Thrombin and thrombin receptor agonist peptide induce tyrosine phosphorylation and tyrosine kinases in the platelet cytoskeleton

Translocation of pp60^{c-src} and Integrin $\alpha_{IIb}\beta_3$ (glycoprotein IIb/IIIa) is not required for aggregation, but is dependent on formation of large aggregate structures

Kevin M. PUMIGLIA* and Maurice B. FEINSTEIN

Department of Pharmacology, School of Medicine, University of Connecticut Health Center, Farmington, CT 06030, U.S.A.

The maximal aggregation of platelets induced by α -thrombin or by the receptor agonist peptide thrombin-(42–47)-peptide (TRP^{42/47}) rapidly increased the pp60^{c-src} associated with the cytoskeleton fraction. There was good correlation between the tyrosine kinase activity and the mass of pp60^{c-src}. Tyrosine kinase activity associated with the cytoskeleton phosphorylated several endogenous cytoskeleton-associated proteins, as revealed by immunoblotting with anti-phosphotyrosine antibody following incubation with ATP *in vitro*. However, with the exception of pp60^{c-src}, few phosphotyrosine-containing proteins were retained in the cytoskeleton in intact platelets when compared with total platelet lysates. Translocation of pp60^{c-src} to the cytoskeleton induced by α -thrombin and TRP^{42/47} is dependent on glycoprotein IIb/IIIa (GPIIb/IIIa)-fibrinogen-mediated aggregation, but does not occur when ristocetin/von Willebrand factor produces GPIb-mediated platelet aggregation. The translocation of GPIIb/

IIIa and pp60^{c-src} to the cytoskeleton is not necessary for aggregation, as it is not seen when clearly visible small to moderate-sized aggregates are initially formed after exposure to thrombin. The linkage of these proteins to the cytoskeleton occurs only after later extensive formation of large aggregates. Translocation of GPIIa/IIIa to the cytoskeleton is not sufficient for the cytoskeletal association of pp60^{c-src}, as the former occurs independently in platelets stimulated with concanavalin A in the absence of aggregation. Linkage of the integrin GPIIb/IIIa and pp60^{c-src} to the internal cytoskeleton structure, and the corresponding tyrosine phosphorylation of certain proteins upon formation of large aggregates, may be an example of mechanochemical transduction by integrin receptors and may represent a structure with the requisite tensile strength to stabilize large platelet aggregates against high shear stresses.

INTRODUCTION

Platelets contain high levels of non-receptor protein tyrosine kinase activity, largely due to the proto-oncogene products pp60^{c-src} (Golden et al., 1986) and pp72^{src} (Ohta et al., 1992), as well as to a number of lesser tyrosine kinases such as pp59^{lyn}, pp62^{src}, pp54/pp58^{lyn} and pp62^{src} (Horak et al., 1990; Huang et al., 1991). Platelet activation by certain agonists is accompanied by increased protein phosphotyrosine content (Ferrell and Martin, 1988; Nakamura and Yamamura, 1989; Golden and Brugge, 1989), and several studies have implicated tyrosine phosphorylation as a major regulator of the activation of phospholipase C (Dhar et al., 1990; Salari et al., 1990), polyphosphoinositide synthesis (Gutkind et al., 1990; Gaudette and Holub, 1990) and secretion (Salari et al., 1990; Nakamura and Yamamura, 1989). Additionally, platelet aggregation has been proposed to be regulated by tyrosine phosphorylation because (1) it is blocked by tyrosine kinase inhibitors (Dhar et al., 1990; Salari et al., 1990); (2) it can be initiated by tyrosine phosphatase inhibitors (Inazu et al., 1990; Pumiglia et al., 1992); and (3) it is accompanied by the increased tyrosine phosphorylation of a unique set of proteins, a process which appears to be regulated by the principle platelet integrin and fibrinogen receptor, glycoprotein (GP) IIb/IIIa (integrin $\alpha_{IIb}\beta_3$) (Ferrell and Martin, 1989; Golden et al., 1990).

Upon contact with compromised vessel walls or exposure to soluble agonists such as thrombin, ADP or thromboxane A₂, platelets that are normally discoid in shape and non-adhesive in

nature become activated to change shape to adhesive spheres with pseudopodia, to secrete granules and to aggregate in the presence of fibrinogen. These changes are believed to be triggered, at least in part, by increased phospholipid hydrolysis and cytoplasmic calcium, as well as by the activation of protein kinase C (Kroll and Shafer, 1989). Many of these phenomena may be directly or indirectly affected by the concomitant reorganization of the cytoskeleton (CSK) which, following activation and subsequent aggregation, contains the bulk of the platelet's abundant actin and myosin, and a number of proteins that are fundamental for adhesion and aggregation, e.g. GPIIb/IIIa, GPIb (Phillips et al., 1980; Kouns et al., 1991), vinculin and talin (Asigee et al., 1990; Kouns et al., 1991). Other important proteins reported to be present in the Triton X-100-insoluble CSK fraction of platelets include enzymes that are potentially regulated by tyrosine phosphorylation, such as phosphatidylinositol kinases and phospholipase C (Nahas et al., 1989; Grondin et al., 1991; Zhang et al., 1992) as well as the major platelet tyrosine kinase pp60^{c-src} (Grondin et al., 1991; Zhang et al., 1992; Horvath et al., 1992).

The co-localization of signalling molecules with the platelet's CSK raises the intriguing possibility that the CSK may be intimately involved in signal transduction, perhaps in response to cell adhesion or aggregation. However, it is unclear if there are functional consequences of the association of these molecules with the CSK. Therefore we sought to better understand the relationship between tyrosine phosphorylation and the platelet CSK. In this study we have examined Triton-insoluble CSK

Abbreviations used: CSK, cytoskeleton; GP, glycoprotein; vWF, von Willebrand Factor; TRP^{42/47}, thrombin receptor agonist peptide [thrombin-(42–47)-peptide]; PMSF, phenylmethanesulphonyl fluoride; t.i.u., trypsin inhibitor unit.

