoxy)-ethane-N,N,N'-triacetic acid; Fura 2-AM, acetoxyethyl ester of Fura 2; PMA, phorbol 12-myristate 13-acetate; PVD, polyvinylidene difluoride.
concentration of 5·10⁻¹⁰ cells/ml, and used within 2 h of isolation. All incubations were performed in capped plastic tubes at 37 °C with gentle shaking (approximately 150 rpm). In some experiments, platelets were radiolabeled by incubation with 1 M ci of [³²P]ATP for 1·5–2 h at 37 °C.

**Platelet Activating Agents**—Human thrombin (approximately 4000 units/ml) was a gift from David Phillips (COR Therapeutics, South San Francisco). Ionomycin was purchased from Calbiochem and phorbol 12-myristate 13-acetate (PMA) from Sigma. Ionomycin and PMA were added as concentrates in Me₂SO to yield a final Me₂SO concentration of 1% (v/v). Platelets treated with these agents were compared with platelets treated with Me₂SO alone.

**Electrophoresis**—Platelet samples were lysed in 0·25 volume of 5 × gel sample buffer to yield 2·3% (w/v) sodium dodecyl sulfate, 10% (w/v) glycerol, 5% (w/v) 2-mercaptoethanol, 55 mM EDTA, 62·5 mM Tris (pH 6·8), mixed vigorously, and promptly immersed in boiling water for 3 min. Lysed samples were subjected to 7·5% polyacrylamide gel electrophoresis (14). Unless otherwise indicated, approximately 50 µg of platelet protein was loaded per lane. Molecular weights were assigned by comparison with pre-stained molecular weight standards (Sigma). By calibration against unstained molecular weight standards (Sigma) and platelet proteins of known molecular weight, we assigned two of the prestained standards masses that varied from 68·0 kDa (bovine albumin) to 200 kDa (phosphorylase b).

**Alkali Treatment**—Even after extensive washing, the background radioactivity remaining on the renatured blots was fairly high, particularly when PVDF membranes were used. This background could be greatly decreased by incubating the radiolabeled, washed PVDF blots with 1 M KOH for 10 min at room temperature. The alkali-treated blots were rinsed several times with water and 10% (v/v) acetic acid and then dried and subjected to autoradiography. Alkali treatment did not markedly alter the pattern of phosphorylation (not shown). Enzymes on the guanidine-treated blots were allowed to renature in 100 mM NaCl, 50 mM Tris, 2 mM dithiothreitol, 2 mM EDTA, 1% (w/v) bovine serum albumin (ICN Radiochemicals), 0·1% (w/v) Nonidet P-40, and 0·05% (w/v) Nonidet P-40, and twice again with Tris-saline, each wash for 10 min at room temperature. Washed blots were incubated with [γ-³²P]ATP. Radioactivity remaining on the renatured blots was fairly high, particularly when PVDF membranes were used. This background could be decreased by alkali treatment and then allowed to dry at room temperature as except that albumin was omitted from the renaturation buffer. Blot lanes were cut into 4-mm strips which were immersed in 100 µl of kinase buffer containing 150 mM HEPES (pH 7·0), 10 mM MgCl₂, 1 mM dithiothreitol, 0·1% (w/v) Triton X-100, 1 µg of histone (Sigma, type HI) per 105 platelets/ml, and 50 µCi of [γ-³²P]ATP/ml. The reaction mixtures were incubated at room temperature for 20 min and then stopped by addition of 0·25 volume of 5 × gel sample buffer. Reaction products were resolved by 10% polyacrylamide gel electrophoresis. Radiolabeled co-migrating with the kinases and co-migrating with the histone bands was quantified by autoradiography and scintillation counting.

