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Thrombin-stimulated immunoprecipitation of phosphatidylinositol 3-kinase from human platelets

(c-*src* gene/protein-serine/threonine kinase)

CHRISTINA A. MITCHELL*†, ANNE BENNETT JEFFERSON*, BRUCE E. BEJECK‡§, JOAN S. BRUGGE¶, THOMAS F. DEUEL*§, AND PHILIP W. MAJERUS*‖

*Washington University School of Medicine, Division of Hematology-Oncology, 660 South Euclid, Box 8125, and §The Jewish Hospital of St. Louis, 216 South Kingshighway Boulevard, St. Louis, MO 63110; and ¶Howard Hughes Medical Institute, University of Pennsylvania, 422 Curie Boulevard, Philadelphia, PA 19104-6148

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ABSTRACT **Growth factors and transforming proteins that activate tyrosine phosphorylation have been shown to cause an increased labeling of 3-phosphate-containing phosphatidylinositols. Turnover correlates with the formation of a complex between phosphatidylinositol 3-kinase, the activated protein-tyrosine kinase, and other proteins thought to participate in transmembrane signaling. When human platelets are treated with thrombin, labeling of 3-phosphate-containing phosphatidylinositols is stimulated with a time course and concentration dependence consistent with a role for these lipids in platelet activation. We now report that when human platelets are stimulated with thrombin, a complex forms between phosphatidylinositol 3-kinase, a protein-serine/threonine kinase, and an uncharacterized platelet membrane protein. The complex is immunoprecipitated from detergent lysates of thrombin-stimulated platelets by a rabbit antiserum prepared against a peptide from the cytoplasmic domain of the mouse platelet-derived growth factor (PDGF) receptor. The antigen is not the PDGF receptor, since complex formation is not stimulated by PDGF and thrombin-induced complexes are not precipitated by another rabbit antiserum against the same peptide or by monoclonal anti-human PDGF receptor antibodies. Formation of the complex is rapid (within 30 sec) and occurs at thrombin concentrations that stimulate platelet aggregation and secretion (50% of maximal complex formation at 0.03 unit of thrombin per ml). We propose that the complex initiates formation of 3-phosphate-containing phosphatidylinositols that may function in platelet activation.**

An early event in thrombin-induced platelet activation is hydrolysis of phosphatidylinositols by phospholipase C. The substrates cleaved include phosphatidylinositol (PtdIns), phosphatidylinositol 4-phosphate [PtdIns(4)*P*], and phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2]. Products of this reaction participate in protein kinase C activation, calcium mobilization, and production of icosanoid mediators (1–7).

An alternative PtdIns pathway was recently identified in fibroblasts (8, 9), neutrophils (10), vascular smooth muscle cells (11), astrocytoma cells (12), and platelets (13). PtdIns 3-kinase initiates the pathway by phosphorylating PtdIns to produce PtdIns(3)*P*. Further phosphorylated forms of 3-phosphate-containing lipids have also been discovered, including PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 (14–18). The identity and structure of PtdIns(3)*P*, PtdIns(3,4)P_2, and PtdIns(3,4,5)P_3 have recently been determined in platelets (54). These lipids are labeled rapidly upon thrombin stimulation, suggesting that they may be important in platelet activation (13, 18).

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PtdIns 3-kinase activity has been shown to associate with growth factor receptors and oncoproteins containing tyrosine kinase activity. These include the polyoma virus middle T antigen/pp60$^{c\text{-}src}$ complex (19, 20), pp60$^{v\text{-}src}$ in Rous sarcoma virus-transformed cells (21), the platelet-derived growth factor (PDGF) receptor (22), the colony-stimulating factor 1 receptor (16), and the epidermal growth factor receptor (23). Mutant forms of the PDGF receptor that are defective in mediating cell proliferation in response to PDGF fail to associate with PtdIns 3-kinase, suggesting that association with PtdIns 3-kinase is essential for cell proliferation (22, 24). Recent studies have demonstrated that PtdIns 3-kinase may form part of a multiprotein complex that includes the activated PDGF receptor, the serine/threonine kinase Raf-1 (25), phospholipase C-γ (26, 27), and the GTPase-activating protein GAP (28).