* To whom correspondence should be addressed.

cores for changes in phosphotyrosine content, tyrosine kinase activity, pp60^{c-src} and GPIIb/IIIa following stimulation with α -thrombin or the peptide agonist for the thrombin receptor, thrombin-(42–47)-peptide (TRP^{42/47}) (Vu et al., 1991). The specificity and potential mechanisms of the association of pp60^{c-src} with the CSK and the relationship of translocation with platelet aggregation are also explored.

MATERIALS AND METHODS

Platelet preparation

Human platelet concentrates were obtained from the American Red Cross (Farmington, CT, U.S.A.) within 24 h of collection. The platelets were treated with 200 μ M aspirin for 20 min to inactivate cyclo-oxygenase, then washed in the presence of 100 nM prostacyclin to prevent platelet activation, and finally resuspended to 1×10^9 platelets/ml at 37 °C for 1 h in a buffer containing 145 mM NaCl, 5 mM KCl, 0.04 mM CaCl₂, 1 mM MgCl₂ and 10 mM Hepes, pH 7.4, prior to further experimentation (Pumiglia et al., 1990).

CSK extraction

The platelet CSKs were obtained from washed platelets that were in several different functional states: (1) unstimulated, (2) stimulated by thrombin or TRP^{42/47} but not aggregated, and (3) aggregated by the same agonists. The experimental conditions were further varied to optimize for the production of either small/moderate- or large-sized aggregates. When large aggregates were desired, the platelet suspensions in 1.5 ml Microfuge tubes or 10 ml polypropylene tubes (for large batch experiments) were exposed to thrombin or TRP^{42/47} and subjected to constant lateral shaking at 96 strokes/min, with a stroke length of 4 cm, in a Magni-whirl temperature-controlled water bath at 37 °C. Alternatively, we employed an Eppendorf thermomixer at a setting of 1000 rev./min with identical results. To obtain small/moderate-sized aggregates, the platelets were stimulated with thrombin and rotated on a clinical rotator at 8 rotations/min at 21 °C for the times indicated. Stimulated platelets were obtained by exposure to thrombin under conditions that avoided or prevented aggregation, either by lack of shaking, or shaking in the presence of EGTA or Arg-Gly-Asp-Ser (RGDS). Platelets were also aggregated by ristocetin using cryoprecipitate as a source of von Willebrand Factor (vWF).

CSKs were obtained by the addition to platelet suspensions of an equal volume of a CSK buffer consisting of 2% Triton X-100, 10 mM EGTA, 10 mM EDTA, 1 mM Na₃VO₄, 1 mg/ml leupeptin, 20 μ M pepstatin A, 2 mM phenylmethanesulphonyl fluoride (PMSF), 0.46 trypsin inhibitor unit (t.i.u.)/ml aprotinin, 2 units/ml hirudin, 100 mM benzamidine and 100 mM Tris/HCl, (pH adjusted to 7.4 with HCl). Triton-insoluble proteins, operationally defined as the CSK (Phillips et al., 1980; Fox et al., 1988), were harvested by centrifugation at 15400 *g* for 5 min. The pellets were washed once with 1 $\times$ CSK buffer, followed by three washes with CSK buffer minus Triton X-100. For the electrophoresis of protein (SDS/PAGE) the washed pellets were boiled and solubilized in a SDS-sample buffer consisting of 1.5% SDS, 2.5% β -mercaptoethanol, 5% glycerol, 10 mM EDTA and 30 mM Tris/HCl (pH 6.8). In some experiments the Triton-soluble supernatant proteins were precipitated with 10% trichloroacetic acid. The pellets obtained by centrifugation (12000 *g* for 5 min) were washed twice with diethyl ether/ethanol (1:1, v/v), once with 0.5 M Tris/HCl (pH 8.0), and finally with

PBS (137 mM NaCl, 2.26 mM KCl, 162 mM Na₂PO₄, 1.4 mM KH₂PO₄, pH 7.4) prior to solubilization of the proteins for SDS/PAGE as described above.

Immunoblotting

Following SDS/PAGE, immunoblotting was performed as previously described (Pumiglia et al., 1990) in order to detect phosphotyrosine or specific proteins. ¹²⁵I-Protein A (30 μ Ci/ μ g; 1 μ Ci/ml) was used to detect bound immunoglobulin by autoradiography. In order to overcome the poor affinity of Protein A for murine IgG₁, a rabbit anti-mouse IgG bridging antibody was used. In some cases immunoblotting was performed with the Enhanced Chemiluminescence (ECL) system (Amersham) used exactly as described by the manufacturer. In all cases control experiments in the absence of primary antibody were negative. Quantification was achieved either by excision of specific protein bands from the Immobilon membranes and counting of the bound ¹²⁵I-Protein A radioactivity by liquid scintillation at 0–80 KeV (Packard TriCarb Minaxi 3000), or by three-dimensional densitometry (Molecular Dynamics Personal Densitometer) of autoradiographs in the linear range of X-ray film response. Direct comparison of the two methods yielded quantitatively equivalent results. In some cases, quantification of immunoblots were also performed by direct counting of ¹²⁵I-Protein A using the AMBIS 400 or Molecular Dynamics Phosphorimager radio-detection system.