**Immunoblotting**—Affinity purified anti-actin binding protein antisera was a gift from Andrew Flint and Daniel Koshland, Jr. (Dept. of Biochemistry, University of California, Berkeley). Proteins were transferred to PVDF membranes as described above, and blots were blocked for 1 h at 37 °C in 5% (w/v) non-fat dry milk in Tris-saline (140 mM NaCl, 10 mM Tris (pH 7·5)), and incubated with antisera (1 µg/ml for anti-actin binding protein antisera; 1:1000 dilution for anti-protein kinase C antisera) in blocking buffer for 2 h at room temperature. Blots were washed twice with Tris-saline, once with Tris-saline plus 0·05% (w/v) Nonidet P-40, and then again with Tris-saline, each wash for 10 min at room temperature. Washed blots were incubated with [γ-³²P]ATP, and subjected to autoradiography.

**Intracellular Calcium Measurements**—Platelet-rich plasma was incubated with Fura 2-AM (Sigma) dissolved in Me₂SO, to yield final concentrations of 4 µM Fura 2-AM and 1% Me₂SO, as described (18, 19). Fura 2-loaded platelets were washed as described above and resuspended at a concentration of 1 × 10⁵ cells/ml. Free intracellular calcium was determined by dual wavelength fluorescence spectroscopy (18) at room temperature without stirring. Maximal fluorescence ratios were determined from cells permeabilized with digitonin in the presence of 1 mM Ca²⁺. Calcium was then chelated with buffered 5 mM EDTA to determine minimum fluorescence ratios.

## RESULTS

**Kinase Activity of Proteins Renatured after Blotting**—Platelet samples were lysed and subjected to denaturing gel electrophoresis. Proteins were transferred to blotting membranes, treated with guanidine, allowed to renature, and assayed for kinase activity by incubation with [γ-³²P]ATP. Radiolabel was detected in at least 14 electrophoretically distinct peptides (Fig. 1), which comprised a major band at 170 kDa, plus two minor species migrating just above it at 200 and 220 kDa; two minor bands at 150 and 116 kDa; three prominent, poorly resolved bands at 82, 86, and 94 kDa; and six closely spaced bands at 66, 60, 56, 52, 48, and 40 kDa. Several other radiolabeled bands could be detected after longer exposure of the autoradiograms (at 240, 70, 68, 46, 39, 36, and 32 kDa, not shown).

Each of the radiolabeled bands represents ³²P covalently bound to protein; each band yield ³²P labeled amino acids upon partial acid hydrolysis (Fig. 2). The most straightforward interpretation is that each band corresponds to an electrophoretically distinct protein kinase. We have designated these putative kinases PK220, PK200, and so on, according to their apparent molecular weights. Roughly equal proportions of ³²P, [³²P]phosphoamino acids, and [³²P]phosphopeptides were recovered in partial acid hydrolysates; equal proportions of the three classes of products are also recovered in partial acid hydrolysates of in vivo labeled phosphoproteins (not shown). The residues phosphorylated by all 14 kinases were predominantly serines and threonines (Fig. 2). Traces of [³²P]phos-
were subjected to gel electrophoresis and blotting. Blotted proteins were allowed to renature and were incubated with \([\gamma^{-32}P]ATP\) for various lengths of time as indicated.

In this linear time regime, it should be possible to measure initial kinase reaction rates even if renaturable phosphatases are present, they are not able to dephosphorylate the sites phosphorylated in vitro on this time scale and are not likely to interfere with assessment of kinase activities.

\[ {^{32}P} \] incorporation increased linearly with increasing amounts of platelet protein over a range of 10–100 \(\mu\)g (Fig. 1). This linearity suggests that most of the \([^{32}P]\) incorporation arises through intramolecular autophosphorylation, or through phosphorylation of the blocking agent.

Changes in in vitro Activity following Thrombin Treatment—Platelets were stimulated with human thrombin (1 unit/ml). Aliquots were taken at various times, and initial reaction rates of the renaturable protein kinases were assessed. As shown in Fig. 3, thrombin markedly increased the activities of ten kinases, with masses of 220, 200, 170, 150, 64, 60, 56, 52, 48, and 40 kDa. The most dramatic increases were found in PK60 and PK56, ranging from 5- to 20-fold in four experiments. The other thrombin-stimulated kinases typically increased 2–4-fold in activity. Some of the increases were transient (e.g. PK64, PK48); others were more sustained (PK170, PK60). Qualitatively similar increases were seen when higher concentrations of ATP were used in the kinase reaction (10 \(\mu\)M, 50 \(\mu\)M/ml).