PtdIns 3-kinase-containing complexes have not been found in platelets. We now demonstrate such a complex containing PtdIns 3-kinase, a protein-serine/threonine kinase, and an uncharacterized platelet membrane protein.

EXPERIMENTAL PROCEDURES

Materials. Carrier-free [^{32}P]orthophosphate (in water) and Ptd[^{3}H]Ins(4)*P* (4.5 Ci/mmol; 1 Ci = 37 GBq) were from DuPont/New England Nuclear. [γ-^{32}P]ATP (5000 Ci/mmol) was from Amersham. Soybean PtdIns was from Sigma. TLC plates were from EM Science. Protein A-Sepharose was from Repligen (Cambridge, MA). All other materials were from Fisher or Sigma. Human thrombin was purified by W. A. Dittman (Duke University) as described (29).

Preparation of Antibodies. A monoclonal antibody against pp60$^{c\text{-}src}$ (Mab 327) was developed as described (30). Monoclonal anti-human PDGF receptor antibody (PR7212) was provided by Daniel Bowen-Pope (31). Monoclonal anti-phosphotyrosine antibodies were developed and used as described (32, 33). Polyclonal anti-phosphotyrosine antibodies were provided by W. A. Dittman. A peptide corresponding to amino acids 958–977 of the mouse PDGF receptor (34) was synthesized, conjugated to thyroglobulin, and used to immunize two rabbits, A and E, as described (35).

Washed Platelets. Platelets were isolated from normal donors and resuspended at 2 × 10^9 per ml in 15 mM Tris, pH 7.4/140 mM NaCl/5 mM glucose (36), and in some cases

Abbreviations: PtdIns, phosphatidylinositol; PtdIns(3)*P*, phosphatidylinositol 3-phosphate; PDGF, platelet-derived growth factor.

†Current address: Monash Medical School, Alfred Hospital, Department of Medicine, Commercial Road, Prahran 3181, Melbourne, Australia.
‡Current address: University of Minnesota, Department of Laboratory Medicine and Pathology, 321 Church Street, Minneapolis, MN 55455.
‖To whom reprint requests should be addressed.

were incubated with $^{32}P_i$ (1 mCi/ml) for 1 hr at room temperature. Stimulation with thrombin was for 3 min at 37°C.

Immunoprecipitation. Platelets were sedimented by centrifugation for 10 sec and immediately lysed at a concentration of 10^{10} platelets per ml in RIPA buffer (20 mM Tris, pH 7.4/50 mM NaCl/50 mM NaF/5 mM EDTA/20 mM sodium pyrophosphate/1 mM phenylmethylsulfonyl fluoride/1 mM sodium orthovanadate/1% Triton X-100). After 25 min on ice the supernatant was clarified by centrifugation and incubated 2–12 hr with antiserum at 4°C. Immunoprecipitation reaction mixtures contained 1–4 × 10^8 platelets per 10–40 μl of antiserum A, antiserum E, or nonimmune serum. After incubation with protein A-Sepharose for 1 hr, the immunoprecipitates were washed three times at 4°C with RIPA buffer and then three times with 100 mM NaCl/10 mM Tris, pH 7.4.

PtdIns Kinase Assay. Washed protein A pellets were incubated in 100 μl in the presence of 20 mM Tris (pH 7.5), 10 mM $MgCl_2$, 5 mM EGTA, 20 μg of sonicated PtdIns, 1 μM ATP, and 10 μCi of [γ ^{32}P]ATP for 25 min at room temperature. The assay was linear over this time. Labeled phospholipids were extracted, separated by TLC, and detected by autoradiography (37).

Silica gel was scraped from TLC plates and phospholipids were deacylated with methylamine. An internal standard of Ptd[^{3}H]Ins(4)*P* was then added and lipids were characterized by HPLC as described (38).