Immunoprecipitation of cytoskeletal pp60^{c-src} and measurement of its tyrosine kinase activity

Sonicated platelet CSKs were solubilized with RIPA buffer as previously described (Huang et al., 1991) for 45 min at 4 °C. After clarification by centrifugation at 15400 *g* for 10 min, the soluble fraction was supplemented with BSA (final concentration 2 mg/ml) and incubated with 2 μ l of monoclonal antibody Mo327 for 90 min. The antibody was then precipitated by addition of 1.2 vol. of Tachisorbin M immunosorbant (goat anti-mouse), incubation for an additional 45 min at 4 °C and brief centrifugation to pellet the immunosorbant. Pellets were washed once with RIPA buffer, once with 500 mM LiCl and 20 mM Tris/HCl (pH 7.8), and once with 10 mM EDTA, 150 mM NaCl and 20 mM Tris/HCl (pH 7.8). Kinase assays were then performed on the immunoprecipitates as described (Huang et al., 1991), using 5 μ g of acid-denatured enolase as substrate. Reactions were terminated by addition of an equal volume of 2 $\times$ SDS-sample buffer. The enolase bands on gels were quantified by liquid scintillation counting after digestion with H₂O₂. Tyrosine kinase activity precipitated by Tachisorbin in the absence of Mo327 was subtracted from the activity of the Mo327 immunoprecipitates.

Tyrosine phosphorylation of proteins in isolated CSKs by endogenous kinase activity

Tyrosine kinase activity was measured in CSK preparations obtained from 5.0 ml portions of platelet suspensions (1×10^9 /ml). The CSK pellets were resuspended in 2.0 ml of buffer consisting of 96 mM KCl, 3.2 mM MgCl₂, 20 mM NaCl, 0.8 mM NaH₂PO₄, 215 μ M CaCl₂, 800 μ M K⁺-EGTA (yielding a free Ca²⁺ of 80 nM), 0.5 mM PMSF, 20 μ M leupeptin, 20 μ M pepstatin A, 0.3 t.i.u./ml aprotinin, 1 mM Na₃VO₄, 20% glycerol and 14 mM Hepes (pH 7.0). The pellets were sonicated for 3 $\times$ 10 s with a micro-tip sonicator (Kontes) to achieve uniform dispersion prior to kinase assays. Assays were conducted in a 50 μ l final

reaction volume consisting of 25 mM Hepes, pH 7.4, 10 mM MgCl_2 , 1 mM Na_3VO_4 and 0.5 mM ATP. The reactions were initiated by the addition of CSK protein equivalent to 2.5×10^7 cells; reactions were allowed to proceed for 3 min at 37 °C and then terminated with 2×SDS-sample buffer. Following SDS/PAGE the proteins were analysed by immunoblotting using antiphosphotyrosine antibody.

Materials

α -Thrombin (3488 units/mg; 1 unit/ml = 7.5 nM) was a gift from Dr. J. Fenton II (New York State Department of Health, Albany, NY, U.S.A.). TRP^{42/47}, corresponding to the peptide sequence SFLLRN of the recently cloned thrombin receptor (Vu et al., 1991), was synthesized for us by Dr. Richard Freer (Medical College of Virginia, Richmond, VA, U.S.A.) Cryoprecipitate was obtained from the American Red Cross. Antibodies for immunoblotting were obtained from the following sources: mouse monoclonal antiphosphotyrosine antibody 4G10 was from UBI (Lake Placid, NY, U.S.A.), mouse monoclonal antibody Mo327 specific for pp60^{c-src} (Lipsich et al., 1983) was a generous gift from Dr. J. Brugge (ARIAD, Cambridge, MA, U.S.A.), and a mouse monoclonal antibody against GPIIb (SZ22) was obtained from Amac (Westbrook, ME, U.S.A.). Rabbit anti-mouse and goat anti-sheep antibodies and ristocetin were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) ¹²⁵I-Protein A (> 30 $\mu\text{Ci}/\mu\text{g}$) was purchased from ICN Radiochemicals (Irvine, CA, U.S.A.). Tachisorbin M was from Calbiochem (La Jolla, CA, U.S.A.). An ECL detection kit was purchased from Amersham (Arlington Heights, IL, U.S.A.). All other chemicals were of reagent grade from various sources.

RESULTS

Analysis of cytoskeletal pp60^{c-src} kinase activity

The Triton-insoluble matrix of the platelet is operationally defined as the CSK, based on biochemical and morphological analysis (Phillips et al, 1980; Fox et al, 1988). Platelets ($1 \times 10^9/\text{ml}$) were: (1) unstimulated, (2) stimulated with 7.5 nM α -thrombin to cause aggregation, or (3) stimulated with thrombin plus 2 mM EGTA or 1 mM RGDS to prevent aggregation. All platelets were shaken as described in the Materials and methods section to obtain large aggregates. The Triton-insoluble cores were analysed for the presence of pp60^{c-src}. Quantitative immunoblotting with antibody Mo327 detected very low levels of pp60^{c-src} in the CSK of unstimulated platelets; i.e. $4.4 \pm 0.95\%$ (mean $\pm$ S.E.M.) of the total pp60^{c-src} (Figure 1a). No appreciable change was detected after stimulation with 7.5 nM α -thrombin if aggregation was blocked by either RGDS (1 mM) (Figure 1a) or EGTA (2 mM) (results not shown). Stimulation of platelets in the absence of shaking (no visible aggregation) also produced little or no detectable change in the pp60^{c-src} content of the CSK. However, under maximal aggregating conditions, there was an approx. 15-fold increase in the amount of pp60^{c-src} in the Triton-insoluble fraction, so that it then represented 40–55% of the total platelet pp60^{c-src}. The remainder of the pp60^{c-src} was accounted for in the Triton-soluble fraction; i.e. under all conditions the sum of the pp60^{c-src} in the Triton-soluble and Triton-insoluble fractions was within 5% of the total pp60^{c-src} content of whole platelet lysate. These findings are in general agreement with recently published reports (Zhang et al., 1992; Horvath et al., 1992; Oda et al., 1992).

