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Thrombin-Dependent Association of Phosphatidylinositol-3 Kinase with $p60^{c\text{-}src}$ and $p59^{fyn}$ in Human Platelets

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Recent studies have shown that ligand-activated growth factor receptors as well as transforming versions of nonreceptor protein-tyrosine kinases physically associate with phosphatidylinositol-3 kinase (PI-3 kinase). Reasoning that PI-3 kinase might also play a role in the normal functions of nonreceptor kinases, we sought to determine whether association with PI-3 kinase might serve as a measure of nonreceptor protein-tyrosine kinase activation under physiological conditions. We found that $p60^{c\text{-}src}$ as well as $p59^{fyn}$, the product of another member of the *src* family of proto-oncogenes, physically associated with a PI kinase activity within 5 s after exposure to thrombin. Furthermore, PI kinase reaction products generated in $p60^{v\text{-}src}$, $p60^{c\text{-}src}$ or $p59^{fyn}$ containing immunoprecipitates were indistinguishible, demonstrating the identity of the associated enzyme as PI-3 kinase. These findings demonstrate a thrombin-dependent interaction between $p60^{c\text{-}src}$ or $p59^{fyn}$ and PI-3 kinase and suggest a role for nonreceptor protein-tyrosine kinases in human platelet signal transduction.

Protein-tyrosine kinases are encoded by roughly half of the proto-oncogenes described to date. These enzymes fall into two distinct categories, the polypeptide growth factor receptors and proteins such as $p60^{c\text{-}src}$ which lack the transmembrane and extracellular domains characteristic of the receptor class. Proteins of the receptor class act as mediators of proliferative signals that include platelet-derived growth factor (PDGF) and epidermal growth factor. Physiological roles for nonreceptor kinases have not yet been uncovered, although abundant concentrations of *src*, *yes*, and *fgr* gene products in postmitotic cells have suggested their involvement in processes unique to fully differentiated cells (6, 7, 19).

Phosphatidylinositol-3 (PI-3) kinase is an enzyme that catalyzes phosphorylation of phosphoinositides at position 3 within the inositol ring. This enzyme has been implicated in the mitogenic response to polypeptide growth factors through its association with the intracellular domain of ligand-bound growth factor receptors (1, 2). PI-3 kinase kinase activity also physically associates with transforming but not with nontransforming versions of the polyomavirus middle T–$p60^{c\text{-}src}$ complex (8, 9, 15, 20–22) and with the protein product of the v-*src* oncogene (4). In this study, we have investigated the association of PI-3 kinase with normal nonreceptor protein-tyrosine kinases expressed in their natural settings.

Expression of $p60^{c\text{-}src}$ and $p59^{fyn}$ in human platelets. Previous studies have demonstrated that $p60^{c\text{-}src}$, the protein product of the c-*src* proto-oncogene, is abundant in human platelets, constituting as much as 0.4% of total platelet protein (5, 6). Treatment of human platelets with thrombin, the physiological activator, produces a dramatic increase in the tyrosine phosphorylation of several proteins (3, 5). However, no increase in the specific activity of the $p60^{c\text{-}src}$ tyrosine kinase has been observed in response to platelet activators (3, 5). In a search for other protein-tyrosine kinases expressed in platelets, we took advantage of our specific antipeptide sera directed against $p55^{c\text{-}fgr}$ and $p59^{fyn}$, both members of the *src* family of nonreceptor tyrosine kinases known to be expressed in hematopoietic cells (7, 11, 12).

