Substrate affinity of the protein tyrosine kinase pp60^{c-src} is increased on thrombin stimulation of human platelets

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Human blood platelets contain high levels of non-receptor protein tyrosine kinases of the Src family, particularly pp60^{c-src}, suggesting an important role for these enzymes in platelet physiology. Indeed, in response to various agonists of platelet function, a number of proteins become phosphorylated at tyrosine residues. However, no enzymic activation of an Src-related tyrosine kinase has yet been shown in platelets. In searching for the kinase(s) responsible, we found that all agonists tested that directly or indirectly activate protein kinase C in platelets (phorbol 12-myristate, 13-acetate, thrombin, vasopressin, collagen, calcium ionophore A23187) increased the overall activity of pp60^{c-src} determined by IgG phosphorylation in an immunocomplex assay in the presence of low ATP concentrations. On the other hand, elevation of cyclic AMP directly by forskolin or indirectly by prostaglandin E₁, or elevation of cyclic GMP by sodium nitroprusside did not significantly affect the activity of the enzyme. To substantiate the

differences in enzyme activity, we determined K_m and V_{max} values of pp60^{c-src} from resting and thrombin-stimulated platelets. Thrombin treatment increased substrate affinity of pp60^{c-src} as indicated by a 2- to 3-fold decrease in the K_m values for ATP and the exogenous protein substrate casein. V_{max} values were only slightly altered under the assay conditions used. To further rule out modifications of pp60^{c-src} in phosphorylation as a probable cause of the changed substrate affinity, we analysed tryptic phosphopeptides of immunoprecipitated, ³²P-labelled pp60^{c-src} of unstimulated and stimulated platelets. The platelet agonists listed above induced an increase in pp60^{c-src} phosphorylation at Ser-12, which is the amino acid phosphorylated by protein kinase C. Surprisingly, we found that elevation of cyclic AMP did not affect ³²P labelling of pp60^{c-src}. On the basis of our data, we suggest that phosphorylation at Ser-12 might be one of the signal-triggering events that cause the increase in substrate affinity of pp60^{c-src}.

INTRODUCTION

Blood platelets constitute a useful system for investigating mechanisms that couple extracellular stimuli to intracellular signal-transduction events. In response to various agonists, platelets undergo adhesion, shape change, secretion and aggregation accompanied by a dramatic increase in phosphorylation of numerous proteins at serine, threonine and tyrosine residues (for reviews, see refs. [1]–[3]). Platelets contain high levels of non-receptor protein tyrosine kinases, of which the most abundant is pp60^{c-src} [2,4], suggesting that it has an important role in platelet physiology. Other members of the Src family tyrosine kinases, i.e. p59^{fyn}, p62^{c-yes}, p59^{hck}, p54/58^{lyn}, are also expressed [2,4–6]. On the other hand, high phosphotyrosine phosphatase activity was found in human platelets [7–9], which may likewise influence the stimulation-induced protein tyrosine phosphorylation. However, the kinases and phosphatases responsible and their specific roles in platelet activation have not been defined.

As it has been shown that the stimulated platelet-derived growth factor (PDGF) receptor forms complexes with proteins involved in signal transduction [10], complex-formation of non-receptor tyrosine kinases with cellular signalling and cytoskeletal elements following platelet stimulation may be considered indirect evidence for kinase activation. Such complexes may exist in platelets. On thrombin stimulation of platelets, pp60^{c-src} and p60^{fyn} associate with phosphatidylinositol 3-kinase [11,12]. In addition, interaction of pp60^{c-src}, phospholipase C, inositol lipid kinase and diacylglycerol kinase with the cytoskeleton has been

found [13,14]. More recently, an association of p59^{fyn}, p54/58^{lyn} and p62^{c-yes} with the p21^{ras} GTPase-activating protein has been reported [15]. Translocation of pp60^{c-src} has also been reported in rabbit platelets stimulated by platelet-activating factor [16]. In resting platelets, p59^{fyn}, p62^{c-yes} and p54/58^{lyn} are physically associated with the transmembrane thrombospondin receptor CD36 (glycoprotein IV) [4], suggesting a possible role for signal-transduction events during platelet activation.

In searching for the tyrosine kinase(s) responsible for the enhanced net protein tyrosine phosphorylation, several laboratories found no enzymic activation of pp60^{c-src} on thrombin stimulation [6,11,17,18]. We report here that pp60^{c-src} is modulated on agonist-induced platelet stimulation with respect to its substrate affinity, and becomes phosphorylated at Ser-12 as a result of activation of the Ca²⁺, phospholipid-dependent protein kinase (PKC).

