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Redistribution of Activated pp60$^{c\text{-}src}$ to Integrin-Dependent Cytoskeletal Complexes in Thrombin-Stimulated Platelets

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Thrombin stimulation of platelets induces a transient increase in the specific activity of pp60$^{c\text{-}src}$ followed by a redistribution of pp60$^{c\text{-}src}$ to the Triton X-100-insoluble, cytoskeleton-rich fraction. Concomitant with the observed increase in pp60$^{c\text{-}src}$ activity was a rapid dephosphorylation of tyrosine 527 in 10 to 15% of pp60$^{c\text{-}src}$ molecules. In addition, we found that pp60$^{c\text{-}src}$ from the Triton-insoluble fraction was phosphorylated on tyrosine 416, the autophosphorylation site which is phosphorylated in activated oncogenic variants of pp60^{src}. Furthermore, in platelets from patients with Glanzmann's thrombasthenia (which are deficient in the integrin receptor GPIIb-IIIa), pp60$^{c\text{-}src}$ was not translocated to the Triton-insoluble fraction, and there was a sustained increase in pp60$^{c\text{-}src}$ activity following thrombin treatment. These results suggest that pp60$^{c\text{-}src}$ is rapidly activated in thrombin-stimulated platelets, potentially by a protein tyrosine phosphatase, before it translocates to a cytoskeletal fraction, where many of its potential substrates are found. The evidence that the cytoskeletal association of pp60$^{c\text{-}src}$ is dependent upon engagement of the integrin receptor GPIIb-IIIa suggests that integrin-cytoskeletal complexes may serve to compartmentalize and anchor activated enzymes involved in signal transduction.

The induction of platelet secretion and aggregation by thrombin and other agonists results in a dramatic increase in tyrosine phosphorylation of multiple proteins. Since none of the known receptors for platelet agonists possess protein tyrosine kinase activity, agonist-induced tyrosine phosphorylation must be mediated by nonreceptor kinases that are activated downstream from agonist-receptor interactions (for a review, see reference 44). Some of these phosphorylation events have been shown to be regulated by the integrin receptor glycoprotein (GP) IIb-IIIa (14, 19) and are proposed to result from signals transduced upon binding of fibrinogen to this platelet surface receptor. The kinases responses responsible for these integrin-dependent and/or -independent tyrosine phosphorylation events have not been identified.

The Src-related tyrosine kinases are candidate enzymes that may be invovled in agonist-induced tyrosine phosphorylation in platelets. Five members of this family (Src, Yes, Fyn, Hck, and two variants of Lyn) have been detected in platelets (24, 26), the most abundant of which is pp60$^{c\text{-}src}$, which represents as much as 0.4% of total platelet protein (18). It has been suggested that activation of this abundant kinase could greatly contribute to agonist-stimulated tyrosine phosphorylation. To date, no increase in the specific activity of pp60$^{c\text{-}src}$ has been detected upon agonist stimulation of platelets (18, 22, 24). However, in other cell types, related protein tyrosine kinases have been shown to be activated after engagement of a variety of membrane receptors. For example, the in vitro tyrosine kinase activity of pp56^{lck} is activated three- to fivefold in T lymphocytes after cross-linking of the CD4 or CD8 receptor (47). This activation accompanies the tyrosine phosphorylation of multiple T-cell proteins. In B lymphocytes, the activity of p56^{blk} is increased five- to eightfold and p58^{lyn} and p60^{fyn} activities are also slightly enhanced after cross-linking of the immunoglobulin M surface receptor (2).

A mechanism for activation of pp60$^{c\text{-}src}$ has been suggested from structural and biochemical studies of transforming variants of Src family members. Several lines of evidence suggest that phosphorylation of tyrosine 527 within the carboxy-terminal regulatory domain of pp60$^{c\text{-}src}$, a domain that is truncated in pp60$^{v\text{-}src}$, regulates the kinase activity of pp60$^{c\text{-}src}$ (5–8, 27, 30). Since phosphorylation of tyrosine 527 suppresses the kinase activity of pp60$^{c\text{-}src}$, an activation mechanism may involve dephosphorylation of tyrosine 527. It has been proposed that pp60$^{c\text{-}src}$ is maintained in a low activity state by an interaction between phosphotyrosine 527 and the Src homology 2 (SH2) domain and that pp60$^{c\text{-}src}$ is activated upon tyrosine 527 dephosphorylation (41). Dephosphorylation of phosphotyrosine 527 could release the SH2 domain, thus allowing it to bind to specific phosphotyrosine-containing proteins (17, 31).

Whereas tyrosine 527 phosphorylation has been shown to have a negative regulatory effect on pp60$^{c\text{-}src}$ kinase activity, phosphorylation of tyrosine 416 within the catalytic domain of pp60$^{c\text{-}src}$ has been shown to positively correlate with kinase activity (5, 29), and most activated oncogenic variants of pp60$^{c\text{-}src}$ are phosphorylated on tyrosine 416 (28). Phosphorylation at this site is not absolutely required for activation nor for transformation by highly oncogenic forms of pp60$^{v\text{-}src}$ (9, 46). However, phenylalanine substitution of tyrosine 416 causes a loss of transforming activity in less-potent transforming variants of pp60$^{c\text{-}src}$ (12). Such phosphorylation may be a consequence, rather than a cause, of activation. Phosphorylation of tyrosine 416 may also be important in the formation of signalling complexes between pp60$^{c\text{-}src}$ and SH2-containing signalling molecules during nomal cellular activation.

Another property that distinguishes pp60$^{c\text{-}src}$ from its activated, oncogenic variants is the ability to associate with the Triton X-100-insoluble cell material. All oncogenic vari-

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ants of pp60^{src} have been shown to be tightly associated with the Triton-insoluble cytoskeletal matrix, and this association correlates with the elevated tyrosine kinase activity of pp60^{src} (17, 23, 35). This association was determined to be dependent on the SH2 domain of pp60v-*src* (17).

These studies motivated us to investigate more thoroughly the role of pp60$^{c\text{-}src}$ in tyrosine phosphorylation of platelet proteins. Here we report that pp60$^{c\text{-}src}$ is rapidly and transiently activated upon thrombin stimulation of platelets and that a fraction of pp60$^{c\text{-}src}$ translocates to the Triton X-100-insoluble, actin-rich cytoskeletal fraction in which a large percentage of the tyrosine-phosphorylated platelet proteins are also found. This cytoskeleton-associated pp60$^{c\text{-}src}$ is phosphorylated on tyrosine 416, consistent with the hypothesis that the cytoskeleton-associated fraction of pp60$^{c\text{-}src}$ is activated. In addition, the association of pp60$^{c\text{-}src}$ with the Triton-insoluble fraction is dependent on the presence of GPIIb-IIIa, the platelet integrin receptor for fibrinogen, suggesting that integrins may, in association with the cytoskeleton, function as a foundation for the building of signalling complexes.