***In Vitro* Protein Kinase Assay.** Protein kinase assays were performed on immunoprecipitates just as PtdIns kinase assays except that the reactions were stopped with the addition of SDS/2-mercaptoethanol sample buffer. After SDS/7.5% PAGE (39), the dried gels were subjected to autoradiography.

Other Methods. Proteins phosphorylated *in vitro* or in platelets were localized by autoradiography, excised from the gel, and subjected to SDS/12.5% PAGE in the presence of *Staphylococcus aureus* V8 protease (Miles) according to Cleveland *et al.* (40). Phosphorylated proteins were extracted from SDS/polyacrylamide gels and precipitated with 20% (wt/vol) trichloroacetic acid, and phospho amino acids were analyzed as described by Lou and Schulman (41).

RESULTS

We initially investigated whether PtdIns 3-kinase activity was altered following thrombin treatment of platelets. Washed human platelets were treated with thrombin (2 units/ml) for 3 min and solubilized in RIPA buffer. PtdIns kinase assays were performed in 0.001% Triton X-100, which does not inhibit platelet PtdIns 3-kinase. Total PtdIns kinase activity was the same in thrombin-stimulated and unstimulated platelets, with no change in the proportions of PtdIns(4)*P* and PtdIns(3)*P* products.

Since platelets have been reported to have small numbers of PDGF receptors (42) and this receptor forms complexes with PTdIns 3-kinase, we attempted to immunoprecipitate PtdIns 3-kinase by using antisera raised against PDGF receptors. Rabbit anti-PDGF receptor antiserum A precipitated PtdIns kinase activity. In numerous experiments analyzed only by measuring the PtdIns*P* produced by immunoprecipitated PtdIns kinase and isolated by chromatography on TLC plates, we found that immunoprecipitates of lysates from thrombin-stimulated platelets had 2- to 17-fold increased PtdIns kinase activity compared with immunoprecipitates from unstimulated cells. The PtdIns*P* products were analyzed by deacylation of the lipids and separation of glycerophosphoinositols by HPLC (Fig. 1). The immunoprecipitate from unstimulated platelets contained approximately equal amounts of PtdIns(3)*P* and PtdIns(4)*P*. The immunoprecipitate from unstimulated platelets contained approximately equel amounts of PtdIns(3)*P* and PtdIns(4)*P*. The immunoprecipitate thrombin-treated cells contained >90% PtdIns(3)P with 6-fold increased PtdIns 3-kinase activity. In three other experiments PtdIns*P* products were deacylated and the glycerophosphoroinositols were analyzed. We found little or no PtdIns(3)*P* in immunoprecipitates with nonimmune serum, somewhat variable amounts in unstimulated platelets with immune serum A, and 4-fold increased PtdIns 3-kinase activity precipitated from thrombin-treated platelet lysates with >90% PtdIns(3)*P* in each case. PtdIns 3-kinase activity immunoprecipitated by antiserum A represented <5% of the total PtdIns 3-kinase activity measured in whole cell lysates.

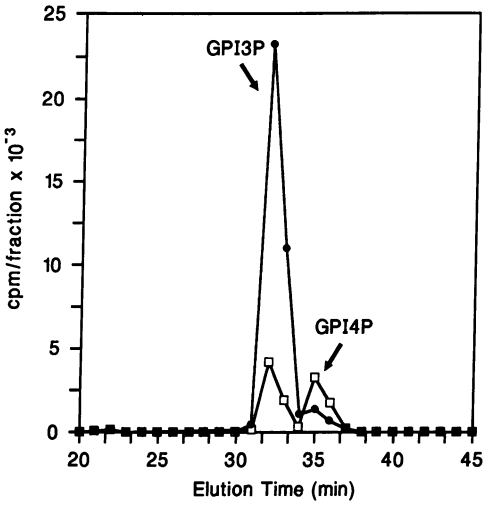

FIG. 1. HPLC analysis of glycerophosphoinositols (GPI) derived from PtdIns*P* products. Deacylated ^{32}P-labeled PtdIns*P* compounds generated from PtdIns kinase assays of immunoprecipitates of thrombin-stimulated (●) or unstimulated (□) platelets were separated by TLC, deacylated, and analyzed by HPLC. Peaks corresponding to the 3-phosphate- and 4-phosphate-containing derivatives are indicated.