MATERIALS AND METHODS

Materials

Thrombin, phorbol 12-myristate, 13-acetate (PMA), [Arg⁸]vasopressin, calcium ionophore A23187, sodium nitroprusside, prostaglandin E₁ (PGE₁), forskolin, leupeptin, antipain, pepstatin A, trypsin treated with Tos-Phe-CH₂Cl (TPCK), rabbit anti-(mouse IgG) serum, Protein A–Sepharose and monoclonal anti-phosphotyrosine antibodies were purchased from Sigma (München, Germany). 6-Aminohexanoic acid was

Abbreviations used: A23187, calcium ionophore A23187; PKC, Ca²⁺, phospholipid-dependent protein kinase; PMA, phorbol 12-myristate, 13-acetate; TBR serum, serum from tumour-bearing rabbits immunoprecipitating pp60^{c-src}; PDGF, platelet-derived growth factor; PGE₁, prostaglandin E₁; mAb, monoclonal antibody; ECL, enhanced chemiluminescence; ACD, acid-citrate-dextrose; RIPA buffer, radioimmune precipitation buffer.

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from Merck (Darmstadt, Germany). The monoclonal antibody that recognizes pp60^{c-src} (MAb 327) and the peroxidase-conjugated sheep anti-(mouse IgG) antibody were from Dianova (Hamburg, Germany). Polyclonal antisera against pp60^{c-src} (TBR sera) were produced as originally described [19]. About 30 % of the TBR sera produced in our laboratory has a remarkable titre in immunoprecipitating the cellular counterpart of the v-src gene product, pp60^{c-src} [20]. Aprotinin was a kind gift from Bayer AG (Leverkusen, Germany). Collagen was purchased from Nycomed (München, Germany). *Staphylococcus aureus* V₈ proteinase was from Boehringer (Mannheim, Germany). Casein was dephosphorylated by the method of Ashby and Walsh [21]. Nitrocellulose was from Schleicher and Schuell (Dassel, Germany). The ECL (enhanced chemiluminescence) Western-blotting detection kit, sheep ¹²⁵I-labelled anti-(mouse IgG) antibody (750–3000 Ci/mmol, 28–111 TBq/mmol), [γ -³²P]ATP (3000 Ci/mmol, 111 TBq/mmol) and [³²P]orthophosphate (carrier-free, 20 mCi/ml) were obtained from Amersham Buchler (Braunschweig, Germany).

Isolation and stimulation of platelets

Human blood was drawn from healthy adult volunteers who had not taken drugs that affect platelet function for 2 weeks before donation. Acid-citrate-dextrose (ACD) was used as an anti-coagulant (6 vol. of venous blood to 1 vol. of ACD). Platelet-rich plasma was obtained by centrifuging the ACD blood at 200 g for 15 min. This was then supplemented with PGE₁ to a final concentration of 100 nM and centrifuged at 400 g for 20 min. The platelet pellets were washed twice at 300 g for 90 s with incubation buffer (137 mM NaCl, 2.7 mM KCl, 5.5 mM glucose, 1 mM MgCl₂, 5.5 mM Hepes, pH 7.4) and suspended at a concentration of 8×10^8 – 10×10^8 cells/ml. Samples (80 μ l) were incubated with biologically active agents at 37 °C in a final volume of 100 μ l. The particular concentrations of the substances used and the incubation times are reported in the text and Figure legends.

Kinase assay and isolation of ³²P-labelled pp60^{c-src}

Stimulated platelets were lysed for 20 min on ice with 100 μ l of lysis buffer [20 mM 6-aminohexanoic acid, 8 mM EDTA, 2 mM phenylmethanesulphonyl fluoride, 80 mM NaF, 400 μ M Na₃VO₄, 40 mM benzamidin, 20 μ M leupeptin, 0.2 mM antipain, 30 μ M aprotinin, 2 % (v/v) Triton X-100, 20 mM Tris/HCl, pH 7.4]. The lysates were centrifuged at 50 000 g for 15 min. The clarified supernatants (50 μ l) were incubated at 4 °C for 2 h with either 5 μ l of MAb 327 (1 mg/ml) and 10 μ l of anti-(mouse IgG) serum or 5 μ l of the undiluted TBR serum together with swollen Protein A-Sepharose (3 mg dry weight per sample). After extensive washing to remove unbound material, the phosphotransferase reaction was started by addition of 20 μ l of a kinase reaction mixture that contained 10 mM MgCl₂, 10 mM MnCl₂ and various concentrations of [γ -³²P]ATP ranging from 20 nM to 80 μ M (3000 Ci/mmol to 1.5 Ci/mmol) as described previously [22]. For kinetic analysis of casein phosphorylation, 0.02–4 mg/ml dephosphorylated casein was included in the reaction mixture. After 20 min at 30 °C, the reactions were stopped by addition of SDS sample buffer and the proteins were analysed by SDS/PAGE. Phosphoproteins were detected by subsequent autoradiography. The IgG band and casein were cut out from the dried gel and radioactivity was determined by Cerenkov counting. Kinase activity was calculated as pmol of phosphate incorporated into IgG or casein/min per mg of extract.