We next considered the possibility that the kinase activity of pp60^{c-src} was altered (activated or inhibited) by its association

with the CSK. The changes in the CSK content (mass) of pp60^{c-src} and its kinase activity were measured by immunoprecipitation with Mo327. Sham immunoprecipitates, in the absence of Mo327, showed no phosphorylation of either enolase or of the autophosphorylated 60 kDa protein which co-migrated with purified pp60^{c-src} (Figure 1b). In Mo327 immunoprecipitates from CSKs of platelets prevented from aggregating using EGTA, enolase phosphorylation was only 1.4-fold higher than in control unstimulated platelets when equal amounts of platelets were compared (Figure 1b). In contrast, in Mo327 immunoprecipitates from the CSKs of thrombin-aggregated platelets enolase phosphorylation was increased 14.9-fold. This increase is in very

Figure 1 Aggregation-dependent translocation of pp60^{c-src} to the CSK

(a) Increased pp60^{c-src} mass associated with the platelet CSK following aggregation. Platelet proteins derived from the CSK (5×10^7 platelet equivalents/lane) or Triton-soluble fraction (TS) (2.5×10^7 platelet equivalents/lane) were separated by SDS/7.5%-PAGE (15 cm) and immunoblotted with Mo327. Platelets ($1 \times 10^9/\text{ml}$) were unstimulated (C), stimulated with 7.5 nM α -thrombin in the presence of 2 mM EGTA (S), or maximally aggregated with 7.5 nM α -thrombin (A). The minor band below pp60^{c-src} is the 50 kDa proteolytic fragment of pp60^{c-src} and constituted less than 5% of the total. (b) Immunoprecipitation of pp60^{c-src} activity from solubilized CSKs. CSKs from platelets stimulated as described above were solubilized and immunoprecipitated with Mo327 or without primary antibody (Sham.). Sedimented proteins were analysed by immunoprecipitation kinase reactions with 5 μg of enolase as described in the Materials and methods section. Each lane contains the reaction products from the immunoprecipitate of 1.25×10^8 platelet equivalents. Following SDS/PAGE (10%; 15 cm), the dried gel was exposed for autoradiography. In similar experiments Mo327 immunoprecipitated a purified pp60^{c-src} standard, which co-migrated with the single 60 kDa phosphoprotein immunoprecipitated from platelets.

Figure 2 CSK-associated protein tyrosine kinase activity phosphorylates endogenous CSK proteins

Tyrosine phosphorylation of endogenous platelet CSK proteins was measured *in vitro*. Sonicated CSK proteins from platelets which were unstimulated (C), stimulated with 7.5 nM α -thrombin in the presence of 2 mM EGTA (S) or stimulated with 7.5 nM α -thrombin under maximally aggregating conditions (A) were incubated with or without 0.5 mM ATP for 3 min at 37 °C. After stopping the reaction, proteins (2.5×10^6 platelet equivalents/lane) were separated by SDS/8% PAGE (8 cm) followed by immunoblotting with anti-phosphotyrosine antibody and 125 I-Protein A. Similar results were obtained in two other experiments.

Figure 3 Tyrosine phosphorylation of platelet CSK proteins

Washed platelets (1×10^9 /ml) were treated as described below under maximally aggregating conditions (see the Materials and methods section). Proteins from the CSK, or whole platelet lysates (WP) (2.0×10^7 platelet equivalents/lane), were separated by gradient SDS/7.5% PAGE and transferred to Immobilon-P. The proteins were immunoblotted with anti-phosphotyrosine antibody and 125 I-Protein A, and radioactivity was analysed with a Molecular Dynamics Phosphorimager. The image was optimized for detection and resolution of whole-platelet samples to allow for quantitative comparison with CSK samples. Lanes represent unstimulated platelets (C); 7.5 nM α -thrombin for 5 min in the presence of 1 mM RGDS (S); 7.5 nM α -thrombin for 5 min (maximally aggregated) (A).

good agreement with the measurements of pp60^{c-src} mass by immunoblot analysis.

In order to assess the specificity of the pp60^{c-src} translocation, we measured the total protein associated with the CSK pellet and compared it with the increase in pp60^{c-src} measured by immunoblotting. The total protein in the Triton-insoluble pellet was

Figure 4 GPIIb-mediated aggregation fails to induce pp60^{c-src} translocation to the CSK

(a) GPIIb/IIIa- and GPIIb-mediated aggregation. Platelet aggregation (3×10^8 platelets/ml) was measured in a lumiaggregometer under stirring conditions (1000 rev./min) following treatment with (1) 7.5 nM α -thrombin or (2) 0.3 mg/ml ristocetin plus 10 μ l of cryoprecipitate (as a source of vWF), in the presence of 1 mM RGDS to block fibrinogen binding to GPIIb/IIIa. (b) Quantification of CSK-associated pp60^{c-src} and GPIIb. In a parallel experiment, platelets were stimulated as indicated in (a) and the CSKs were extracted after 5 min and analysed after immunoblotting with Mo327 or SZ22 by ECL: unstimulated (Con), stimulated with thrombin (THR) or stimulated with ristocetin/vWF (Risto). CSK-associated pp60^{c-src} (□) and GPIIb (■) were quantified by densitometry and data are expressed as percentages of the same proteins detected in an equivalent fraction of whole-cell lysate. Identical results were obtained in a separate experiment.

increased approx. 2-fold, in good agreement with previously published data for the cytoskeletal fraction of aggregated platelets (Zucker and Masiello, 1983; Grondin et al., 1991; Zhang et al., 1992). In the same platelets the increase in pp60^{c-src} associated with the CSK was 15-fold; hence there was an approx. 7.5-fold specific enrichment in the CSK pp60^{c-src} content.