Immunoprecipitations performed on lysates from control and thrombin-treated platelets using antisera against other platelet antigens did not immunoprecipitate PtdIns kinase activity. These included polyclonal antisera against glycoproteins GPIIb and GPIIIa (43) and monoclonal antisera against the insulin-like growth factor I receptor (44) and insulin receptor (45).

Thrombin Dependence of Immunoprecipitation. Studies of the time course and concentration-dependence of thrombin-stimulated PtdIns kinase immunoprecipitation demonstrated that formation of complexes occurred in parallel with thrombin-induced platelet activation. Nearly maximal complex formation was seen within 30 sec of thrombin addition, and complexes persisted for at least 10 min (data not shown). Complex formation was half-maximal with thrombin at 0.03 unit/ml (Fig. 2). These responses are similar to those found when measuring other parameters of thrombin-dependent platelet activation.

***In Vitro* Protein Kinase Assay of Immunoprecipitates.** Other studies have shown that PtdIns 3-kinase forms complexes with receptors or oncoproteins that have intrinsic protein-tyrosine kinase activity (16, 21–23). We therefore carried out *in vitro* protein kinase assays on immunoprecipitates from platelet lysates. No exogenous protein substrates were added in these assays. No protein kinase activity was observed in immunoprecipitates using nonimmune serum or antiserum E. In contrast immunoprecipitates made using antiserum A contained protein kinase activity that was markedly increased in lysates from thrombin-stimulated platelets. The major phosphorylated protein in the immunoprecipitate had an apparent molecular mass of 60 kDa (Fig. 3, lanes 7 and 8). As the tyrosine kinase pp60$^{c\text{-}src}$ has been demonstrated to be

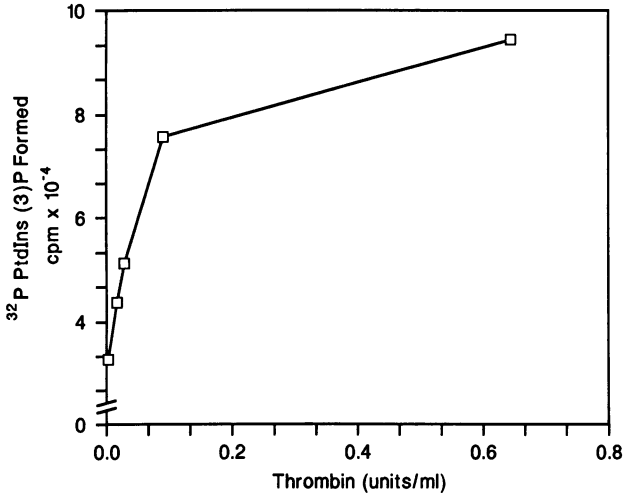

FIG. 2. Thrombin dependence of PtdIns 3-kinase immunoprecipitation. Platelets (2×10^9 per ml) were treated with the indicated concentrations of thrombin for 3 min. Immunoprecipitates (4×10^8 platelets and 40 μl of antiserum A) were used for PtdIns kinase assays.

one of the major proteins phosphorylated *in vitro* in platelet membranes (46), the presence of a 60-kDa phosphorylated protein in immunoprecipitates with antiserum A suggested that this protein might be pp60$^{c\text{-}src}$. *In vitro* protein kinase reactions on immunoprecipitates of platelet lysates prepared using a monoclonal antibody against pp60$^{c\text{-}src}$ (Mab 327) are shown in lane 5. Note that the 60-kDa phosphorylated protein from antiserum A immunoprecipitates migrates slightly faster than pp60$^{c\text{-}src}$. Partial V8 proteolytic peptide maps showed that the 60-kDa phosphoprotein immunoprecipitated by antiserum A was distinctly different from pp60$^{c\text{-}src}$ (Fig. 4). Partial V8 proteolysis was also used to compare the 60-kDa phosphoprotein from antiserum A immunoprecipitates with other members of the *src* family that are expressed in platelets, including the products of *fyn* (47), *lyn*, *hck*, and *yes* (Min Mei Huang, personal communication). The partial proteolytic pattern generated from these immunoprecipitated proteins also was distinct from that of the protein precipitated