Platelets, obtained at the National Institutes of Health blood bank from healthy human donors, were washed and suspended at a final concentration 5×10^8 cells per ml in a buffer containing 137 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5.6 mM glucose, 5 mM NaHEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid sodium, pH 7.4), 0.1% bovine serum albumin (essentially fatty acid free; Sigma Chemical Co., St. Louis, Mo.), and 1 U of apyrase (grade V; Sigma) per ml (3). Platelets or fibroblasts were lysed in a buffer containing 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM Na_4VO_3, 1% Nonidet P-40, 10% glycerol, 20 mM Tris (pH 8.0), 0.1 mM leupeptin, 1% Trasylol, and 1 mM phenylmethylsulfonyl fluoride for 20 min at 4°C. Insoluble material was removed by centrifugation at 4°C for 20 min at 10,000 × *g*. Soluble lysate containing 200 μg of total cellular protein was incubated with 3 μl of the anti-Src monoclonal antibody (MAb) 327 (kindly provided by J. Brugge) or anti-Fyn peptide antibodies (10). Immunoprecipitates were recovered with the aid of *Staphylococcus aureus* protein A bound to Sepharose beads (Pharmacia Fine Chemicals, Piscataway, N.J.). For anti-Src MAb 327, beads were precoated with a rabbit anti-mouse affinity-purified polyclonal antiserum (Organon Teknika). Immune complex kinase assays using rabbit muscle enolase (Sigma) as an exogenous substrate were performed as described previously (7).

Whereas no $p55^{c\text{-}fgr}$ was detected (data not shown), $p60^{c\text{-}src}$ and $p59^{fyn}$ kinase activities were readily observed in platelet lysates (Fig. 1). In control experiments, low levels of $p60^{c\text{-}src}$ and $p59^{fyn}$ were detected in NIH 3T3 fibroblasts, and increased activity was observed in lysates of NIH 3T3 cells transfected with c-*src* (18) or *fyn* (10) expression plasmids (Fig. 1). The specificity of the immunoprecipitations was further demonstrated by competition with the corresponding $p59^{fyn}$ peptide and by immunoprecipitation with an MAb directed against the myeloid-specific protein CD11b (Fig. 1).

Platelet preparations were treated for times ranging from 5 to 180 s with purified human thrombin (3,000 U/mg of protein; >99% α-thrombin), a kind gift from John Fenton, New York State Department of Health. The concentration

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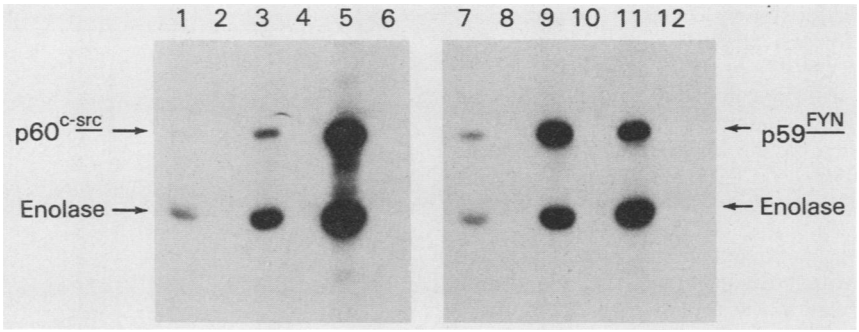

FIG. 1. Detection of $p60^{c\text{-}src}$ and $p59^{fyn}$ kinase activity in platelet extracts. Lysates of NIH 3T3 fibroblasts (lanes 1, 2, 7, and 8) and NIH 3T3 cells overexpressing $p60^{c\text{-}src}$ (lanes 3 and 4) or $p59^{fyn}$ (lanes 9 and 10) as well as human platelets (lanes 5, 6, 11, and 12) were immunoprecipitated with anti-$p60^{src}$ MAb 327 (lanes 1, 3, and 5), anti-CD11b (lanes 2, 4, and 6), or anti-$p59^{fyn}$ antibody (lanes 7 to 12) and assayed for kinase activity. In some cases (lanes 8, 10, and 12), antibody was incubated with excess homologous peptide before immunoprecipitation. Locations for $p60^{c\text{-}src}$, $p59^{fyn}$, and enolase are also shown.

used (1 U/ml) was sufficient to cause maximal platelet shape change and aggregation, as determined by microscopic examination. Under these conditions, we observed significant increases in tyrosine phosphorylation of several proteins but no change in the in vitro kinase activities of either $p60^{c\text{-}src}$ or $p59^{fyn}$ (data not shown). These findings are consistent with the results of previous studies (3, 5; I. D. Horak, M. L. Corcoran, P. A. Thompson, L. M. Wahl, and J. B. Bolen, Oncogene, in press).