For isolation of ³²P-labelled pp60^{c-src}, platelets were labelled

with [³²P]orthophosphate (2 mCi/ml) in incubation buffer for 60 min at 37 °C. Platelets were treated as described above, and the clarified supernatant (60 μ l) was used to isolate ³²P-labelled pp60^{c-src} as described recently [23].

Protein determination

Protein was determined by the Coomassie Blue dye-binding assay of Bradford [24].

Immunoblot procedures

For detection of pp60^{c-src} with MAb 327, isolated platelets were stimulated with thrombin (1 unit/ml) for 30 s, lysed and centrifuged as described above. To 100 μ l of the clarified supernatant, 30 μ l of SDS sample buffer was added. The pellet was resuspended in 50 μ l of SDS sample buffer. For immunoblot analysis of phosphotyrosine-containing proteins, SDS sample buffer was added immediately to the platelets after stimulation. Proteins were separated on SDS/polyacrylamide gels (11 % polyacrylamide), electrotransferred to nitrocellulose (0.8 mA/cm², 1.5 h) and processed with the ECL Western-blotting system to detect pp60^{c-src} according to the manufacturer's protocol. Proteins were probed with MAb 327 at a final dilution of 1 μ g/ml in Tris-buffered saline, pH 7.5, (10 mM Tris/HCl, 137 mM NaCl, 0.01 % sodium azide) supplemented with 0.1 % (v/v) Tween, and then incubated with sheep anti-(mouse IgG) antibody conjugated to horseradish peroxidase. Blots were exposed to Kodak X-Omat AR film for a period of 30 s to 3 min. Detection of phosphotyrosine-containing proteins was performed by a modified protocol described by Kamps and Sefton [25] using 1 % BSA instead of 5 %. The anti-phosphotyrosine antibody and the ¹²⁵I-labelled anti-(mouse IgG) antibody were used at a final concentration of 1 μ g/ml and 1 μ Ci/ml respectively. The radioactive bands were visualized by autoradiography for 1–3 days on Kodak X-Omat AR film.

One-dimensional partial-peptide mapping

Partial proteolysis of isolated pp60^{c-src} was performed by the method of Cleveland et al. [26]. The immunoprecipitated ³²P-labelled 60 kDa bands were cut out from SDS/polyacrylamide gels and digested with 100 or 500 ng of V₈ proteinase from *Staphylococcus aureus* per sample for 45 min. The resulting phosphopeptides were separated by SDS/PAGE on 15 % gels and visualized by autoradiography using an intensifying screen for 5 days at –70 °C.

Two-dimensional tryptic phosphopeptide maps

After autoradiography, the immunoprecipitated ³²P-labelled 60 kDa bands were excised from dried SDS/polyacrylamide gels, digested with trypsin [27], and the resulting phosphopeptides separated in two dimensions on cellulose plates as described recently [23]. Electrophoresis at pH 1.9 was first performed in the horizontal dimension followed by ascending chromatography. Plates were exposed to Kodak X-Omat AR films for 5–10 days with intensifying screens at –70 °C.

Phosphoamino acid analysis

One-dimensional separation and identification of ³²P-labelled amino acids were performed as described previously [23].

Calculation of kinetic constants

To calculate K_m and V_{max} of the phosphotransferase reactions,

the data of the non-saturated curves were fitted by the non-linear least-squares method using the equation:

$$V_o = V_{con} \cdot [S] + \frac{V_{max} \cdot [S]}{K_m + [S]}$$

where V_{con} (constant) represents the non-saturable 'conversion rate' [28].