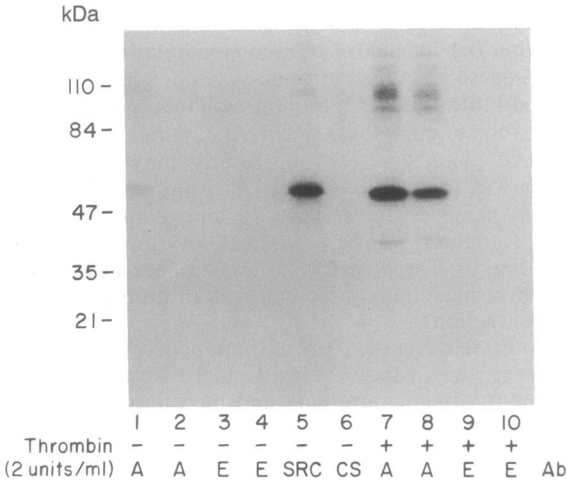

FIG. 3. Protein kinase activity in immunoprecipitates. Platelets (2×10^9/ml) were incubated with thrombin (2 units/ml) for 3 min. Immunoprecipitates obtained from 2×10^8 platelets with the indicated antibody (Ab) were assayed for endogenous protein kinase activity. Products were analyzed by SDS/7.5% PAGE and autoradiography. Lanes 1, 2, 7, and 8, antiserum A; lanes 3, 4, 9, and 10, antiserum E; immunoprecipitations; lane 5, anti-pp60$^{c\text{-}src}$ monoclonal antibody (Mab 327); lane 6, nonimmune control serum (CS).

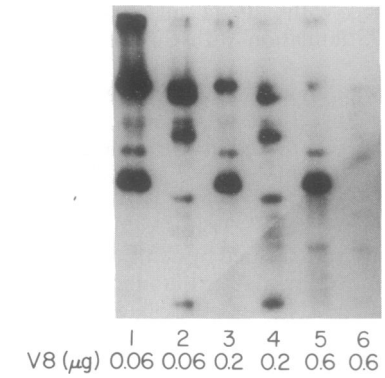

FIG. 4. *S. aureus* V8 protease digestion of 60-kDa phosphoproteins. The 60-kDa phosphorylated proteins were excised from gels and subjected to SDS/PAGE in the presence of the indicated concentrations of V8 protease. Lanes 1, 3, and 5, pp60$^{c\text{-}src}$ (from lane 5 of Fig. 3); lanes 2, 4, and 6, pp60 band (from lanes 7 and 8 of Fig. 3).

with antiserum A, indicating that the 60-kDa phosphoprotein immunoprecipitated by antiserum A is not one of these *src*-like proteins. Anti-pp60$^{c\text{-}src}$ Mab 327 did not detect pp60$^{c\text{-}src}$ when the antiserum A immunoprecipitates containing both thrombin-stimulated PtdIns 3-kinase activity and protein-serine/threonine kinase activity were probed in an immunoblot assay. Phospho amino acid analysis of the 60-kDa protein phosphorylated in protein kinase assays of antiserum A immunoprecipitates showed primarily phosphothreonine and no phosphotyrosine (Fig. 5).