MATERIALS AND METHODS

Platelet preparation and activation. Venous blood was obtained from healthy, drug-free volunteers and platelets were prepared as previously described (19). The gel-filtered platelets were maintained in Walsh buffer (137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 5.6 mM glucose, 1 mg of bovine serum albumin per ml, 3.3 mM NaH_2PO_4, 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] [pH 7.4]) and adjusted to a final concentration of 4×10^8 platelets per ml. For in vivo labeling experiments, the platelets were maintained in a phosphate-free Walsh buffer and incubated for 2 h with 1 mCi of carrier-free $^{32}PO_4$ per ml of platelets (ICN, Costa Mesa, Calif.). Platelets were activated with bovine α-thrombin (0.1 U/ml; Chrono-log, Havertown, Pa.) at 37°C under constant stirring conditions at 1,000 rpm in a lumi-aggregometer (Chrono-log).

Platelet lysis and fractionation. After the platelets were activated for the prescribed period, activated and control platelets were immediately lysed with an equal volume of 2× Triton buffer (2% Triton X-100, 2 mM EGTA, 100 mM Tris [pH 7.2], 500 μg of leupeptin per ml, 0.2 mM phenylmethylsulfonyl fluoride, 2.0 mM benzamidine, 2.0 mM Na_3VO_4 [Fisher], aprotinin [FBA Pharmaceuticals, West Haven, Conn.] at 200 Kallikrein inactivator units/ml) for 5 min. This and all subsequent steps were performed at 4°C. The lysates were clarified by centrifugation at 16,000 × *g* for 5 min. The supernatant (Triton-soluble fraction) was removed, and the pellet was homogenized in RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 158 mM NaCl, 10 mM Tris [pH 7.2], 1 mM EGTA, 250 μg of leupeptin per ml, 0.1 mM phenylmethylsulfonyl fluoride, 1.0 mM benzamidine, 1.0 mM Na_3VO_4, aprotinin at 100 Kallikrein inactivator units/ml) for 30 min. This lysate was then clarified by centrifugation at 16,000 × *g* for 5 min. The supernatant (Triton-insoluble/RIPA-soluble fraction) was removed, and the pellet (Triton-insoluble/RIPA-insoluble) was solubilized in 1× SDS-sample buffer (2% SDS, 1.0% β-mercaptoethanol, 66 mM Tris [pH 7.5], 10 mM EDTA).

Immunoprecipitation of platelet fractions. Platelet lysates were incubated with one of two monoclonal antibodies (MAbs) to pp60$^{c\text{-}src}$ (LA074; Quality Biotech, Inc., Camden, N.J., or MAb 327 [33]) at 4°C for 120 min. Activated *Staphylococcus aureus* (Calbiochem, La Jolla, Calif.) bound with rabbit anti-mouse immunoglobulin G (Cappel, Durham, N.C.) was then added for an additional 40 min. The immunoprecipitate was then washed three times with ice-cold wash buffer (1% Triton X-100, 50 mM NaCl, 10 mM Tris [pH 7.5], 1 mM EGTA, 1 mM Na_3VO_4) for the Triton-soluble fraction or with RIPA buffer for the Triton-insoluble/RIPA-soluble fraction and divided in half for both an in vitro kinase assay and an anti-Src immunoblot.

In vitro kinase assay. The immunoprecipitates were washed once with low-salt buffer (100 mM NaCl, 5 mM $MnCl_2$, 10 mM Tris [pH 7.4]) and then preincubated with 25 μl of kinase reaction buffer (10 mM Tris [pH 7.4], 5 mM $MnCl_2$) for 5 min at 30°C. The kinase reaction was started with the addition of 5 μCi of [λ-^{32}P]ATP (1 Ci = 37 GBq; ICN), 1 μM ATP, and 5 μg of enolase (final concentrations). The reaction was terminated after 2 min with the addition of an equal volume of 2× SDS-sample buffer and heated at 100°C for 5 min. Samples were subjected to electrophoresis on SDS-7.5% polyacrylamide gels, dried, and quantitated with a Phosphorimager (Molecular Dynamics, Sunnyvale, Calif.).

Immunoblotting of phosphotyrosine-containing proteins. Immunoblot assays were performed essentially as described previously (48). Immunoblots of Src immunoprecipitates were probed with the Src MAb LA074 and then by ^{125}I-labeled goat anti-mouse immunoglobulins. The bound radioactivity was quantitated with a Phosphorimager. Phosphotyrosine-containing proteins were detected on immunoblots probed with a mixture of two antiphosphotyrosine MAbs, 4G10 (a gift from T. Roberts) and PY20 (ICN) and goat anti-mouse immunoglobulin-horseradish peroxidase (Bio-Rad Laboratories, Richmond, Calif.), using a chemiluminescence detection kit (ECL; Amersham).

Cleavage of pp60$^{c\text{-}src}$ proteins. pp60$^{c\text{-}src}$ immunoprecipitated from in vivo $^{32}PO_4$-labeled platelets with LA074 was cleaved with V-8 protease or CNBr exactly as previously described (43). In some experiments, the V_2 fragment from the V-8 protease digest was excised from the gel and subjected to CNBr digestion. Samples were quantitated with a Phosphorimager.

RESULTS

Activity of pp60$^{c\text{-}src}$ in Triton-soluble platelet fraction. Previous attempts to detect an increase in pp60$^{c\text{-}src}$ kinase activity from agonist-stimulated platelets were performed with excessive concentrations of thrombin (1 to 10 U/ml), and most measurements were made at time points relatively late in the activation process (30 s or later) (18, 22, 24). Since the activation of tyrosine phosphorylation and other events (such as the conformational change in GPIIb-IIIa) can occur as early as 5 s after thrombin stimulation (16, 18), we reexamined the activity of pp60$^{c\text{-}src}$ at earlier time points after stimulation using lower concentrations of thrombin.

pp60$^{c\text{-}src}$ was immunoprecipitated from Triton-soluble lysates of platelets treated with 0.1 U of thrombin per ml. The specific activity of enolase phosphorylation was assayed by immunocomplex kinase assays. A two- to fourfold increase in the specific activity of pp60$^{c\text{-}src}$ was detected 5 to 15 s after thrombin stimulation. The specific activity then returned to the level detected in unstimulated platelets (Fig. 1A). This activation of pp60$^{c\text{-}src}$ was observed when platelets were lysed in 1% Triton buffer but was not detected when platelets were lysed in RIPA buffer (for buffer components, see Materials and Methods). The detection of pp60$^{c\text{-}src}$ activation was also dependent on both platelet and agonist con-