The experimental basis for the use of this equation was the finding that control phosphotransferase assays performed in the absence of enzyme (without platelet extracts) resulted in the ³²P content of the casein bands being higher than the background radioactivity measured in areas of the gels above or below casein. Calculation of this unspecific phosphorylation resulted in a linear correlation between ATP concentration and 'enzyme activity' (not shown), which may simply be due to the aforementioned higher-than-background phosphorylation or to non-enzymic tyrosine phosphorylation [29] that might occur during heating of the samples for 5 min at 96 °C in sample buffer.

RESULTS

Within 30 s of stimulation with 1 unit/ml thrombin, we found an increased tyrosine phosphorylation of platelet proteins, as previously described by several laboratories [17,18,30–32]. Proteins with molecular masses of 180, 140, 120, 100, 85, 80, 67, 60, 56 and 33 kDa on SDS/polyacrylamide gels have been identified by immunoblot analysis using a monoclonal anti-phosphotyrosine antibody (Figure 1). Discrepancies among various reports may be due to isolation procedures of platelets or different antibodies used.

To investigate the kinase(s) responsible for tyrosine phosphorylation, we focused our investigations on pp60^{c-src}, as it is the major and best-characterized member of the Src family in human platelets. We studied the influence of various biologically active agents on enzyme activity of antibody (TBR IgG)-immobilized pp60^{c-src} in an *in vitro* standard kinase immunocomplex assay system (see references in ref. [33]). As can be seen in Table 1, the agonists of platelet function tested

Figure 1 Time-dependence of thrombin-induced protein tyrosine phosphorylation in intact platelets

Isolated platelets were incubated for the indicated times with 1 unit/ml thrombin. After lysis, proteins were analysed by immunoblotting with an anti-phosphotyrosine antibody as described in the Materials and methods section. The positions of molecular-mass markers are shown on the left. Arrows on the right indicate the positions of proteins that undergo tyrosine phosphorylation in response to thrombin.

Table 1 pp60^{c-src} tyrosine kinase activity of human platelets stimulated by various biologically active agents

Isolated platelets were incubated for 30 s at 37 °C with various agents. The reactions were terminated with an equal volume of ice-cold lysis buffer. The clarified 50 000 g supernatants were used for further analysis. Kinase activity was determined by measuring TBR IgG phosphorylation as described in the Materials and methods section using 20 nM ATP. The percentage values are determined relative to untreated controls (= 100%). The data are expressed as mean values ± S.D. for the number of experiments given in parentheses.

Agents	pp60 ^{c-src} activity (%)
Buffer (control)	100
Thrombin (1 unit/ml)	199 ± 37 (8)
PMA (100 ng/ml)	207 ± 23 (6)
A23187 (150 nM)	160 ± 10 (3)
Collagen (40 µg/ml)	170 ± 19 (4)
[Arg ⁸]vasopressin (1 µM)	160 (2)
PGE ₁ (250 nM)	80 ± 10 (3)

Figure 2 Distribution of pp60^{c-src} in supernatants and pellets of lysed, resting and thrombin-stimulated platelets

Isolated platelets were incubated for 30 s with thrombin (1 unit/ml) or buffer for control, lysed and centrifuged as described in the Materials and methods section. Supernatants and remaining pellets were analysed for pp60^{c-src} with MAb 327 by Western blotting using the ECL blotting system. The resulting autoradiogram is presented. Lanes 1 and 2, supernatants; lanes 3 and 4, pellets; lanes 1 and 3, controls; lanes 2 and 4, thrombin-stimulated; the arrow indicates the 60 kDa position.

Table 2 Concentration-dependent effect of thrombin on pp60^{c-src} tyrosine kinase in human platelets

Isolated platelets were stimulated for 30 s with increasing concentrations of thrombin. Termination of the reactions and determination of kinase activity were as described in Table 1. All percentage values of kinase activity are determined relative to untreated controls (= 100%). Data are expressed as mean values ± S.D. for the number of experiments given in parentheses.

Thrombin concentration (units/ml)	pp60 ^{c-src} activity (%)
0.0	100
0.01	138 ± 20 (4)
0.1	137 ± 9 (3)
0.2	160 (2)
0.5	165 ± 40 (4)
1	199 ± 37 (8)
5	235 ± 39 (3)
10	294 ± 50 (7)

Figure 3 Determination of K_m and V_{max} of pp60^{c-src} from resting and thrombin-stimulated platelets