Thrombin-dependent association of a PIK with $p60^{c\text{-}src}$ and $p59^{fyn}$. Several potentially important substrates for stimulated PDGF receptor have been reported recently. These include the c-*raf* proto-oncogene product, $p70^{raf}$ (16), phospholipase Cγ (13), GTPase-activating protein (14), and PI-3 kinase (1, 2). PI-3 kinase has also been shown to associate with $p60^{c\text{-}src}$ when activated by polyomavirus middle T antigen (8, 9, 15, 20–22) and with $p60^{v\text{-}src}$ (8), an oncogenic version of the *src* translational product. We reasoned that assays for associated substrates may be a more sensitive means of detecting the activation of nonreceptor kinases

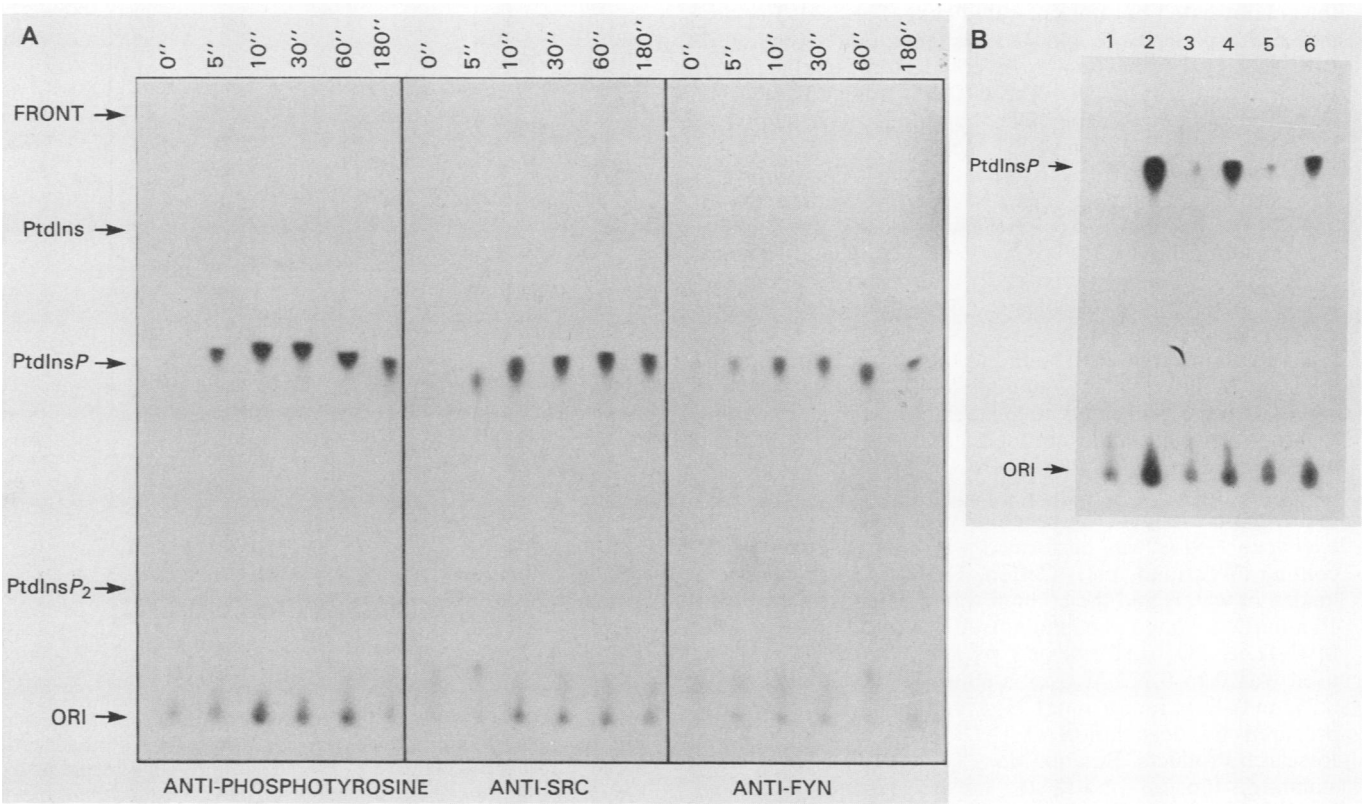

FIG. 2. Immunoprecipitation of PIK activity by antibodies directed against phosphotyrosine, $p60^{c\text{-}src}$, or $p59^{fyn}$. (A) Platelets were treated with thrombin for times ranging from 5 to 180 s and lysed immediately. Protein extracts were immunoprecipitated with the antisera indicated and assayed for PIK activity as described in the text. (B) Platelets treated with thrombin for 30 s were lysed, and 900 μg of protein extract was immunoprecipitated with antiphosphotyrosine serum in the presence (lane 1) or absence (lane 2) of phosphotyrosine, anti-CD11b (lane 3), MAb 327 (lane 4), or anti-$p59^{fyn}$ in the presence (lane 5) or absence (lane 6) of homologous peptide. Immunoprecipitates were assayed for PIK activity as described in the text.

than in vitro kinase reactions. Thus, we tested lysates from thrombin-treated cells for phosphatidylinositol kinase (PIK) activity associated with $p60^{c\text{-}src}$ or $p59^{fyn}$.