Tyrosine phosphorylation of endogenous proteins by CSK-associated tyrosine kinases

The results described above clearly demonstrate an increase in pp60^{c-src} and tyrosine kinase activity in the CSK of maximally aggregated platelets. It therefore seemed likely that endogenous CSK substrates for this activity might exist. This was tested by analysing the ability of the cytoskeletal tyrosine kinase activity to utilize endogenous CSK proteins as substrates *in vivo*. The CSKs were isolated in the absence of tyrosine phosphatase inhibitors so that they contained very low levels of phosphotyrosine. The Triton-insoluble proteins were washed and sonicated into suspension, and then incubated with or without 0.5 mM non-radioactive ATP and immunoblotted with anti-phosphotyrosine antibody. In the absence of ATP there was little or no detectable phosphotyrosine (Figure 2). However, when ATP was added, tyrosine phosphorylation in CSKs from an equal number of platelets, measured as the protein bound 125 I-protein A, was increased by only 1.4 ± 0.3 -fold in thrombin-activated (non-aggregated) platelets, but by 12.9 ± 1.5 -fold after aggregation (Figure 2). The finding that several CSK proteins could be tyrosine-phosphorylated *in vitro* when pp60^{c-src} was translocated to this fraction suggested that these same proteins might be substrates for the enzyme in intact platelets.

Figure 5 The formation of aggregate complexes is not sufficient to induce $pp60^{c-src}$ translocation to the platelet CSK

Small to moderate-sized aggregates were formed under 'low aggregating' conditions (see the Materials and methods section). Platelets fixed with 1% formaldehyde were visualized by differential interference contrast microscopy and micrographs were taken (final magnification $200\times$; scale bar represents $20\mu m$). (a) Unstimulated platelets showed a characteristic discoid shape and were predominantly single. While a few doublets and triplets can be seen, further observation revealed they were not stable associations. A small percentage ($\sim 5\%$) were associated in a stable fashion with other platelets. (b) After stimulation with 7.5 nM α -thrombin for 3 min under 'low aggregating' conditions, most of the platelets were associated in aggregate complexes ranging in size from small (2–5 platelets; indicated by the short arrow) to larger (5 to ≥ 15 platelets, as denoted by the long arrow) aggregates.

Aggregation-dependent tyrosine phosphorylation and the CSK

To test the possibility of aggregation-dependent tyrosine phosphorylation we probed the Triton-insoluble proteins with anti-phosphotyrosine antibody and compared this with the whole platelet fraction, matched for equivalent platelet number. Figure 3 demonstrates that when platelets were stimulated in the absence of aggregation (blocked with either EGTA or RGDS, or no stirring) there was a detectable increase in phosphotyrosine in several proteins, most notably those at 56, 65, 68, 70, 80, 100, 116, 130 and 140 kDa. Thus there are several proteins which undergo aggregation-independent tyrosine phosphorylation. This is in agreement with the findings of Ferrell and Martin (1989), but in contrast to a more recent report which implied that aggregation was essential for all of the enhanced tyrosine phosphorylation produced by thrombin (Golden et al., 1990).

Figure 6 Quantification of aggregation, $pp60^{c-src}$ and GPIIb in the CSK

(a) Platelets were stimulated under low aggregating conditions and fixed with 1% formaldehyde at the time points indicated. The extent of aggregation quantified microscopically is expressed by giving the percentage of single (non-aggregated) platelets (□). Values represent the means $\pm$ S.E.M. of three separate fields (100–200 platelets/field). In parallel experiments the GPIIb (●) and $pp60^{c-src}$ (▲) in the CSK fraction were quantified relative to the amounts present in the total platelet lysate and compared with the degree of aggregation. Identical results were observed in several similar experiments. (b) Western blots of $pp60^{c-src}$ and GPIIb associated with the CSK fraction as compared with one-half of the total platelet (WP) content are shown for various time points after stimulation under low aggregating conditions and for 3 min under maximal aggregating conditions (Max). Under maximal aggregating conditions, GPIIb represented 16% and $pp60^{c-src}$ represented 38% of the total after 3 min.

When platelets were allowed to maximally aggregate, enhanced tyrosine phosphorylation was seen in several protein bands, i.e. at 80, 96, 100 and 116 kDa. This is in good agreement with the aggregation-dependent tyrosine phosphorylation previously reported (Ferrell and Martin, 1989; Golden et al., 1990).

The equivalent CSK fraction of these platelets was analysed for phosphotyrosine-containing proteins. Under resting and stimulated conditions, few or no tyrosine-phosphorylated proteins were detected. In some experiments a protein at ~ 130 kDa was detected in the Triton-insoluble fraction of stimulated platelets; however, the proportion of this protein compared with the whole platelet band was quite low ($< 10\%$). In maximally aggregated platelets a 60 kDa tyrosine-phosphorylated protein was clearly visible and represented about 50% of the 60 kDa phosphotyrosine in the whole platelet sample. Measurement of the phosphotyrosine content in the 60 kDa band of whole platelet lysates revealed no difference in the stimulated and aggregated condition; thus the increase in the CSK fraction is due to translocation rather than enhanced tyrosine phosphorylation of an existing CSK protein. This band is most likely $pp60^{c-src}$, as it co-migrates with authentic $pp60^{c-src}$. Furthermore, the amount of phosphotyrosine in this CSK band closely paralleled the mass of immunoprecipitated CSK $pp60^{c-src}$.