Isolated platelets were stimulated for 30 s with thrombin (1 unit/ml) and lysed, and the kinase activity of TBR-IgG-immobilized pp60^{c-src} was determined by casein phosphorylation as described in the Materials and methods section. (a) Dependence on ATP concentration. The ATP concentration was adjusted by adding increasing amounts of unlabelled ATP containing 1 μ Ci of [γ -³²P]ATP. The final casein concentration was 2.0 mg/ml. ●, Resting platelets; ▲, thrombin-stimulated platelets. (b) The curve of pp60^{c-src} activity of resting platelets determined from (a). ●, is separated graphically into the non-saturable linear portion and the saturable portion calculated by the equation given in the Materials and methods section. (c) The calculated saturation curves for ATP shown as double-reciprocal plots. (d) Dependence on casein concentration. Various amounts of casein were present and a final ATP concentration of 20 μ M for the phosphotransferase reaction. The calculated saturation curves analogous to (b) are shown as double-reciprocal plots.

(vasopressin, collagen, the phorbol ester PMA, the calcium ionophore A23178 and thrombin) induced an overall increase in TBR IgG phosphorylation between 1.6- and 2.0-fold compared with resting platelets, indicating stimulation-dependent stable alterations in the enzyme. Serine- or threonine-specific kinases did not participate in the increased TBR IgG phosphorylation, as tyrosine residues were identified by phosphoamino acid analysis to be exclusively phosphorylated (results not shown). The antagonist of platelet function, PGE₁, did not increase TBR IgG phosphorylation. Identical results were obtained with a monoclonal anti-pp60^{c-src} antibody (MAb 327) (results not shown).

As it is known that pp60^{c-src} is translocated from the plasma membrane to the platelet cytoskeleton on thrombin treatment [13,14], we had to prove that differences in enzyme activity were

not simply due to different amounts of pp60^{c-src} in the extracts used. Therefore we analysed the clarified extracts of resting and thrombin-stimulated platelets for pp60^{c-src} by immunoblotting. Under the two conditions, equal amounts of pp60^{c-src} protein were present in the supernatants (Figure 2), indicating that the cytoskeletal association of pp60^{c-src} must be disrupted under our lysis conditions, which differ from those used for preparing cytoskeletal fractions [13,14]. In the pellets, only very small amounts of pp60^{c-src} were detectable.

The time- and concentration-dependence of thrombin stimulation was studied in more detail. Overall kinase activity maximally increased about 2-fold within 10 s after thrombin treatment (1 unit/ml), corresponding to a rapid increase in protein tyrosine phosphorylation in intact platelets. Although an additional increase in tyrosine phosphorylation of a variety of

Figure 4 Isolation and partial-peptide mapping of ^{32}P -labelled pp60^{c-src} from resting and thrombin-stimulated platelets

(a) Isolation of ^{32}P -labelled pp60^{c-src}. pp60^{c-src} was isolated from the supernatants of ^{32}P -labelled platelets as described in the Materials and methods section with either TBR serum (lanes 1 and 2) or MAb 327 (lanes 3 and 4) of unstimulated (lanes 1 and 3) and thrombin-stimulated platelets (lanes 2 and 4). (b) One-dimensional partial-phosphopeptide analysis of ^{32}P -labelled pp60^{c-src}. The ^{32}P -labelled 60 kDa bands isolated with TBR sera were excised and rerun on a 15% polyacrylamide gel after digestion with 100 ng (lanes 1 and 4) or 500 ng (lanes 2 and 3) *Staphylococcus aureus* V₈ proteinase as described in the Materials and methods section. ^{32}P -labelled pp60^{c-src} of unstimulated (lanes 1 and 2) and 1 unit/ml thrombin-stimulated (lanes 3 and 4) platelets are shown. V1–V4 represent the characteristic phosphopeptides of pp60^{c-src} cleaved by V₈ proteinase.

proteins was still evident for 3–10 min during thrombin stimulation (Figure 1), no further increase in pp60^{c-src}-dependent TBR IgG phosphorylation was observed *in vitro* during an ensuing stimulation period of 10 min (results not shown). The concentration-dependent stimulation of platelets revealed a 1.4-fold increase in TBR IgG phosphorylation at 0.01 unit/ml thrombin and was increased to 3-fold by 10 units/ml thrombin (Table 2).