It was difficult to assess changes in the activity of the three kinases clustered between 80 and 100 kDa, in part because they were not well resolved from each other and in part because there was a good deal of variability in their responses to thrombin. However, PK82 usually showed a decrease in its activity. (These changes are not apparent in Fig. 3 but are in Fig. 4.) PK68 and PK94 sometimes showed modest changes in activity following thrombin stimulation, but these findings were inconsistent (Figs. 3 and 4).

Regulation by Calcium and Phorbol Esters—Intact platelets were treated with the calcium ionophore ionomycin in the presence of 1 mM Ca\(^{2+}\), or with the phorbol ester PMA, or with combinations of the two agents, and the activities of the renaturable kinases were assessed. Fig. 4a shows the response of these kinases to various doses of ionomycin in the absence or presence of enough PMA to maximally activate protein kinase C (50 ng/ml). Fig. 4b shows responses to various doses of PMA in the absence and presence of 100 nM ionomycin.

\[ \text{Time and Concentration Dependence of } {^{32}P} \text{ Labeling} \]

Renatured, blotted proteins were overlaid with \([\gamma^{-32}P]ATP\) for various lengths of time, and the time dependence of \([^{32}P]\) labeling was determined. As shown in Fig. 1, \([^{32}P]\) incorporation into each band increased linearly with time through 30 min. In this linear time regime, it should be possible to measure initial kinase reaction rates even if renaturable phosphatases were to co-migrate with the kinases.

Possible interference from renaturable phosphatases was investigated further with a pulse-chase experiment. Renatured, blotted proteins were overlaid with \([\gamma^{-32}P]ATP\) for 10 min, after which the label was chased with 1 mM nonlabeled ATP. Reactions were terminated by dousing the blots in 1 M KOH for 10 min. No decrease in labeling was apparent through 60 min of chase (not shown). Thus, if any co-migrating, renaturable phosphatases are present, they are not able to dephosphorylate the sites phosphorylated in vitro on this time scale and are not likely to interfere with assessment of kinase activities.

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did not by itself elevate intracellular Ca\(^{2+}\), and did not alter the levels of intracellular Ca\(^{2+}\) effected by ionomycin (not shown).

One minor kinase band, PK150, was activated by PMA in an ionomycin-independent fashion (Fig. 4b). PK150 activation was half-maximally effected by roughly 5 ng/ml PMA (8 nM).

A number of other kinases were dramatically activated by the two agents together, but not by either individually (Fig. 4). These kinases included four major bands, PK170, PK60, PK56, and PK52, and two minor bands, PK220 and PK200. PK64 was also stimulated by combinations of PMA and ionomycin (not shown), although the effects were transient and were not always apparent (Fig. 4 versus Fig. 8). Relatively modest doses of ionomycin (roughly 100 nM) caused maximal activation of these kinases in the presence of PMA, and the lowest doses tested (10 nM) caused significant activation (Fig. 4a). In the presence of 100 nM ionomycin, 5 ng/ml PMA (8 nM) caused half-maximal activation of these kinases (Fig. 4b).

PK82 generally decreased in activity in response to PMA, and PK94 and PK86 showed variable modest changes in activity in response to combinations of PMA and ionomycin (Fig. 4).

Effects of Activating Agents on Protein Phosphorylation in Vivo—To compare the effects of these activating agents on kinase activity in vitro with their effects on protein phosphorylation in intact platelets, platelets were radiolabeled with \(^32\)P, treated with various doses of the ionomycin and PMA, or with thrombin, and analyzed by gel electrophoresis and autoradiography (Fig. 5). The protein kinase C-mediated phosphorylation of pleckstrin was half-maximally stimulated by 5 ng/ml PMA (Fig. 5b) and was very slightly elevated by 500 nM ionomycin in the absence of PMA (Fig. 5a). A variety of other unidentified \(^32\)P-phosphoprotein bands increased in intensity in response to PMA as well (Fig. 5b). The concentration of PMA required to stimulate the phosphorylation of pleckstrin (as well as the phosphorylation of the other unidentified bands) was comparable to the concentration required to activate PK150 (as well as the phosphorylation of the other unidentified bands) was comparable to the concentration required to activate PK150 (Fig. 4b), and to that required to activate PK220, PK200, PK170, PK64, PK50, PK56, PK52 in the presence of ionomycin (Fig. 4b).