Immunoprecipitates were washed three times with cold phosphate-buffered saline containing 1 mM Na_4VO_3 and 1% Nonidet P-40, twice with cold 0.5 M LiCl–100 mM Tris (pH 7.6), once with cold 100 mM NaCl–1 mM EDTA–10 mM Tris (pH 7.6), and once more with 20 mM HEPES (pH 7.6). PIK activity was assayed essentially as described previously (1). Briefly, reaction mixtures contained immunoprecipitates and phosphatidylinositol (PtdIns) (Sigma) at a final concentration of 0.2 mg/ml. Phosphorylation was initiated by addition of 10 μCi [γ-^{32}P]ATP and $MgCl_2$ to final concentrations of 1 μM and 5 mM, respectively. Before thrombin treatment, very little PtdIns was further phosphorylated when immunoprecipitates containing $p60^{c\text{-}src}$ or $p59^{fyn}$ were used as potential sources of PIK (Fig. 2A). In contrast, by 10 s after thrombin treatment, readily detectable phosphatidylinositol phosphate (PtdInsP) was observed under the same conditions. Neither anti-CD11b monoclonal antibody nor anti-$p59^{fyn}$ preincubated with homologous peptide immunoprecipitated detectable PIK activity (Fig. 2B). Since previous studies have demonstrated that PIK activity is not an intrinsic property of protein-tyrosine kinases (4, 17), we conclude that an enzymatically active PIK rapidly associates with both $p60^{c\text{-}src}$ and $p59^{fyn}$ as a function of thrombin activation.

To determine whether detection of PIK activity was dependent on tyrosine phosphorylation, antiphosphotyrosine immunoprecipitates were also assayed for PIK activity. PIK activity was immunoprecipitated from lysates of thrombin-treated but not untreated cells (Fig. 2A). Furthermore, phosphotyrosine blocked the immunoprecipitation of PIK activity by the Sepharose-conjugated antiphosphotyrosine antibody (Fig. 2B). These findings demonstrate that thrombin-induced tyrosine phosphorylation plays an obligatory role in the detection of PIK activity.