Measuring overall kinase activity of immobilized pp60^{c-src} by phosphorylation of TBR IgG in the presence of 20 nM ATP provides only limited kinetic information about the enzyme from stimulated platelets. Therefore we determined K_m and V_{max} values of the immobilized enzyme using casein as an exogenous substrate to substantiate the differences in enzyme properties between resting and thrombin-stimulated platelets (Figure 3). We found only small differences in the V_{max} values, varying up to 1.3-fold. The affinity of the enzyme for ATP, however, was 2- to 3-fold higher compared with resting platelets. The K_m values for ATP of resting and thrombin-stimulated platelets were $5.6 \pm 0.3 \mu\text{M}$ and $1.9 \pm 0.4 \mu\text{M}$ respectively. The data are mean values $\pm$ S.D. of three independent experiments. These results directly demonstrate that it is primarily the substrate affinity of the pp60^{c-src} enzyme that is altered during thrombin stimulation. Additional studies with increasing amounts of casein up to 2 mg/ml in the presence of 20 μM ATP confirmed these results (Figure 3d). Thrombin stimulation was accompanied by an approx. 2-fold decrease in K_m values for casein (for example, from 0.09 to 0.04 mg/ml). As expected, the V_{max} values were again only slightly altered.

Isolation of pp60^{c-src} from extracts of ^{32}P -labelled platelets, by using either a monoclonal antibody (MAb 327) or polyclonal antiserum (TBR sera), demonstrated that thrombin increases the incorporation of ^{32}P into the protein by about 2-fold (Figure 4a).

Partial V₈ proteinase degradation of the 60 kDa phosphoprotein yielded the typical V1–V4 phosphorylated fragments that are characteristic of pp60^{c-src} (Figure 4b). The location of the thrombin-induced phosphorylation appeared to be in the V1 fragment and its secondary V3 and V4 cleavage products which represent the N-terminal part of pp60^{c-src} [34,35]. To characterize the affected phosphorylation site(s) further, we analysed tryptic phosphopeptides of ^{32}P -labelled isolated pp60^{c-src} (Figure 5). In resting platelets (Figure 5a), the prominent Ser-17 and Tyr-527 phosphorylation sites and the minor Tyr-416 autophosphorylation site were detected by comparison with previously published tryptic phosphopeptides of pp60^{c-src} from human platelets [17]. All of the platelet agonists used in our study led to an additional phosphorylation at Ser-12, which is the amino acid within the pp60^{c-src} molecule shown to be phosphorylated by PKC [36].

Direct elevation of cyclic AMP by forskolin (Figure 5j) or indirect elevation by PGE₁ (Figure 5i) did not affect ^{32}P labelling of pp60^{c-src}. No enhanced phosphorylation was observed at Ser-17, which is thought to be preferentially phosphorylated by the cyclic AMP-dependent kinase (for a review, see ref. [37]). Elevation of cyclic GMP by sodium nitroprusside had no effect on ^{32}P labelling of pp60^{c-src} compared with controls (Figure 5k).

To address the question of whether another member of the Src family besides pp60^{c-src} contributed in the immunocomplex kinase assay to the observed changes in substrate affinity and phosphorylation pattern, we analysed mixed tryptic phosphopeptides of the 60 kDa phosphoproteins isolated by using either MAb 327 or TBR serum. As shown in Figure 5(d), no additional phosphopeptide appeared compared with the patterns of separately analysed phosphopeptides of the 60 kDa phosphoproteins isolated with MAb 327 or TBR IgG. These results indicate that no other 60 kDa phosphoprotein besides pp60^{c-src} is isolated with TBR IgG from thrombin-stimulated platelets.

DISCUSSION

Platelets possess unusually high activities of a structurally uniform type of pp60^{c-src} [5,23] and respond to a variety of biologically active agents with rapid changes in protein tyrosine phosphorylation (for a review, see ref. [3]; [6], [17] and [18]). The role of protein tyrosine phosphorylation in platelet activation, however, has not been established. A number of proteins localized at the plasma membrane are tyrosine-phosphorylated *in vitro* [5,22,38], some of them probably by pp60^{c-src} [22], but their identities are still unknown. It is also not known whether the proteins that are tyrosine-phosphorylated in intact platelets are identical with substrates phosphorylated *in vitro*, only that they are comparable in relative molecular mass on SDS/polyacrylamide gels. Recently, we established that the glycoproteins IIb and IIIa are substrates of purified pp60^{c-src} *in vitro* [39]. In platelet membranes, only glycoprotein IIIa is phosphorylated on tyrosine residues [40], but this has not yet been confirmed in intact platelets [41].