We also labeled intact platelets with $^{32}P_i$, treated them with thrombin, and immunoprecipitated extracts from the cells with antiserum A (Fig. 6*A*). The major phosphoprotein immunoprecipitated under these conditions had a molecular mass of 97 kDa and was phosphorylated on serine (Fig. 6*B*). Although the major phosphoprotein radiolabeled in the *in vitro* kinase assay was 60 kDa, no protein having this electrophoretic mobility was radiolabeled in whole platelets, suggesting either that the 60-kDa protein is not a phosphoprotein *in vivo* or that only a very small amount of this protein is present in the complex. While this experiment does not address whether the 97-kDa protein contains the epitope recognized by antiserum A, one can conclude that this

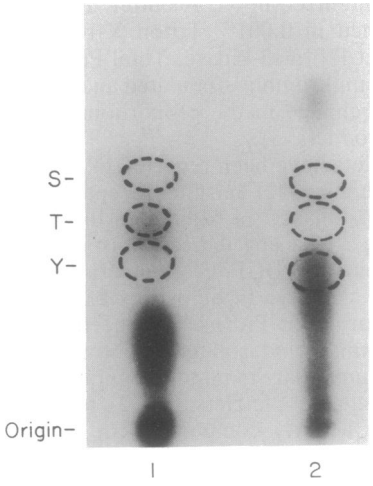

FIG. 5. Phospho amino acid analysis of 60-kDa phosphoproteins extracted from gels. Lane 1, 60-kDa phosphoprotein from *in vitro* kinase assays of antiserum A immunoprecipitates; lane 2, pp60$^{c\text{-}src}$ from *in vitro* kinase assays of Mab 327 immunoprecipitates. S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine.

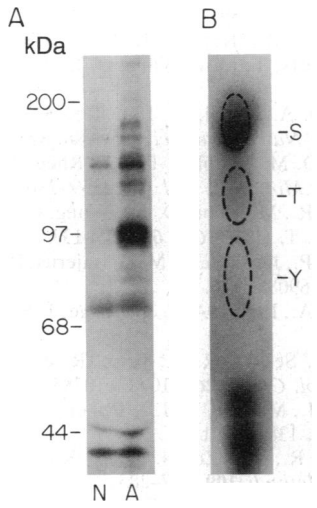

FIG. 6. Antiserum A-associated proteins phosphorylated in intact platelets. (*A*) ^{32}P-labeled platelets were stimulated with thrombin (2 units/ml) and cell extracts were immunoprecipitated with nonimmune serum (lane N) or antiserum A (lane A). Immunoprecipitates were analyzed by SDS/PAGE and autoradiography. (*B*) Phospho amino acid analysis of the 97-kDa phosphoprotein immunoprecipitated from thrombin-stimulated platelets by antiserum A was performed as in Fig. 5.

phosphoprotein is a major component of the immune complex.

Previous reports on the association of PtdsIns 3-kinase activity with receptors or oncoproteins have also shown that a phosphotyrosine-containing protein has a critical role in the formation of the complex (48–51). We therefore analyzed entire reaction mixtures from *in vitro* protein kinase assays of antiserum A immunoprecipitates and found them to contain phosphoserine and phosphothreonine but no phosphotyrosine (data not shown). We also found no phosphotyrosine by immunoblotting antiserum A immunoprecipitates with anti-phosphotyrosine antiserum (32). When pp60$^{c\text{-}src}$ was immunoprecipitated from platelets by Mab 327, autophosphorylated *in vitro*, and subjected to phospho amino acid analysis, we found only phosphotyrosine (Fig. 5). Therefore, the absence of phosphotyrosine in antiserum A immunoprecipitates was not due to an inability to recover phosphotyrosine under the conditions used. Anti-phosphotyrosine immunoprecipitates from platelet lysates were also assayed for PtdIns kinase activity. PtdIns kinase activity was increased 2- to 4-fold with thrombin treatment of platelets. However, these immunoprecipitates contained 10–20 times less PtdIns kinase activity compared with antiserum A immunoprecipitates.