Phosphorylation of myosin light chain-1, which is carried out by both protein kinase C and myosin light chain kinase (4, 22, 23), was slightly elevated in ionomycin-treated cells, although only at ionomycin concentrations which led to a slight increase in pleckstrin phosphorylation as well (Fig. 5a). Myosin light chain-1 phosphorylation was more greatly elevated in cells treated with PMA and greatest in cells treated with both agents (Fig. 5, a and b).

No other phosphoprotein bands appeared to be more heavily phosphorylated in response to PMA and ionomycin than they were in response to maximal concentrations of PMA alone. Similarly, no bands were more heavily phosphorylated in response to thrombin than they were to PMA. This finding suggests that the targets of the synergistically activated protein kinases are relatively scarce proteins.

Electrophoretic Separation of the Species Phosphorylated in Vitro—Kinetic evidence (see above) suggests that most of the phosphorylation represents either intramolecular phosphorylation or phosphorylation of proteins absorbed onto the blot from the albumin blocking solution. The second possibility is of particular interest, because it could provide a way to monitor the activities of the kinases toward exogenous substrates. This possibility was explored by incubating renatured blotted proteins with \([\gamma\text{-}32\text{P}])\text{ATP, eluting the species present in the regions of each prominent radiolabeled band, and}
subjecting the eluted species to gel electrophoresis and autoradiography. As shown in Fig. 6, the majority (70–90%) of the radiolabel present in each of the eluted bands co-migrated with the kinase activity. Presumably a large proportion of this represents autophosphorylation of the kinases. Each eluted band also contained a series of radiolabeled proteins that migrated between 48 and 70 kDa. One of these bands (band 1 in Fig. 6) co-migrated with the prominent Coomassie Blue-stained albumin band. The others corresponded to minor Coomassie Blue-stained protein bands, of uncertain identity, that were present in the blocking solution.

Platelet agonists were found to cause changes in the rates of both autophosphorylation and exogenous substrate phosphorylation. As shown in Fig. 6, the activity of PK220 toward albumin (denoted protein 1) increased after treatment with TPA plus ionomycin. Likewise, the activity of PK170 toward several substrates (proteins 1–5) increased. In a portion of the blot containing PK86 and PK82, the kinase activity toward several exogenous substrates decreased. PK60 activity toward substrates 1 and 5 increased after TPA/ionomycin stimulation, and PK52 and PK48 increased in activity toward substrates 1 and 2. Overall, the activity of the protein kinases towards exogenous substrates correlated well with their total in vitro activity. Similar results were found in experiments comparing control platelets with thrombin-stimulated platelets (not shown).

Histone Phosphorylation—The ability of the kinases to phosphorylate albumin suggested that they might be able to phosphorylate other exogenous proteins as well. To investigate this possibility, we subjected blotted platelet proteins to renaturation as described above, except that albumin was omitted from the renaturation buffer and the blocking step was omitted. The blot was cut into slices, and the phosphorylation of histone by proteins on the slices was assessed. Histone kinase activity was largely confined to two regions: the region around PK170, and region extending from about 40 to 48 kDa (Fig. 7). The 170-kDa histone kinase activity increased about 2-fold after TPA/ionomycin stimulation, whereas the 40–48-kDa activity was unchanged.
alone proteins were allowed to renature in the absence of albumin. Slices by of each lane were incubated with \([\gamma-^{32}P]ATP\) and histone, and the counting. and apparent autophosphorylation were assessed by scintillation counting.