Identification of the PIK associated with $p60^{c\text{-}src}$ and $p59^{fyn}$. The PIK associated with activated PDGF receptor and with $p60^{v\text{-}src}$ has been shown to phosphorylate the 3 position of the inositol ring of PtdIns to yield phosphatidylinositol 3-phosphate. This activity is distinct from that of PI-4 kinase, which phosphorylates the 4 position of the inositol ring to yield phosphatidylinositol 4-phosphate (21). In an effort to identify the PIK bound to the nonreceptor tyrosine kinases in thrombin-activated platelets, PtdInsP reaction products were subjected to deacylation and analyzed by high-performance liquid chromatography (HPLC). PtdInsP was scraped from thin-layer chromatography plates, extracted with $CHCl_3$-methanol (1:1), and deacylated with methylamine reagent as previously described (1). Anion-exchange HPLC was performed with a Partisphere SAX5 column (Whatman, Inc., Clifton, N.J.). The samples were loaded in water, and the column was washed with water for 10 min. Deacylated phosphoinositides were eluted with 0.42 M $(NH_4)H_2PO_4$ (pH 3.8) at 1 ml/min. The linear gradient used was 0 to 0.252 M over a 60-min period, followed by 0.252 to 0.42 M for 10 min. [^{3}H]glyceroinositol 4-phosphate, prepared by deacylation of [^{3}H]PtdInsP (Dupont, NEN Research Products, Boston, Mass.), was used as an internal standard. Inositol phosphate, inositol bisphosphate, and inositol trisphosphate were obtained commercially. We used immunoprecipitates containing $p60^{v\text{-}src}$ expressed in fibroblasts as a known source of PI-3 kinase (4). Glycerolinositol 3-phosphate was the major form of deacylated PtdInsP produced in immunoprecipitates containing $p60^{v\text{-}src}$ (Fig. 3A). As expected, only small amounts of phosphatidylinosi-