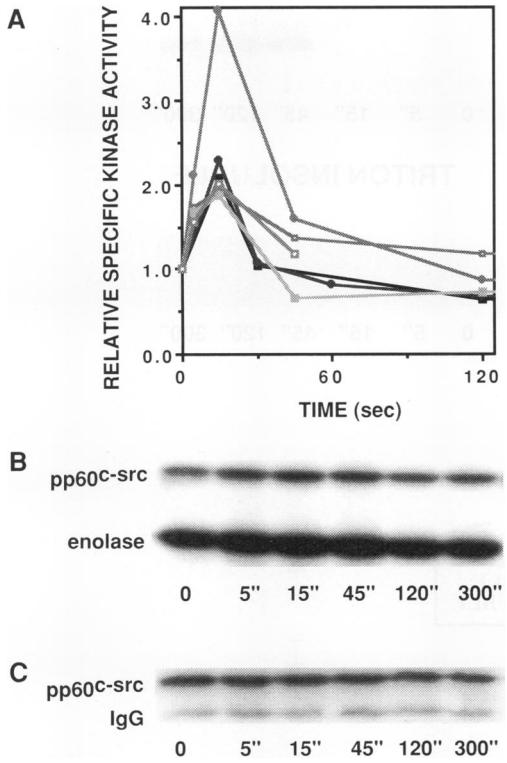

FIG. 1. pp60$^{c\text{-}src}$ specific kinase activity in Triton X-100-soluble fractions. Platelets (4×10^8/ml) stimulated with α-thrombin (0.1 U/ml) for the indicated times (0 to 300 s) were lysed in buffer containing 1% Triton X-100. pp60$^{c\text{-}src}$ was immunoprecipitated from the Triton-soluble lysate with pp60$^{c\text{-}src}$ MAbs 327 or LA074. After being washed, immunoprecipitates were divided equally; one half was subjected to an in vitro kinase assay with enolase as an exogenous substrate, and the other half was used for a quantitative immunoblot assay. Enolase phosphorylation and pp60$^{c\text{-}src}$ protein levels were assayed as described in Materials and Methods. The specific activity of pp60$^{c\text{-}src}$ was determined by measuring the incorporation of $^{32}PO_4$ into enolase relative to the protein level of pp60$^{c\text{-}src}$ in individual samples. This value was then set relative to the specific activity of pp60$^{c\text{-}src}$ in the unstimulated (0 s) immunoprecipitable samples. The graph in panel A represents results from six independent trials. The autoradiograms from one representative trial are shown in panels B (kinase assay) and C (immunoblot). The autoradiograms shown in this figure, and in subsequent figures, were scanned on a Hewltt-Packard Scanjet Plus scanner and printed on a Linotronic 230 linotype printer. IgG, immunoglobulin G.

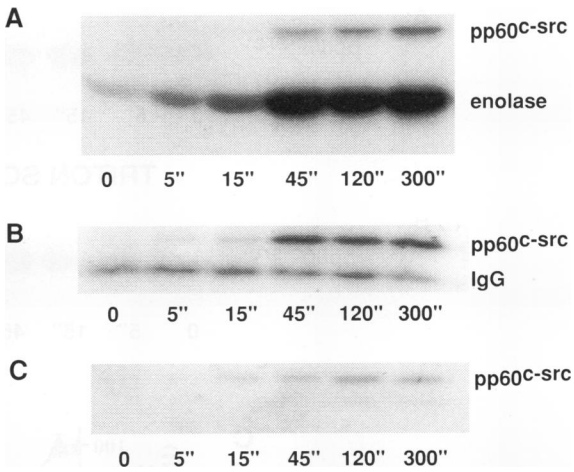

FIG. 2. pp60$^{c\text{-}src}$ kinase activity in Triton X-100-insoluble/RIPA-soluble fractions. Platelets were stimulated, lysed, and fractionated as described in the legend to Fig. 1. Triton-insoluble pellets were then lysed in RIPA buffer for 30 min. pp60$^{c\text{-}src}$ was immunoprecipitated from this Triton-insoluble/RIPA-soluble lysate with either pp60$^{c\text{-}src}$ MAb 327 or LA074. The Triton-insoluble/RIPA-insoluble pellet was heated in SDS sample buffer as described in Materials and Methods. After being washed, the immunoprecipitates were split in half and subjected to either in vitro kinase or immunoblot assays as described in the legend to Fig. 1. The autoradiograms from one of six trials are shown in panels A (kinase assay with enolase as an exogenous substrate) and B (quantitative immunoblot assay). Total pp60$^{c\text{-}src}$ detected in the Triton-insoluble/RIPA-insoluble pellets is shown in panel C. Numbers on bottom show time in seconds. IgG, immunoglobulin G.

centration since it was not observed when a higher concentration of thrombin (1 U/ml or greater) or of platelets (10^9 platelets per ml) was used. We surmise that under these conditions, the activation of pp60$^{c\text{-}src}$ may take place so rapidly that it is not detectable in our assay system. In addition, the inclusion of short-lived platelet activation inhibitors such as prostaglandin E_1 and apyrase to block premature activation of platelets during the preparation steps may contribute to an optimal detection of kinase activation.

Activity of pp60$^{c\text{-}src}$ in Triton-insoluble/RIPA-soluble platelet fraction. Recent work from this laboratory and others suggested that upon platelet stimulation pp60$^{c\text{-}src}$ translocates from the Triton-soluble supernatant to the Triton-insoluble pellet (11, 15a, 20, 25). In addition, activated oncogenic variants of pp60^{src} expressed in fibroblasts associate with the Triton-insoluble cellular matrix. To examine the level of pp60$^{c\text{-}src}$ activity in the Triton-insoluble fraction, we lysed pellets from resting and stimulated platelets in RIPA buffer to further solubilize the cytoskeletal-associated proteins. The level of pp60$^{c\text{-}src}$ activity immunoprecipitated from this Triton-insoluble/RIPA-soluble fraction was found to rapidly (within 5 s) and dramatically (as high as 30-fold at 300 s) increase after thrombin stimulation of platelets (Fig. 2A). We were unable to immunoprecipitate detectable amounts of pp60$^{c\text{-}src}$ from the Triton-insoluble pellet from resting platelets (Fig. 2B), suggesting that pp60$^{c\text{-}src}$ translocates to the Triton-insoluble cytoskeletal fraction only after platelet activation.