Figure 7 Translocation of GPIIb/IIIa to the CSK induced by concanavalin A does not promote translocation of pp60^{c-src}

Whole platelets (2.5×10^6 platelet equivalents/lane) or CSK samples from the same platelets (5×10^6 platelet equivalents/lane) were separated by SDS/PAGE (8%, 8 cm) and immunoblotted with SZ22 or Mo327 to detect GPIIb and pp60^{c-src} respectively. Lanes represent: 1, whole platelets; 2, unstimulated platelets; 3, platelets stimulated with 7.5 nM α -thrombin without stirring; 4, stimulation with 7.5 nM α -thrombin under maximally aggregating conditions; 5, stimulation with 50 μ g/ml concanavalin A without stirring. In all cases incubations were for 5 min at 37 °C.

Enhancement of the association of pp60^{c-src} with the CSK by TRP^{42/47} but not by ristocetin/vWF

We considered the possibility that the association of pp60^{c-src} with the CSK might result from artifactual trapping in a fibrin meshwork, or an association between pp60^{c-src} and Triton-insoluble fibrin. To test this, platelets were aggregated with TRP^{42/47}, a peptide agonist of the recently cloned thrombin receptor (Vu et al., 1991) which stimulates and aggregates platelets in a fashion identical to thrombin, but has no capacity to cleave fibrinogen to fibrin (Vu et al., 1991; Vassallo et al., 1992). TRP^{42/47} also produced an aggregation-dependent translocation of pp60^{c-src} to the Triton-insoluble CSK which was indistinguishable from that produced by thrombin (results not shown).

Additionally we incubated platelets (3×10^8 /ml) with exogenous fibrinogen (240 μ g/ml) and 7.5 nM thrombin under non-stirring conditions. Under these conditions there was appreciable generation of insoluble fibrin, as indicated by the visible increase in the Triton-insoluble protein, but there was no increase in CSK pp60^{c-src}. Furthermore, recent studies have reported translocation of pp60^{c-src} to the CSK using phorbol 12-myristate 13-acetate and U46619 (Oda et al., 1992; Horvath et al., 1992), agents which do not generate fibrin. Taken together, these experiments conclusively demonstrate that: (1) the generation of fibrin by thrombin is neither necessary nor sufficient for the translocation of pp60^{c-src} to the CSK; and (2) the association of pp60^{c-src} with the CSK is not an artifact due to association with, or trapping by, insoluble fibrin.

We also considered the possibility that platelet aggregation somehow made pp60^{c-src} inherently less soluble or less accessible to the Triton buffer. Additionally, it was important to define if specific association of pp60^{c-src} with the CSK might take place under any conditions in which platelets undergo homologous cross-linking of adhesive surface glycoproteins. We therefore used ristocetin plus cryoprecipitate, as a source of vWF, to induce platelet aggregation. This process is mediated by GPIb, a membrane protein that has also been proposed to be a high-affinity thrombin receptor (Harmon and Jamieson, 1986) and is

known to become associated with the CSK following aggregation (Fox et al., 1988). RGDS was also included in the medium. This peptide has no effect on the GPIb/vWF-mediated agglutination process (Figure 4), but it prevents vWF and fibrinogen (present in large amounts in the cryoprecipitate) from binding to GPIIb/IIIa (Plow et al., 1985). It was important to inhibit the latter because recent experiments have shown that the binding of vWF to GPIb can subsequently induce the activation of GPIIb/IIIa (Savage et al., 1992). Under these conditions we found that thrombin and ristocetin produced equal changes in light transmittance, as measured in an aggregometer (Figure 4a), indicative of the same degree of platelet aggregation. However, only after thrombin-induced aggregation was a significant amount of pp60^{c-src} or GPIIb associated with the CSK (Figure 4b). This result, and the ability of TRP^{42/47} to cause aggregation-dependent translocation of pp60^{c-src}, demonstrates that the process is mediated by the G-protein-coupled receptor (Vu et al., 1991) rather than by GPIb, and that the translocation of pp60^{c-src} may be specific for integrin (GPIIb/IIIa)-mediated aggregation.

Relationship between aggregate size and pp60^{c-src} translocation

In order to better understand the relationship between the association of pp60^{c-src} with the CSK and the aggregation of platelets, we utilized a protocol consisting of a lower platelet concentration (3×10^8 /ml) coupled with gentle rotation at room temperature (see the Materials and methods section) in order to slow the aggregation process. After stimulation of 7.5 nM thrombin, duplicate samples were subjected to extraction of the CSK or were fixed with 1% formaldehyde and examined by Nomarski differential interference contrast and bright field microscopy for the presence of platelet aggregates. This microscopic method is more sensitive for the detection of the initial aggregation process than is light transmittance in an aggregometer (Leung, 1984). Unstimulated preparations (Figure 5a) were $95.5 \pm 0.4\%$ discoid single platelets. Following stimulation with thrombin there was a rapid disappearance of single platelets. At 3 min, platelets formed very fine but clearly visible aggregates. By microscopy (Figure 5b), $78.8 \pm 2.0\%$ of platelets were in aggregate complexes ($\leq 15 \mu$ m diameter) ranging from two to approx. 15 platelets. Despite the extensive formation of small aggregates, pp60^{c-src} and GPIIb/IIIa were undetectable in the CSK (Figure 6) even when the stimulation was carried out for 10 min. The lack of translocation of pp60^{c-src}/GPIIb to the CSK despite widespread aggregation of platelets was demonstrable at both higher (27 nM) and lower (1.67 nM) doses of α -thrombin (results not shown), thus it is not dose-dependent. It is noteworthy that under these low-aggregating conditions, aggregation-dependent tyrosine phosphorylation was not seen; rather, the profile of proteins closely resembled the pattern seen with thrombin stimulation when aggregation was blocked (results not shown; refer to Figure 2). When the same platelets were stimulated at 37 °C and stirred at 1000 rev./min both pp60^{c-src} and GPIIb/IIIa were readily detected in the CSK fraction after 3 min (Figure 6). However, under these conditions the platelets had formed aggregates which were clearly much larger to the eye. Microscopically these aggregates were fewer but much larger, i.e. $\geq 100 \mu$ m diameter (results not shown).