Calpain Activation—Strong platelet activating agents can raise intracellular calcium levels sufficiently to activate calpain I (20). Calpain activation could generate active protein kinases from less active precursor proteins. We therefore assessed calpain activation in thrombin-, PMA-, ionomycin-, and PMA plus ionomycin-treated platelets, by immunoblotting with antiserum against actin-binding protein (20). Actin-binding protein is a 260-kDa protein which is hydrolyzed by calpain in vitro and in vivo to yield fragments of 200, 100, and 91 kDa (20). As shown in Fig. 8, the antiserum recognized a prominent 260-kDa protein in lysates from control platelets. Traces of a 200-kDa immunoreactive band could be seen on heavily exposed autoradiograms. Various agonists caused marked changes in the activity of renaturable kinases without causing an increase in the intensity of the minor 200-kDa band or appearance of lower molecular weight bands. Similar results were obtained by immunoblotting with antiserum against another calpain substrate, spectrin (not shown). Calpain can be activated by lysing platelets with 1% (w/v) Triton X-100 in the presence of calcium (24). Triton X-100 treatment caused the disappearance of the 260-kDa actin binding protein band and the appearance of prominent 200- and 100-kDa immunoreactive proteins, as reported previously (20). Concomitantly, Triton X-100 treatment decreased the activity of most of the renaturable kinases (Fig. 8). In similar experiments, an 82-kDa band recognized by anti-protein kinase C antiserum was found not to be proteolyzed in response to thrombin, PMA, ionomycin, or Triton X-100 (not shown). Thus, calpain activation is neither necessary nor sufficient for activation of the renaturable kinases.

DISCUSSION

When denatured platelet proteins are blotted, treated with guanidine, allowed to renature, and overlaid with \([\gamma-^{32}P]ATP\), radiolabel is incorporated into various regions of the blot. The radiolabel represents phosphoryl residues transferred from the \(\gamma\)-position of ATP to seryl and threonyl residues. The proteins responsible for this transfer are therefore protein kinases or are catalytic subunits of oligomeric protein kinase holoenzymes. We cannot formally exclude the possibility that the denaturation-renaturation procedure allows enzymes which do not normally function as protein kinases to acquire this function but we consider this unlikely. For example, whereas 100 ng of protein kinase C was readily detectable as a renatured kinase, no renaturable kinase activity was found in 20-µg samples of rabbit muscle creatine kinase, fructose-6-phosphate kinase, myokinase, or pyruvate kinase (not shown).

Platelets apparently contain a large number of serine/threonine-specific protein kinases. Fourteen electrophoretically distinct species are routinely detected after renaturation, and several others are detected on more heavily exposed autoradiograms. Three of the renaturable kinases, PK94, PK86, and PK82, have masses that are similar to those of two known platelet protein kinases, protein kinase C (80 kDa (25)) and myosin light chain-1 kinase (90-105 kDa (4, 26)). Thus at least 11 of the kinases appear not have been previously identified in platelets. It is not known if any of these novel platelet kinases are related to known kinases from other tissues, or if any are related to each other.

Some kinases go undetected in the renaturation assay, either because they fail to renature or because they are incapable of phosphorylating the species to which they have access.
New Thrombin-stimulated Protein Kinases

on blots. For example, only traces of tyrosine kinase activity were evident after renaturation, even though at least one tyrosine kinase, pp60

It appears that the predominant phosphorylation reaction carried out by the renatured kinases is intramolecular auto-

In most cases (10 out of 14) the renaturable platelet kinases exhibit increased initial reaction rates as a consequence of in vivo changes. The most direct explanation is that the kinases are covalently modified by agonist treatment such that their catalytic activities are increased. Alternative hypotheses seem less likely. One such hypothesis is that platelet activation leads to an increased availability of substrates to the kinases in vitro. For example, thrombin and other agonists could cause dephosphorylation of the kinases at their putative autophos-

The observed changes in kinase activity presumably arise through covalent modification. One possible activating modi-

We suspect that these changes in specific activity are brought about by changes in phosphorylation. The kinases could be stimulated by phosphorylation, by either autophos-

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