The observation that loss of pp60$^{c\text{-}src}$ activity from the Triton-soluble fraction coincided with the appearance of pp60$^{c\text{-}src}$ in the Triton-insoluble fraction suggested that this insoluble fraction might contain the activated fraction of pp60$^{c\text{-}src}$. However, the specific activity of the RIPA-soluble pp60$^{c\text{-}src}$ solubilized from the Triton-insoluble fraction was similar to that found in the Triton-soluble fraction (data not shown). This direct comparison of the specific activity of pp60$^{c\text{-}src}$ from the Triton-soluble and -insoluble fractions may be misleading since it was necessary to solubilize the Triton-insoluble pellet with RIPA buffer. To determine whether RIPA buffer altered the activity of platelet pp60$^{c\text{-}src}$ extracted in Triton buffers, we immunoprecipitated pp60$^{c\text{-}src}$ from Triton-soluble lysates of resting and activated platelets. The immunoprecipitates were divided equally, and one half was washed with Triton buffer and the other half with RIPA buffer. In the RIPA-washed samples, there was no detectable difference in the specific activity of pp60$^{c\text{-}src}$ immunoprecipitated from resting or thrombin-treated platelets,

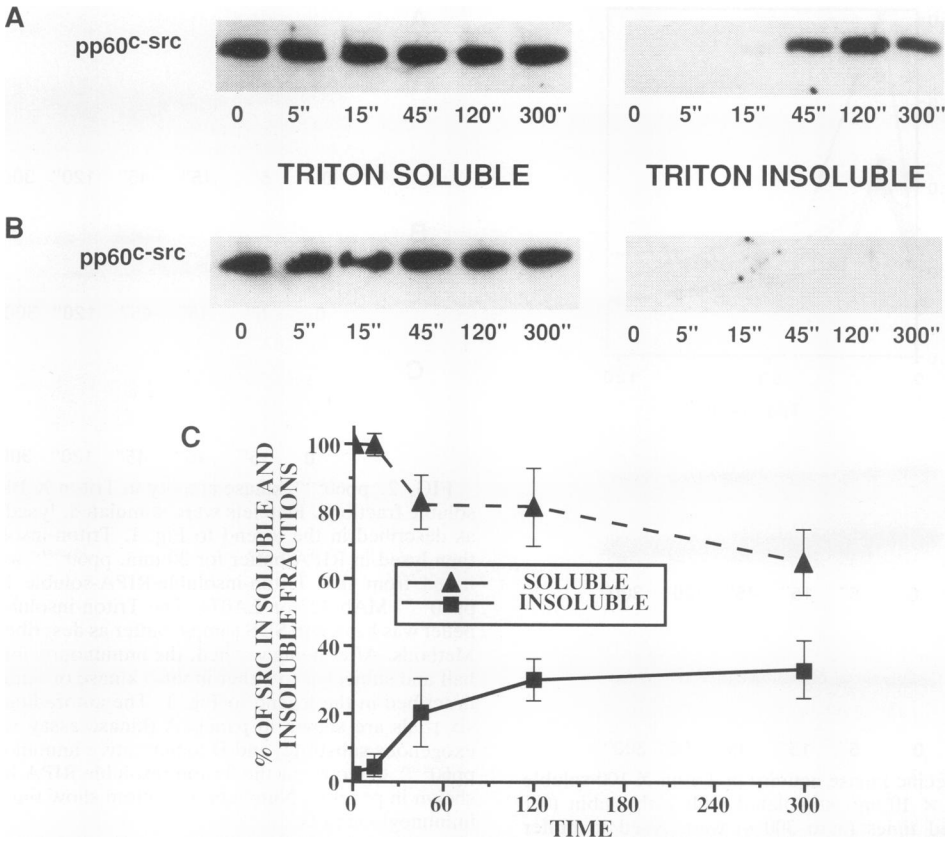

FIG. 3. pp60$^{c\text{-}src}$ protein levels in Triton X-100-soluble and -insoluble fractions. Platelets (4×10^8/ml) stimulated with α-thrombin (0.1 U/ml) for the indicated times (0 to 300 s) were lysed in Triton X-100-containing buffer as described in Materials and Methods. The Triton-soluble and Triton-insoluble lysates were solubilized in SDS-sample buffer and subjected to SDS-polyacrylamide gel electrophoresis. The samples were immunoblotted with biotinylated MAb 327–streptavidin-horseradish peroxidase and detected with an ECL chemiluminescence kit. The immunoblot in panel A shows the protein levels of pp60$^{c\text{-}src}$ in the Triton-soluble and Triton-insoluble fractions from 1×10^7 and 4×10^7 platelets, respectively. The immunoblot in panel B shows an identical time course fractionation experiment with platelets from a patient with Glanzmann's thrombasthenia. The graph in panel C shows the level of pp60$^{c\text{-}src}$ in the Triton-soluble and -insoluble fractions from four independent trials. The data were quantitated with a Molecular Dynamics Phosphorimager as described in Materials and Methods.

whereas the Triton-washed pp60$^{c\text{-}src}$ immunoprecipitates from platelets stimulated with thrombin for 15 s displayed a characteristic 2.4-fold-higher specific activity than resting platelets (data not shown). This suggests that RIPA buffer may affect the specific activity of pp60$^{c\text{-}src}$.

Redistribution of pp60$^{c\text{-}src}$ from the Triton-soluble to the Triton-insoluble platelet fraction. To determine the percentage of pp60$^{c\text{-}src}$ that redistributes to the Triton-insoluble fraction under our experimental conditions, Triton-soluble and Triton-insoluble fractions from resting and thrombin-stimulated platelets were directly solubilized in SDS-sample buffer. After electrophoresis and transfer to nitrocellulose, the immunoblots were probed with pp60$^{c\text{-}src}$ MAb 327 (Fig. 3A). pp60$^{c\text{-}src}$ was not detected in the Triton-insoluble fraction of unstimulated plateletes in contrast to a recent study by Horvath et al. (25). We believe that the different method of platelet isolation used by these researchers may cause the premature activation of these platelets, resulting in the premature redistribution of pp60$^{c\text{-}src}$ to the Triton-insoluble fraction. Alternatively, the gel filtration of platelets used in our study may cause pp60$^{c\text{-}src}$ to dissociate from the Triton-insoluble cytoskeletal fraction. pp60$^{c\text{-}src}$ was first detected in the Triton-insoluble fraction 45 s after thrombin stimulation, and the levels of pp60$^{c\text{-}src}$ in this fraction increased throughout the time course of thrombin treatment. Under the conditions used here, only 20 to 30% of the total pp60$^{c\text{-}src}$ was detected in the Triton-insoluble fraction in platelets stimulated with thrombin for 2 to 5 min. This increase in pp60$^{c\text{-}src}$ protein levels in the Triton-insoluble fraction paralleled a 20 to 30% decrease in pp60$^{c\text{-}src}$ in the Triton-soluble fraction (Fig. 3C).