Incorporation of GPIIb/IIIa into the CSK is not sufficient to induce translocation of pp60^{c-src}

As there is a good correlation between the translocation of pp60^{c-src} and GPIIb/IIIa to the CSK (Figures 4 and 6) we

reasoned that these two proteins might be tightly associated with each other. To test this possibility we used the lectin concanavalin-A, which has been shown to induce an association of GPIIb/IIIa with the platelet CSK even under non-aggregating conditions (Painter and Ginsberg, 1982). After treatment with 50 $\mu\text{g/ml}$ concanavalin A for 5 min there was a marked association of GPIIb with the CSK (Figure 7), equivalent to that seen following maximal aggregation with 7.5 nM α -thrombin; however, pp60^{c-src} was not translocated to the CSK.

DISCUSSION

From these experiments we are able to draw several major conclusions. (1) The proto-oncogene tyrosine kinase pp60^{c-src} associates with the platelet CSK in a specific and aggregation-dependent fashion. (2) Several CSK proteins are substrates for CSK-associated tyrosine kinase activity, which increases upon maximal aggregation. (3) Only a minor fraction of the total tyrosine-phosphorylated proteins in the platelets are associated with the CSK. (4) The association of pp60^{c-src} with the platelet CSK is not a generalized phenomenon of platelet-platelet contact, as agglutination of platelets by ristocetin/vWF (mediated by GPIb) failed to induce pp60^{c-src} translocation. (5) Translocation of pp60^{c-src} to the CSK is not necessary for initial platelet aggregation, and aggregation alone is not sufficient to produce translocation of pp60^{c-src}, since extensive formation of small to moderate-sized aggregates was not accompanied by translocation of pp60^{c-src}. (6) The appearance of pp60^{c-src} in the platelet CSK is a relatively late event in the platelet activation cascade, correlating well with the formation of very large aggregates and with aggregation-dependent tyrosine phosphorylation of some platelet proteins. (7) The association of pp60^{c-src} and GPIIb/IIIa with the CSK, which occur concurrently with the formation of large aggregates, may be independent events, since the association of GPIIb/IIIa with the CSK was induced by concanavalin A without an accompanying change in pp60^{c-src}.

The association of pp60^{c-src} with the CSK is aggregation-dependent, as: (1) it is blocked by RGDS, a tetrapeptide which competitively blocks fibrinogen binding, as well as by EGTA and EDTA, which remove bivalent cations required for fibrinogen binding; and (2) it does not occur in the absence of stirring. Under the latter condition fibrinogen binding is normal, but at a platelet concentration of 3×10^8 no significant aggregation occurs (Painter and Ginsberg, 1982). Additionally, incubation of platelets with RGDS alone, which is capable of inducing an active form of the GPIIb/IIIa molecule (Du et al., 1991), did not increase translocation of pp60^{c-src} to the CSK. These data argue that fibrinogen binding is a necessary event, in that it is required for aggregation, but that neither fibrinogen binding nor the activation of the GPIIb/IIIa molecule to a state competent to bind fibrinogen is sufficient to induce the translocation of pp60^{c-src}. It should also be noted that the secretion of granules, calcium mobilization and pleckstrin phosphorylation occurred normally in platelets stimulated by thrombin in the absence of aggregation (results not shown). Therefore none of these initial platelet responses are regulated by the association of pp60^{c-src} with the CSK, nor are any of these responses sufficient to elicit the translocation of pp60^{c-src}. Moreover, pp60^{c-src} translocation occurred in aspirinized platelets in the presence of ADP scavengers, indicating that this process is not dependent upon the release of secondary mediators.

During the preparation of this paper, several other reports of pp60^{c-src} associating with the platelet CSK have appeared (Grondin et al., 1991; Zhang et al., 1992; Horvath et al. 1992;

Oda et al., 1992). Zhang et al. (1992) in their preliminary report of this phenomenon found only partial inhibition by RGDS, suggesting independence from fibrinogen binding to GPIIb/IIIa. One potential reason for this discrepancy is that at the high platelet concentrations [$(3-5) \times 10^8/\text{ml}$] used in their study the secreted fibrinogen would be better able to compete against the lower RGDS concentration (0.5 mM) they employed; hence aggregation may not have been completely blocked. Horvath et al. (1992) and Oda et al. (1992) found a strict aggregation-dependence for the association of pp60^{c-src} with the CSK by similar criteria as presented here. Our quantitative estimates of pp60^{c-src} in the aggregated CSKs are in close agreement with previous estimates (Horvath et al., 1992). Both Oda et al. (1992) and Horvath et al. (1992) found evidence for inhibition by cytochalasin D, suggesting this process also required ongoing actin polymerization.

The physical translocation of pp60^{c-src} to the CSK is accompanied by an increase in the CSK-associated tyrosine kinase activity as determined by two assays: (1) immunoprecipitation of solubilized CSK pp60^{c-src} with Mo327 to measure changes in its activity and mass, and (2) total tyrosine kinase activity measured by *in vitro* phosphorylation of endogenous CSK proteins in the presence of ATP. The increased tyrosine kinase activity measured by both methods correlated extremely well with the increased mass of pp60^{c-src}. Thus no significant alteration (activation or inhibition) of pp60^{c-src} activity was detected following its translocation to the CSK. However, at this time we cannot rule out the possibility that the procedure necessary to solubilize the CSK-associated pp60^{c-src} for immunoprecipitation disrupts a potential regulatory element or affects the pp60^{c-src} molecule itself.