In investigating the tyrosine kinase(s) responsible for stimulation-dependent protein tyrosine phosphorylation in platelets, we have determined that substrate affinity of pp60^{c-src} kinase increases approx. 2- to 3-fold on stimulation of platelets with thrombin (Table 2). This is accompanied by an increase in phosphorylation of the enzyme at Ser-12 (Figure 5). The K_m values of immobilized pp60^{c-src} from platelets that we calculated agree with those determined for purified pp60^{c-src} from human platelets (K_m for ATP 2–4 μM [20,23,42]; K_m for casein 2.4 μM or 0.07 mg/ml [23]). Elevation of intracellular cyclic AMP levels by the platelet antagonist PGE₁, however, seems neither to

Figure 5 Two-dimensional tryptic phosphopeptide maps of pp60^{c-src} from resting and stimulated platelets

³²P-labelled platelets were stimulated for 30 s with various agents, lysed and pp60^{c-src} was isolated with MAb 327 or TBR serum as described in the Materials and methods section. Samples were analysed by SDS/PAGE and autoradiographed. The 60 kDa bands were excised from the gels, digested with trypsin, and the resulting phosphopeptides separated in two dimensions and autoradiographed as described in the Materials and methods section. (a) Control; (b) thrombin (1 unit/ml)-stimulated; (c) pp60^{c-src} isolated exclusively with MAb 327; (d) equal mixture, in terms of Cerenkov radiation, of pp60^{c-src} isolated with MAb 327 and TBR serum; (e) PMA (100 ng/ml)-stimulated; (f) A23187 (150 nM)-stimulated; (g) collagen (40 µg/ml)-stimulated; (h) [Arg⁸]vasopressin (1 µM)-stimulated; (i) PGE₁ (250 nM)-stimulated; (j) forskolin (10 µM)-stimulated; (k) sodium nitroprusside (100 µM)-stimulated; (l) schematic presentation of phosphopeptides and phosphoamino acid composition of pp60^{c-src} identified by comparison with published fingerprints of ³²P-labelled pp60^{c-src} [17,36]. The arrows in the autoradiographs indicate in each case the peptide containing phosphorylated Ser-12.

influence enzyme properties nor to induce an increase in ³²P labelling of pp60^{c-src} (Table 1 and Figure 5i).

pp60^{c-src} is not the only protein tyrosine kinase of the Src family that is modulated during thrombin treatment of human platelets. We have also observed alterations in the enzymic properties of p59^{fyn} (U. Liebenhoff and P. Presek, unpublished work). The abundance of p59^{fyn} in human platelets is approx. 5- to 10-fold lower than that of pp60^{c-src} [4,6], which does not

necessarily imply that p59^{fyn} plays only a minor role in tyrosine phosphorylation events. We cannot exclude the possibility that the other members of the Src family present in human platelets (p62^{c-yes}, p59^{hck}, p54/58^{lyn} [2,4]) become modulated on platelet stimulation.

Other laboratories have not detected any alteration in enzymic properties of pp60^{c-src} or p59^{fyn} in thrombin-treated platelets [6,11,17,18]. It has been proposed that the conformation of

activated pp60^{c-src} may be sensitive to platelet lysis and immunoprecipitation conditions [6,18]. In contrast with our results with platelets (Table 1 and Figure 5), Gould and Hunter [43] reported no activation of antibody-immobilized pp60^{c-src} from fibroblasts that had been stimulated with vasopressin and PMA, although, as we observed in platelets, the enzyme did become phosphorylated at Ser-12. They isolated pp60^{c-src} with radioimmune precipitation (RIPA) buffer containing ionic detergents (SDS and sodium deoxycholate), which might be one reason why changes in substrate affinity of the enzyme have not been previously detected [17,36,43]. On the other hand, additional alterations in the phosphorylation of pp60^{c-src}, as reported for PDGF-stimulated fibroblasts, might have functional consequences which could be monitored even when RIPA buffer was used for cell lysis [43,44]. Another important consideration is the concentrations of ATP used in immunocomplex kinase assays. We detected alterations in enzyme activity with low ATP concentrations (0.02–1 μ M); others found no changes using 40- to 1000-fold higher ATP concentrations [11,17,18]. Such differences in substrate concentration might well explain differences in the ability to measure changes in an enzyme property such as substrate affinity. PDGF-induced activation of pp60^{c-src} in fibroblasts has also been measured in the presence of relatively low ATP concentrations of 0.3–2 μ M [43,44], which does not allow changes in $V_{\max}$ and K_m to be distinguished.