Since thrombin induction of tyrosine phosphorylation of several platelet proteins was previously shown to be dependent on platelet aggregation mediated by the integrin receptor GPIIb-IIIa, we examined the detergent solubility profile of pp60$^{c\text{-}src}$ in platelets genetically deficient in surface expression of functional GPIIb-IIIa (from donors with Glanzmann's thrombasthenia). Thrombin stimulation of Glanzmann's platelets induces secretion of alpha and dense granules, but platelet aggregation is inhibited because of the absence of GPIIb-IIIa, the integrin receptor for fibrinogen, which mediates platelet-to-platelet aggregation. Our studies revealed that thrombin stimulation of GPIIb-IIIa-deficient platelets did not cause a redistribution of pp60$^{c\text{-}src}$ to the Triton-insoluble fraction (Fig. 3B). Although pp60$^{c\text{-}src}$ was not detected in this Triton-insoluble fraction from these platelets, many of the abundant cytoskeletal proteins (i.e., actin, myosin, actin-binding protein) were found to redistrib-

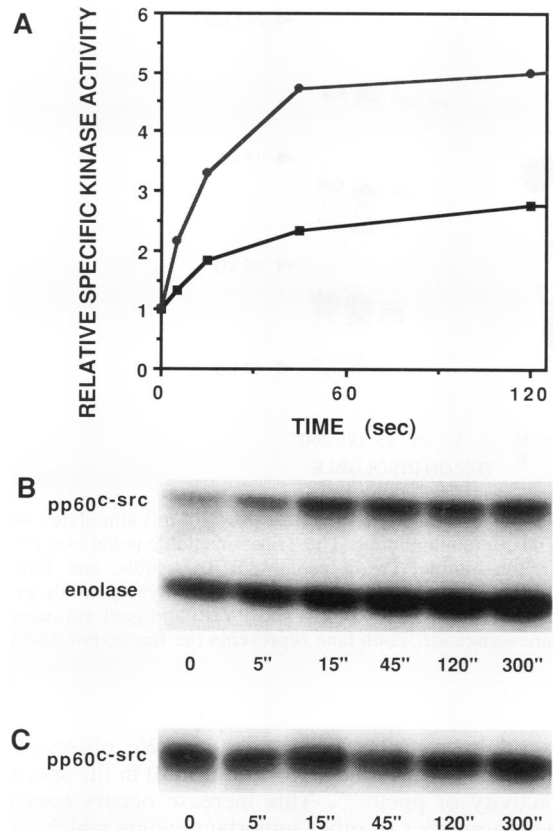

FIG. 4. pp60$^{c\text{-}src}$ specific kinase activity in Trition X-100-soluble fractions from thrombin-stimulated platelets from patients with Glanzmann's thrombasthenia. Platelets (4×10^8 ml) from patients with Glanzmann's thrombasthenia were stimulated and lysed, and pp60$^{c\text{-}src}$ was immunoprecipitated from the lysate exactly as described in the legend to Fig. 1. The relative specific kinase activity for two trials was determined as described in the legend to Fig. 1 and is shown in panel A. The autoradiograms from one trial (showing the threefold increase in the relative specific kinase activity at 300 s) are shown in panels B (kinase assay with enolase as an exogenous substrate) and C (immunoblot assay).

ute to this fraction (data not shown). This result suggests that the association of pp60$^{c\text{-}src}$ with the Triton-insoluble fraction, like the induction of tyrosine phosphorylation of a number of platelet proteins (14, 19), is GPIIb-IIIa dependent.

Activity of pp60$^{c\text{-}src}$ in Glanzmann's thrombasthenia platelets. Since the thrombin-induced redistribution of pp60$^{c\text{-}src}$ is altered in GPIIb-IIIa-deficient platelets, we examined whether the activation of pp60$^{c\text{-}src}$ is also GPIIb-IIIa dependent and whether the absence of a translocation of pp60$^{c\text{-}src}$ affected the detection of activated pp60$^{c\text{-}src}$ in the Triton-soluble fraction (Fig. 4). We observed a three- to sixfold increase in the specific activity of pp60$^{c\text{-}src}$ in the Triton-soluble fraction of thrombin-stimulated platelets from thrombasthenic patients. pp60$^{c\text{-}src}$ activity was near maximal at 45 s after thrombin stimulation and was maintained at this level throughout the rest of the time course studied (Fig. 4A and B). This sustained activation is clearly distinct from the transient activation of pp60$^{c\text{-}src}$ observed in normal platelets (Fig. 1A and B). These data suggest that pp60$^{c\text{-}src}$ is activated prior to, or independent of, fibrinogen binding to GPIIb-IIIa and that the loss of detection of activated pp60$^{c\text{-}src}$ in the Triton-soluble fraction from normal platelets may result from the movement of the activated pp60$^{c\text{-}src}$ to the Triton-insoluble fraction.

Appearance of phosphotyrosine-containing proteins in the Triton-insoluble platelet fraction. Since translocation of pp60$^{c\text{-}src}$ to the Triton-insoluble platelet fraction may facilitate the agonist-induced increase in tyrosine phosphorylation, we used antiphosphotyrosine immunoblots to examine tyrosine-phosphorylated proteins in platelet fractions that contain different levels of pp60$^{c\text{-}src}$. The immunoblots of Triton-soluble, Triton-insoluble/RIPA-soluble, and Triton-insoluble/RIPA-insoluble platelet fractions all showed a thrombin-induced increase in phosphotyrosine-containing proteins (Fig. 5). Two major phosphotyrosine-containing protein bands with apparent molecular masses of 60 and 120 kDa were detected in the Triton-soluble fraction of unstimulated platelets. The 60-kDa band which reacts with MAbs to pp60$^{c\text{-}src}$ has been shown to be tyrosine phosphorylated exclusively on tyrosine 527 in resting platelets (13), while the identity of the 120-kDa band, which appears to contain multiple phosphotyrosine-containing proteins, has yet to be determined. The Triton-insoluble fractions of unstimulated platelets (Triton-insoluble/RIPA-soluble and -insoluble) do not have detectable levels of phosphotyrosine-containing proteins. However, high levels of phosphotyrosine-containing proteins were detected in thrombin-stimulated platelets, many of which appear in both Triton-soluble and Triton-insoluble fractions. At this time we are unable to determine whether the phosphotyrosine-containing proteins found in the Triton-insoluble fractions are phosphorylated in the soluble fraction before translocating to the insoluble fractions, or whether they are present in the insoluble fraction and only phosphorylated when the tyrosine kinase(s) also translocates. It is worth noting that the level of phosphotyrosine in the Triton-insoluble fraction correlates well with the presence of pp60$^{c\text{-}src}$ in this fraction (compare Fig. 3A with Fig. 5).

Analysis of phosphorylation state of pp60$^{c\text{-}src}$. The negative regulation of pp60$^{c\text{-}src}$ kinase activity is proposed to occur via phosphorylation at tyrosine 527 (6, 8). One mechanism of activation of pp60$^{c\text{-}src}$ may involve dephosphorylation at this site by a protein tyrosine phosphatase (PTP). In thrombin-treated platelets, we consistently observed a slight decrease in phosphotyrosine content of both the 60- and 120-kDa bands on antiphosphotyrosine immunoblots (i.e., Fig. 5, 5s), suggesting that a PTP(s) might be activated at this time. In resting platelets, tyrosine 527 is the only phosphorylated tyrosine residue, and thrombin stimulation causes a twofold increase in the phospholabeling of tyrosine 527 (13). If the stoichiometry of phosphorylation of tyrosine 527 is nearly 100%, as it appears to be in fibroblasts (6), this twofold increase in phospholabeling of tyrosine 527 could reflect the turnover of unlabeled PO_4 for $^{32}PO_4$ at this site. This would suggest at least a transient activation of pp60$^{c\text{-}src}$.