Several groups have reported a similar wave of tyrosine phosphorylation of a specific subset of proteins concurrent with GPIIb/IIIa-dependent aggregation (Ferrell and Martin, 1989; Golden et al., 1990). We suggest that the translocation of pp60^{c-src} to the CSK may be involved in the observed aggregation-dependent tyrosine phosphorylation for the following reasons: (1) several of these proteins (100 and 96 kDa) are good substrates for tyrosine kinase activity in CSKs isolated from aggregated platelets; (2) the association of pp60^{c-src} with the CSK and aggregation-dependent tyrosine phosphorylation are similarly dependent upon maximal aggregation; and (3) cytochalasin D, which did not prevent aggregation, did inhibit both pp60^{c-src} translocation and aggregation-dependent tyrosine phosphorylation (Oda et al., 1992). Other tyrosine kinases may also play a role, such as pp125^{fak}, a recently described non-*src* family kinase associated with focal adhesions in fibroblasts (Shaller et al., 1992). Stimulation-induced tyrosine phosphorylation and activation of pp125^{fak} are absent from platelets obtained from patients with Glanzmann's thrombasthenia, which lack GPIIb/IIIa (Lipfert et al., 1992). Platelets with this defect also do not show aggregation-dependent tyrosine phosphorylation of other proteins (Ferrell and Martin, 1989). Interestingly, pp125^{fak} undergoes increased tyrosine phosphorylation in *v-src*-transformed cells (Guan and Shalloway, 1992), raising the possibility that this kinase is a substrate for pp60^{c-src}.

In our studies we did not find a significant proportion of the platelet tyrosine-phosphorylated proteins in the CSK. Rather, pp60^{c-src} was the predominant protein reacting with anti-phosphotyrosine antibodies when equal amounts of whole platelets and CSKs were loaded on gels and radioactivity exposure was optimized for detection of the total phosphotyrosine. Oda et al. (1992) reported the presence of tyrosine-phosphorylated proteins, in addition to pp60^{c-src}, in the platelet CSK. However, the levels were not directly compared

with the levels in whole platelets and no quantitative comparison was made. In fact, if the signal of a band at 42 kDa in their report is used for comparison, it seems that the detection of tyrosine-phosphorylated proteins in the CSK required significant overexposure (relative to the whole-platelet lysates). We have also detected similar tyrosine-phosphorylated proteins in the CSK fraction when autoradiographs were overexposed (results not shown). Additionally, we also detected tyrosine-phosphorylated proteins with identical molecular masses to those reported by Oda et al. (1992) (i.e. 115, 100, 96 and 70 kDa) in the CSK by our *in vitro* tyrosine kinase assay (see Figure 2). Taken collectively, the data indicate that these proteins, which are identical in molecular mass to several which undergo enhanced tyrosine phosphorylation following aggregation, are capable of being tyrosine-phosphorylated by CSK-associated tyrosine kinase activity. However, our data indicate that only a small fraction of the total population of these proteins is stably associated with the Triton-insoluble CSK.

Oda et al. (1992) found that induction of primary aggregation by adrenaline in aspirinized platelets was not sufficient to induce the translocation of pp60^{c-src}. However, when platelets were stimulated with adrenaline in the absence of aspirin, secondary aggregation occurred and *src* recruitment to the CSK was observed. It was not clear from these studies whether the difference was due to an additional signal (e.g. phospholipase C activation) caused by thromboxane production, or whether it was the result of the aggregation state (primary versus secondary). However, in our studies using thrombin as the sole agonist (in presence of aspirin and apyrase), but varying the aggregation conditions, we found that aggregation *per se* is not sufficient for the translocation of pp60^{c-src}. Rather, the translocation of pp60^{c-src} and aggregation-dependent tyrosine phosphorylation are processes that occur late in aggregation, somehow related to very large aggregate size. Furthermore, the translocation of pp60^{c-src} may be specific for integrin-mediated large aggregate formation, rather than a generalized response to homologous cross-linking of any surface-adhesive glycoproteins, e.g. GPIb.

The formation of large platelet aggregates is also a prerequisite for the translocation of GPIIb/IIIa to the CSK. Although the concurrent translocation of pp60^{c-src} and GPIIb/IIIa was aggregation-dependent, their association with the CSK was not necessary for the initial aggregation process. Rather, the association of both proteins with the CSK occurred only after the growth of the aggregates has reached some critical stage. Whether pp60^{c-src} and GPIIb/IIIa actually associate with each other remains to be seen. No condition examined to date has resulted in translocation of pp60^{c-src} to the CSK without a concurrent attachment of GPIIb/IIIa. Hence the linkage to the integrin molecule may be an obligatory event. However, several attempts to co-precipitate these two proteins have been unsuccessful (Ferrell and Martin, 1989; Golden et al., 1990). In addition, our findings with concanavalin A show that the linkage of GPIIb/IIIa to the CSK is not sufficient to produce a similar association of pp60^{c-src}.

In many cell types integrin molecules have been implicated as mechanochemical transducers (see Ingbar, 1991, for a review) whereby external forces (e.g. shear stress) are converted into intracellular signals via integrin links to the CSK. It is interesting to speculate that the increased stress on GPIIb/IIIa and/or other platelet integrins, resulting from increased aggregate size, might convey a signal that is necessary for the translocation of pp60^{c-src}. The increased tyrosine phosphorylation of CSK or loosely

associated proteins may affect the linkages of membrane integrins to the internal CSK, which could impart the increased tensile strength necessary for the stabilization of large platelet aggregates in the presence of high shear stresses encountered in blood vessels.

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