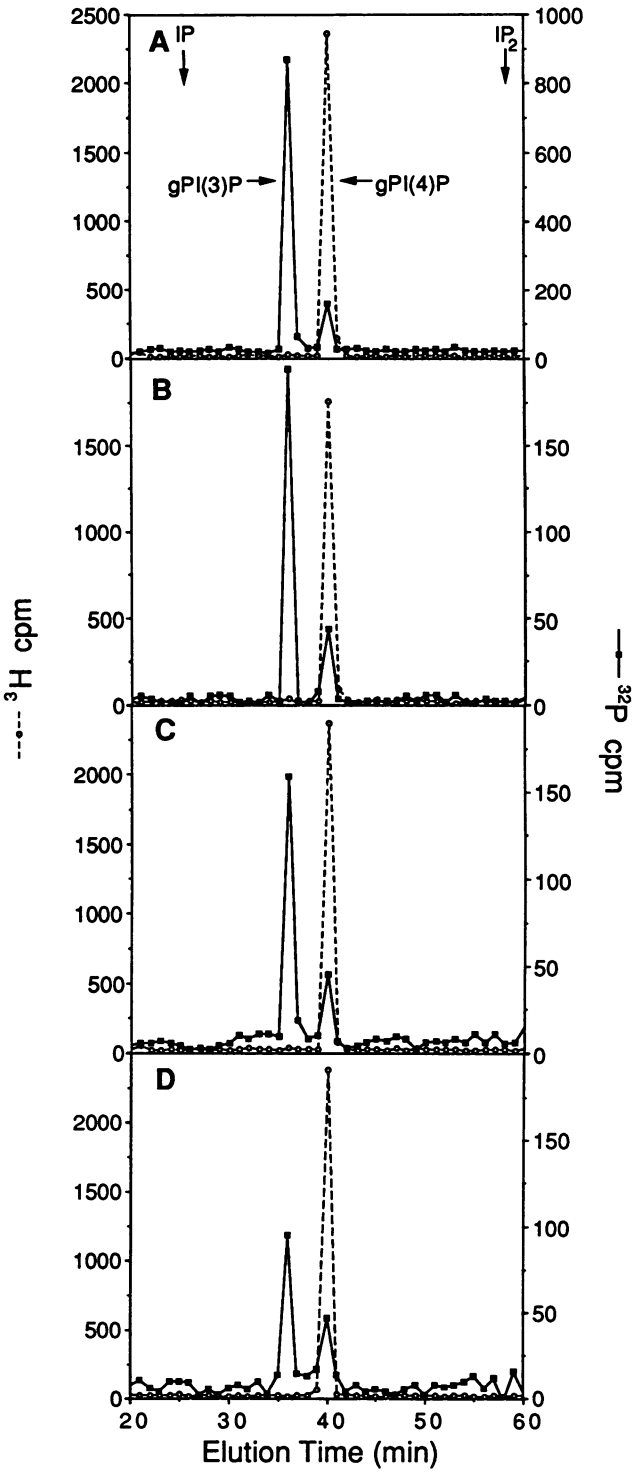

FIG. 3. Identification of phosphorylated products produced in PIK reactions. Elution profiles represent deacylated PtdInsP produced in reactions catalyzed by extracts of thrombin-treated platelets immunoprecipitated with antibodies that recognize phosphotyrosine (B), $p60^{c\text{-}src}$ (MAb 327) (C), or $p59^{fyn}$ (D). As a control, a lysate of fibroblasts expressing $p60^{v\text{-}src}$ was immunoprecipitated with MAb 327 and used as a known source of PI-3 kinase activity (A). Elution times for inositol phosphate (IP) and inositol bisphosphate (IP_2) are also shown. gPI(3)P, Glyceroinositol 3-phosphate; gPI(4)P, glyceroinositol 4-phosphate.

tol 4-phosphate were detected (Fig. 3A). Nearly identical HPLC profiles were observed when PtdInsP was generated from platelet extracts immunoprecipitated with antibodies against phosphotyrosine, $p60^{c\text{-}src}$, or $p59^{fyn}$ (Fig. 3B to D). These findings identify the PIK associated with platelet $p60^{c\text{-}src}$ or $p59^{fyn}$ as PI-3 kinase.

Previous studies have shown that a number of platelet proteins are phosphorylated on tyrosine residues as a consequence of exposure to platelet-activating agents (3, 5). In this study, we have confirmed rapid tyrosine phosphorylation of platelet proteins in response to thrombin and have demonstrated thrombin-dependent association of active PI-3 kinase with $p60^{c\text{-}src}$ and $p59^{fyn}$. Other studies have revealed the appearance of phosphatidylinositol 3,4-bisphosphate in thrombin-treated platelets, suggesting that thrombin induces PI-3 kinase activity (20). Several possibilities may account for these findings. PI-3 kinase may be activated either upon tyrosine phosphorylation by $p60^{c\text{-}src}$ and $p59^{fyn}$ or by virtue of its physical association with tyrosine-phosphorylated proteins, including $p60^{c\text{-}src}$ and $p59^{fyn}$. In any case, our findings demonstrate that thrombin either directly or indirectly influences the biochemical properties of both $p60^{c\text{-}src}$ and $p59^{fyn}$. Since thrombin activation is thought to be mediated by cell surface receptors, the immediate changes in biochemical properties of $p60^{c\text{-}src}$ and $p59^{fyn}$ imply a functional linkage between the extracellular environment and kinases of the *src* family.

Accumulating evidence has suggested that PI-3 kinase may play an important role in mitogenic stimulation by growth factors and their receptors. Receptors for PDGF (1, 2) associate with PI-3 kinase upon activation by PDGF. Furthermore, deletion mutants of the PDGF receptor interkinase domain are impaired for mitogenic signaling and for their ability to associate with PI-3 kinase (2). Thus, ligand-dependent association of PI-3 kinase with normal versions of nonreceptor kinases demonstrates a previously unknown similarity with receptor kinases and suggests that protein-tyrosine kinases of both classes may use a common biochemical pathway for manifestation of their normal functions.

Finally, our findings have implications regarding the mechanism of transformation by nonreceptor protein-tyrosine kinases. When activated by polyomavirus middle T antigen (8, 9, 15, 20–22) or by transforming mutations (4), the *src* translational product associates with PI-3 kinase activity. In addition, PI-3 kinase associates with activated forms of the *fyn* translational product (unpublished observations). Thus, PI-3 kinase association with normal $p60^{c\text{-}src}$ and $p59^{fyn}$ under physiological conditions represents a property shared with transforming versions of these proteins. These findings raise the possibility that transformation by $p60^{v\text{-}src}$ and related proteins involves constitutive activation of a pathway normally mediated by nonreceptor kinases in postmitotic cells.

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