The phosphorylation state of pp60$^{c\text{-}src}$ was analyzed by V-8 protease digestion of pp60$^{c\text{-}src}$ immunoprecipitated from RIPA-soluble and Triton-soluble ^{32}P-labeled platelet fractions. We observed a slight (13.5% ± 2.0%) decrease in $^{32}PO_4$ labeling of the carboxy-terminal (V_2) fragment that contains tyrosine 527 (Fig. 6). This decrease was transient, occurring at 5 to 15 s after thrombin stimulation, after which a steady increase in phosphorylation of this fragment was observed (Fig. 6A and B). ^{32}P incorporation into amino-terminal fragments of pp60$^{c\text{-}src}$ did not decrease during the 5- and 15-s time points; rather, we detected a continual increase throughout the time course observed. Although this apparent dephosphorylation of the carboxy-terminal frag-

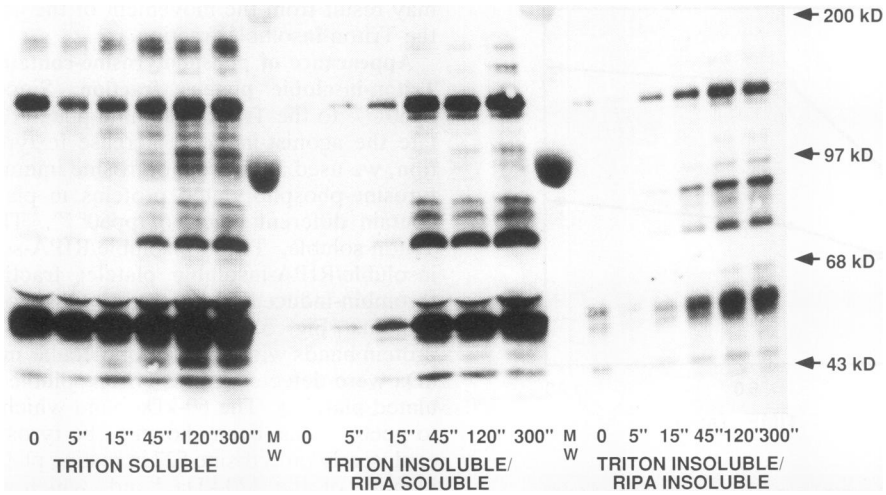

FIG. 5. Antiphosphotyrosine immunoblot of platelet fractions from thrombin-stimulated platelets. Platelets (4×10^8/ml) stimulated with α-thrombin (0.1 U/ml) for the times indicated (0 to 300 s) were lysed in Triton X-100-containing buffer. The Triton-insoluble pellet was lysed in RIPA buffer and fractionated as described in Materials and Methods. The Triton-soluble, Triton-insoluble/RIPA-soluble, and Triton-insoluble/RIPA-insoluble samples were solubilized in SDS-sample buffer and subjected to electrophoresis on 7.5% polyacrylamide gels, transferred to nitrocellulose, and immunoblotted with a combination of antiphosphotyrosine MAbs (4G10 and PY20) and goat anti-mouse immunoglobulin-horseradish peroxidase before detection with an ECL chemiluminescence kit. Each lane represents the fractionated lysate from 1.5×10^7 platelets. kD, kilodaltons.

ment was minimal, with the decrease ranging from 10 to 15%, it was reproducibly detected in four experiments. In addition, the increase in the amino-terminal phosphorylation and the decrease in the carboxy-terminal phosphorylation was observed in the same samples. Finally, at 5 and 15 s after thrombin stimulation, the 60-kDa protein observed on antiphosphotyrosine immunoblots (which immunoreacts with MAbs to pp60$^{c\text{-}src}$) was found to contain only 85% of the phosphotyrosine immunoreactivity observed in resting platelets (data not shown), suggesting that dephosphorylation of phosphotyrosine residues is taking place immediately after thrombin stimulation.

Triton-soluble and -insoluble pp60$^{c\text{-}src}$ fractions were subjected to cleavage with CNBr to determine the site(s) of phosphorylation. All the incorporated ^{32}P from the V_2 fragment of the Triton-soluble pp60$^{c\text{-}src}$ was detected in the 4-kDa fragment that contains tyrosine 527 (Fig. 6C). No phosphorylation was detected in the 10-kDa fragment that contains tyrosine 416. However, ^{32}P incorporation was detected in the 10-kDa fragment (tyrosine 416) as well as in the 4-kDa fragment (tyrosine 527) of pp60$^{c\text{-}src}$ immunoprecipitated from the Triton-insoluble/RIPA soluble fraction of thrombin-stimulated platelets (Fig. 6D). Phosphorylation at tyrosine 416 has been detected in activated oncogenic variants of pp60$^{c\text{-}src}$ (28), and the activity of pp60$^{c\text{-}src}$ is reduced 50% by substitution of this tyrosine with phenylalanine (40). Therefore, tyrosine 416 is thought to be necessary for full activation pp60$^{c\text{-}src}$'s kinase activity. In this analysis, we were unable to discern whether individual pp60$^{c\text{-}src}$ molecules are phosphorylated on both of these tyrosine residues, or if half the ^{32}P-labeled molecules are phosphorylated on tyrosine 416, while the other half are phosphorylated on tyrosine 527.

DISCUSSION

In an attempt to understand the physiological role of pp60$^{c\text{-}src}$, we reexamined the effect of agonist stimulation of platelets on its tyrosine kinase activity. We observed a transient and small increase (two to fourfold) in the specific kinase activity of pp60$^{c\text{-}src}$. This increase occurs concurrently with a number of other important events which take place in platelets; these include a conformational change in GPIIb-IIIa which allows it to bind fibrinogen (16), an activation of phospholipase C, which catalyzes the hydrolysis of inositol phospholipids (33), platelet shape change which is directly linked to reorganization of the actin cytoskeleton (4), and the association of phosphatidylinositol 3-kinase with pp60$^{c\text{-}src}$ (22). Such rapid responses of platelets to thrombin stimulation emphasize the plausibility of this early activation of pp60$^{c\text{-}src}$.

The increase in pp60$^{c\text{-}src}$ specific activity after thrombin treatment is modest compared with the 10- to 20-fold increase in specific activity observed when pp60$^{c\text{-}src}$ is treated with phosphatase (7) or when complexed with polyomavirus middle tumor antigen (1). However, if only a small fraction (10%) of the total pp60$^{c\text{-}src}$ pool was activated 20- to 40-fold, this could account for the 2- to 4-fold activation observed here. Given the high levels of pp60$^{c\text{-}src}$ in platelets, activation of only a small fraction of this population could have a significant effect on protein tyrosine phosphorylation.