We found no 180-kDa protein corresponding in molecular mass to the PDGF-β receptor in *in vitro* kinase assays of either A or E immunoprecipitates or in immunoprecipitates of ^{32}P-labeled platelets (results not shown). Further results suggested that antiserum A was not immunoprecipitating with PDGF receptor but rather was crossreacting with another platelet protein. Antiserum A immunoprecipitates of PDGF (10–200 ng/ml)-stimulated platelets did not demonstrate increased PtdIns kinase activity, and the peptide used as immunogen failed to block immunoprecipitation of PtdIns kinase from thrombin-stimulated platelets. In contrast, comparable studies performed on PDGF (50 ng/ml)-stimulated NIH 3T3 cells demonstrated that both A and E immunoprecipitated equivalent PtdIns 3-kinase activity, and immunoprecipitation was blocked by the peptide immunogen. Finally, a monoclonal antibody to the human PDGF receptor did not immunoprecipitate PtdIns 3-kinase from unstimulated or thrombin-stimulated platelets (results not shown). In order to localize the platelet antigen immunoprecipitated by antiserum A, platelets were sonicated and separated into membrane and soluble fractions and subjected to solubilization with RIPA buffer. All immunoprecipitated PtdIns kinase activity was in the membrane fraction (data not shown). These experiments suggest that the protein immunoprecipitated by antiserum A is not the PDGF receptor but is some other membrane-associated protein whose association with platelet serine/threonine kinases and PtdIns 3-kinase is stimulated by thrombin.

DISCUSSION

We have immunoprecipitated PtdIns 3-kinase by using an antibody raised against amino acids 958–977 of the mouse PDGF-β receptor (34). Coimmunoprecipitated with the PtdIns 3-kinase was a protein-serine/threonine kinase. Complex formation was stimulated in lysates from thrombin-treated platelets.

While antiserum A used in the experiments was obtained by immunizing a rabbit with a peptide from the PDGF receptor, the antigen does not appear to be the PDGF receptor. The amino acid sequence of the mouse PDGF-β receptor used as the immunogen (residues 958–977) does not resemble that of other known receptors or tyrosine kinases that form complexes with PtdIns 3-kinase. No specific proteins were recognized by antiserum A in immunoblot assays or by Coomassie staining after SDS/PAGE; however, a 97-kDa phosphoprotein was immunoprecipitated from ^{32}P-labeled platelets, suggesting that a 97-kDa protein is one component of the complex. Antiserum A may be directed against PtdIns 3-kinase or the serine/threonine kinase present in the complex, provided that one of these proteins or some other protein in the complex can respond to stimulation of platelets by thrombin. The stimulation of complex formation by thrombin suggests the possibility that antiserum A may recognize the thrombin receptor itself. Further studies will require purification of the platelet antigen(s) that complexes with the PtdIns 3-kinase.

Our work also demonstrates that a serine/threonine kinase is active during the formation of the immune complex. Whether the 60-kDa protein phosphorylated in intact platelets represents the autophosphorylated kinase or a substrate phosphorylated by an associated kinase remains to be determined. There are at least two other examples of serine/threonine kinases associating with PtdIns 3-kinase and cellular receptors. One recent study (48) reported the association of a serine/threonine kinase with complexes containing PtdIns 3-kinase, the activated PDGF receptor, and an 85-kDa phosphoprotein that may be the PtdIns 3-kinase (48, 53). Another report (25) demonstrates that the 74-kDa serine/threonine kinase protooncogene product Raf-1 associates with the activated PDGF receptor and shows increased serine/threonine kinase activity upon complex formation.

Immunoprecipitation of thrombin-stimulated PtdIns kinase with antiserum A does not depend on tyrosine phosphorylation. Immunoprecipitations using several anti-phosphotyrosine antibodies demonstrated low levels of PtdIns kinase activity comparable to that observed by Gutkind *et al.* (52). While a role for tyrosine phosphorylation in the formation of complexes in platelets is impossible to exclude, phosphotyrosine-containing proteins were not detected in the complexes. Thus the mechanism of formation of complexes containing PtdIns 3-kinase in platelets may be different from those described in other systems.

Previous studies of complex formation of PtdIns 3-kinase with receptors have concentrated on receptors that evoke growth responses. Stimulation of complex formation in response to thrombin in platelets implies that 3-phosphate-

containing inositol phospholipids have a function not involving cell proliferation.

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