All agonists of platelet function used in this study cause an increase in phosphorylation of pp60^{c-src} at Ser-12 (Figure 5), the major site phosphorylated by PKC [37]. Our data from human platelets confirm the observation of Gould and Hunter [43] for fibroblasts that activators of phosphatidylinositol turnover cause phosphorylation of pp60^{c-src} at Ser-12. Our data are also consistent with those of Ferrell and Martin [17], who reported that thrombin stimulation of human platelets results in phosphorylation of Ser-12. This group, however, also observed an increase in ³²P labelling of Tyr-416 and Tyr-527. In our hands, there is no consistent change in the ³²P content of these residues with respect to the different agonists employed. In the case of collagen and vasopressin, there may be a slight increase in labelling of pp60^{c-src} at Tyr-416 and Tyr-527; for thrombin, PMA and A23187, there may be a decrease in labelling of Tyr-527. Given that phosphorylation of pp60^{c-src} on Tyr-527 has been reported to be almost stoichiometrically complete in unstimulated cells [36,37], a detectable increase in ³²P labelling of Tyr-527 on cell stimulation may indicate either simple changes in phosphate turnover with no functional consequences or that net dephosphorylation at this site occurred, which is reflected by removal of unlabelled and incorporation of ³²P-labelled phosphate. Such a dephosphorylation, however, should result in an increased $V_{\max}$ value for pp60^{c-src}, as has been reported previously [45,46]. On the other hand, as only 5–10% of pp60^{c-src} molecules are phosphorylated at Tyr-416 under basal conditions [37], we should also expect an increase in $V_{\max}$ on phosphorylation of this residue, as has been shown with purified pp60^{c-src} [23]. For collagen and vasopressin, we have not determined the kinetic constants in detail; as noted, however, we did not observe an increase in $V_{\max}$ on treatment of platelets with thrombin. Furthermore, we have no evidence that, on platelet stimulation, tyrosine residues in the N-terminal region of the molecule become phosphorylated, which might have a modulatory effect on kinase activity or enzymic properties as suggested for fibroblasts [43,44,47].

It has been concluded that Ser-17, the major site of serine phosphorylation in pp60^{c-src}, is phosphorylated by the cyclic AMP-dependent protein kinase [37,48,49]. This conclusion was not confirmed in our study of platelet pp60^{c-src}; we did not find

phosphate incorporation to be enhanced at Ser-17 after treatment with PGE₁ or forskolin, although Ser-17 is phosphorylated less than 60% in unstimulated cells [37].

The question of whether the observed increase in Ser-12 phosphorylation of pp60^{c-src} accounts for the increase in substrate affinity must be addressed. Ser-12 is unphosphorylated in unstimulated cells and may be phosphorylated up to 100% when cells are stimulated by agents that activate PKC [36,43,50]. Studies with antibody-immobilized pp60^{c-src} isolated from fibroblasts and subsequently phosphorylated *in vitro* by purified PKC at Ser-12 failed to demonstrate an alteration in $V_{\max}$ or K_m for the phosphorylation of any substrate by pp60^{c-src} [36]. In contrast, in our study, modification of pp60^{c-src} occurred during agonist treatment of the intact platelets before antibody immobilization. Collagen and vasopressin caused less phosphorylation of Ser-12 than thrombin, PMA, or A23187, but had an effect on the level of TBR IgG phosphorylation of the same order of magnitude as the other agents. As we cannot conclude from these data anything about the size of change in the K_m and/or $V_{\max}$ values caused by these compounds, this result does not refute the idea that Ser-12 is important for pp60^{c-src} signalling; rather, it might be that a certain level of phosphorylation of this residue is all that is required to produce the increase in substrate affinity that we have demonstrated for thrombin.

The relationship of PKC activation to protein tyrosine phosphorylation in intact platelets remains unclear. Nevertheless, the onset of tyrosine phosphorylation seems to follow PKC activation [32] and the resulting Ser-12 phosphorylation of pp60^{c-src}; therefore this latter event might be responsible for the observed increase in pp60^{c-src} affinity for protein substrates. Furthermore, we can speculate that Ser-12 phosphorylation might be important for translocation and the physical interaction of pp60^{c-src} with cellular proteins, which has been reported for phosphatidylinositol 3-kinase [11,12] and elements of the cytoskeleton [13,14]. For pp60^{c-src}, it is still unknown which platelet proteins are involved in cellular signalling as a consequence of activation of the enzyme. The increase in the substrate affinity of pp60^{c-src} during platelet activation makes this enzyme a good candidate for involvement in signal-transduction pathways that trigger specific protein tyrosine phosphorylation.

Note added in proof (received 9 July 1993)

While this manuscript was under review, Clark and Brugge reported that pp60^{c-src} is activated on thrombin stimulation of platelets [51].

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