The results of this study strongly support the possibility that the activated population of pp60$^{c\text{-}src}$ is translocated to the Triton-insoluble cytoskeletal fraction. First, the loss of pp60$^{c\text{-}src}$ activity from the Triton-soluble fraction paralleled the appearance of pp60$^{c\text{-}src}$ in the Triton-insoluble fraction, consistent with the hypothesis that activated pp60$^{c\text{-}src}$ redistributes to the Triton-insoluble fraction. Our data suggest that pp60$^{c\text{-}src}$'s translocation to the Triton-insoluble cytoskeletal fraction occurs after activation of the kinase. One possible model would have pp60$^{c\text{-}src}$ activated at 5 to 45 s, then translocating to the cytoskeletal fraction at 45 to 300 s. Second, the sustained three- to sixfold activation of pp60$^{c\text{-}src}$ from thrombasthenic platelets, which occurs in the absence of translocation to the Triton-insoluble fraction, strongly suggests that the transient nature of the activation may result

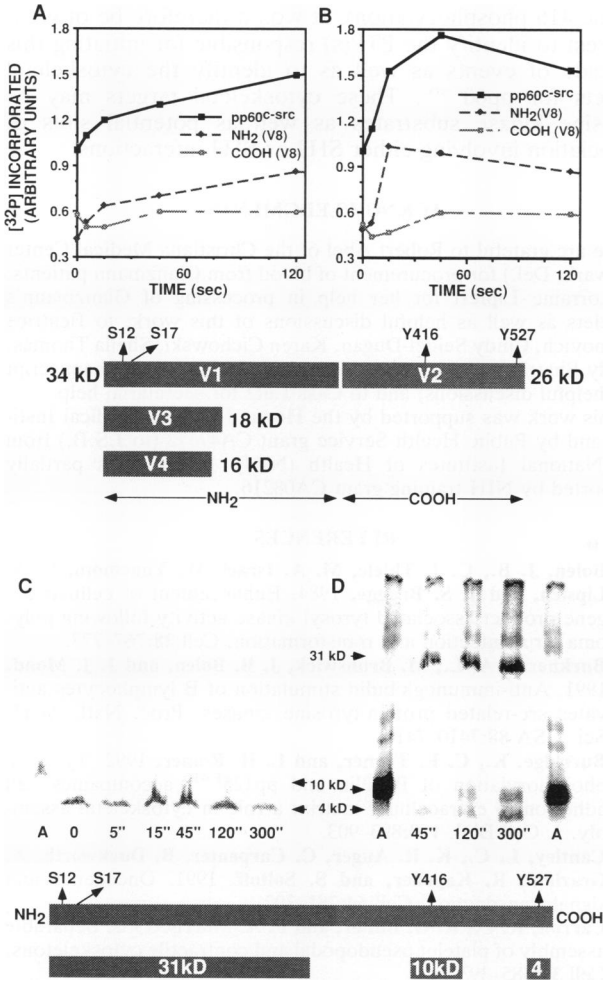

FIG. 6. Analysis of phosphorylation state of pp60$^{c\text{-}src}$. In vivo $^{32}PO_4$-labeled platelets were stimulated with α-thrombin (0.1 U/ml) for 0 to 300 s and lysed in RIPA buffer (A) or Triton X-100-containing lysis buffer (B) as described in Materials and Methods. pp60$^{c\text{-}src}$ was immunoprecipitated from the lysates with MAb LA074 and subjected to electrophoresis on 7.5% polyacrylamide gels. The 60-kDa (kd) band was excised from the gel and subjected to limited proteolysis with V-8 protease as described in Materials and Methods. The amount of $^{32}PO_4$ incorporated into pp60$^{c\text{-}src}$ or its amino- and carboxy-terminal proteolytic fragments (NH_2 and COOH, respectively) was quantitated in panels A and B with a Molecular Dynamics Phosphorimager. The ^{32}P incorporated into pp60$^{c\text{-}src}$ in unstimulated platelets was arbitrarily set as 1.0, and the sum of the amino-terminal plus the carboxy-terminal peptide fragments of pp60$^{c\text{-}src}$ from these unstimulated platelets was set equal to 1.0. The data shown are from two representative experiments (of four trials). Shown below panels A and B is a diagram of the aminoterminal (V_1, V_3, and V_4) and carboxy-terminal (V_2) V-8 proteolytic digestion products for pp60$^{c\text{-}src}$ as well as the major sites of serine (S-12 and S-17) and tyrosine (Y-416 and Y-527) phosphorylation. The CNBr cleavage of pp60$^{c\text{-}src}$ into amino-terminal (31-kDa) and carboxy-terminal (10- and 4-kDa) fragments is shown diagramatically below panels C and D. The V_2 peptide fragments from the Triton-soluble fraction in panel B were excised from the gel and subjected to CNBr cleavage. The autoradiogram of these CNBr-cleaved V_2 fragments is shown in panel C. pp60$^{c\text{-}src}$ which had been $^{32}PO_4$ radiolabeled in an in vitro kinase reaction (see Materials and Methods) was also subjected to CNBr cleavage. This sample (lane A) was used as a marker for the 10-kDa CNBr fragment which includes tyrosine 416, the major site of in vitro autophosphorylation. The 4-kDa CNBr fragment includes tyrosine 527, the major site of in vivo tyrosine phosphorylation. In panel D, pp60$^{c\text{-}src}$ was immunoprecipitated with LA074 directly from the Triton-insoluble/RIPA-soluble platelet fraction, subjected to electrophoresis on 7.5% polyacrylamide gels, excised from these gels, and subjected to CNBr cleavage. The 31-kDa CNBr fragment corresponds to an amino-terminal fragment of pp60$^{c\text{-}src}$ which contains two sites of serine phosphorylation. This 31-kDa fragment is not contained within the V_2 fragment of pp60$^{c\text{-}src}$ and is therefore not present in the autoradiogram shown in panel C.

from pp60$^{c\text{-}src}$ redistribution. Finally, the observation that Triton-insoluble pp60$^{c\text{-}src}$ is phosphorylated on tyrosine 416 suggests that pp60$^{c\text{-}src}$ in this fraction is activated. How this activation and subsequent movement occur is relevant to the question of pp60$^{c\text{-}src}$'s role in both platelet activation and cellular transformation.

The translocation of activated pp60$^{c\text{-}src}$ to the Triton-insoluble fraction is consistent with previous studies of activated, oncogenic mutant variants of pp60^{src}. All oncogenic variants of pp60^{src} have been shown to be tightly associated with the Triton-insoluble cytoskeletal matrix, and this association correlates with the elevated tyrosine kinase activity of pp60^{src} (17, 23).

Our observation that the carboxy-terminal V-8 fragment of pp60$^{c\text{-}src}$ is dephosphorylated transiently during the same period (from 5 to 15 s after thrombin stimulation) when pp60$^{c\text{-}src}$ kinase activity is observed to be maximally activated suggests that the observed increase in specific activity of pp60$^{c\text{-}src}$ might result from activation of a PTP activity that dephosphorylates pp60$^{c\text{-}src}$ at tyrosine 527. We have partially purified a PTP activity from platelets capable of dephosphorylating tyrosine 527 (unpublished observations). In addition, the apparent decrease in antiphosphotyrosine immunostaining of pp60$^{c\text{-}src}$ at 5 and 15 s after thrombin stimulation suggests that such a PTP might be active at this time point. In polyomavirus-infected cells, activated pp60$^{c\text{-}src}$ is also not phosphorylated on tyrosine 527 (5). These data suggest a common mechanism to activate pp60$^{c\text{-}src}$ and perhaps other Src family kinases. For example, the interaction between a Src-related kinase, pp56^{lck}, and CD45, a PTP highly expressed in nucleated cells of hematopoietic origin, appears to play a critical role in T-cell receptor-mediated signalling (for a review, see reference 45). pp56^{lck} is activated upon dephosphorylation by CD45 in vitro, and activation of pp56^{lck} does not occur in T-cell mutants that lack CD45 (36–38).

In addition to tyrosine 527 dephosphorylation of pp60$^{c\text{-}src}$, the phosphorylation of tyrosine 416 is thought to positively affect both the kinase activity and oncogenic potential of pp60$^{c\text{-}src}$ (12, 30, 40). In platelets, we observed phosphorylation of tyrosine 416 exclusively in the Triton-insoluble cytoskeletal-associated fraction of pp60$^{c\text{-}src}$. The phosphorylation of pp60$^{c\text{-}src}$ at tyrosine 416 implies that the cytoskeleton-associated pp60$^{c\text{-}src}$ is activated, since activated mutants of pp60^{src} are phosphorylated on tyrosine 416 in vivo (for a review, see reference 28).

In a working model based on the data reported here, pp60$^{c\text{-}src}$ would be rapidly dephosphorylated at tyrosine 527 upon thrombin stimulation in platelets, causing an activation of its kinase activity. The dephosphorylation would release the pp60$^{c\text{-}src}$ SH2 domain, which is believed to associate with phosphorylated tyrosine 527 in the unactivated kinase (3, 41). The dephosphorylated pp60$^{c\text{-}src}$ would now be able to associate with the reorganizing cytoskeleton via its SH2 domain, which has been shown to be necessary for cytoskeletal association of pp60$^{v\text{-}src}$ (17). In addition, the Src homol-

ogy 3 (SH3) domain may play an accessory role to SH2 in cytoskeletal association (31), and SH2-containing proteins in the cytoskeletal matrix (e.g., tensin [10]) could also complex with pp60$^{c\text{-}src}$ by binding to phosphotyrosine 416. pp60$^{c\text{-}src}$ would then be poised to phosphorylate cytoskeletal substrates, which could potentially affect cytoskeletal reorganization as well as activate other signalling molecules involved in events leading to increased adhesion and clot retraction.

The evidence that pp60$^{c\text{-}src}$ was unable to associate with the cytoskeletal fraction from thrombasthenic platelets lacking functional GPIIb-IIIa suggests that this integrin receptor may be necessary for assembly of the Triton-insoluble cytoskeletal complex that pp60$^{c\text{-}src}$ binds to in thrombin-treated platelets. In addition, Horvath et al. (25) have shown that incubation of platelets with the peptide RGDS or with EDTA, both of which prevent fibrinogen binding to GPIIb-IIIa, prevents the thrombin-induced cytoskeletal association of pp60$^{c\text{-}src}$. Integrin receptors in other cells associate with cytoskeletal proteins including talin and α-actinin which bind to actin microfilaments. Similar integrin-cytoskeletal complexes could serve to anchor pp60$^{c\text{-}src}$ and other signalling molecules in platelets. GPIIb-IIIa becomes associated with the core actin cytoskeleton in aggregated platelets, possibly through interactions with talin, vinculin, and α-actinin (15, 39). Our preliminary studies show that three Src-like kinases (Fyn, Lyn, and Yes), the p85 subunit of phosphatidylinositol 3-kinase, and the GTPase-activating protein of Ras, all of which contain SH2 and SH3 domains, are also present in the Triton-insoluble fraction of activated platelets (5a). Phosphatidylinositol 3-kinase activity as well as diacylglycerol kinase have been shown to redistribute to the Triton-insoluble fraction after platelet activation (20, 49). These data suggest that integrin-cytoskeleton interactions may compartmentalize or stabilize complexes required for transduction of signals initiated by integrin receptors.

Tyrosine phosphorylation has been implicated in events triggered by integrin receptors in platelets and other cell types. Thrombin-stimulated tyrosine phosphorylation of several proteins is dependent on platelet-to-platelet aggregation mediated by fibrinogen binding to GPIIb-IIIa (14, 19), and spreading of platelets on a fibrinogen matrix induces tyrosine phosphorylation of multiple cellular proteins (22a). In addition, spreading of NIH 3T3 cells on fibronectin or cross-linking of β1 integrins on the human carcinoma KB cell line also causes an induction of tyrosine phosphorylation (21a, 32). Integrin-stimulated tyrosine phosphorylation may be mediated in part by the protein tyrosine kinase pp125^{FAK} (42), which is activated and phosphorylated on tyrosine in thrombin-treated platelets in a GPIIb-IIIa-dependent manner (33a). In addition, pp125^{FAK} is phosphorylated in NIH 3T3 cells, KB cells, or chicken embryo fibroblasts after integrin engagement (2a, 21, 31a, 42a). However, our data showing GPIIb-IIIa-dependent association of pp60$^{c\text{-}src}$ with the Triton-insoluble cytoskeleton suggest that pp60$^{c\text{-}src}$ may also be responsible for at least some of the integrin-dependent tyrosine phosphorylation events in platelets.

Although the exact nature of these molecular interactions, especially those which require tyrosine phosphorylation, remains to be elucidated, it seems apparent that integrin-cytoskeletal complexes may serve as a framework for formation of signalling complexes. In addition, the observations made here suggest for the first time that normal cellular processes involving the activation of pp60$^{c\text{-}src}$ may involve similar alterations in the properties of this kinase as those that are altered in oncogenic variants of pp60^{src} (i.e., cytoskeletal association, tyrosine 527 dephosphorylation, tyrosine 416 phosphorylation). It would therefore be of great interest to identify the PTP(s) responsible for initiating this cascade of events as well as to identify the cytoskeletal targets for pp60$^{c\text{-}src}$. These cytoskeletal targets may be tyrosine kinase substrates as well as potential sites of association involving either SH2 or SH3 interactions.

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