Evidence for two distinct phosphatidylinositol kinases in fibroblasts

Implications for cellular regulation

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Phosphatidylinositol (PtdIns) kinase activities from non-transformed and polyoma-middle-T-transformed murine fibroblasts were examined. Both normal and transformed 3T3 fibroblasts have two PtdIns kinases, which can be separated by anion-exchange chromatography. One of these activities (Type I) has a K_m for ATP of 10 μ M, is resistant to inhibition by adenosine, AMP or ADP, and is inhibited by non-ionic detergents. The other activity (Type II) has a somewhat higher K_m for ATP (35 μ M) and is inhibited competitively by ADP, AMP and adenosine at concentrations suggesting regulation of this activity by the energy charge of the cell. The Type II PtdIns kinase is activated by non-ionic detergents. We have previously reported the specific association of a PtdIns kinase activity with polyoma-middle-T immunoprecipitates [Whitman, Kaplan, Schaffhausen, Cantley & Roberts (1985) *Nature* (London) **315**, 239–242; Kaplan, Whitman, Schaffhausen, Raptis, Garcea, Pallas, Roberts & Cantley (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 3624–3628]. Comparison of the immunoprecipitated PtdIns kinase with the activities identified by ion-exchange chromatography indicates that it is the Type I enzyme which specifically associates with the middle-T/pp60^{c-src} complex. This PtdIns kinase activity is separable from both middle T and pp60^{c-src}. Type I PtdIns kinase also associates with pp60^{v-src} immunoprecipitates from Rous-sarcoma-virus-transformed cells. Furthermore, this PtdIns kinase appears to co-precipitate with partially purified platelet-derived growth factor (PDGF) receptor. The amount of this activity found in anti-phosphotyrosine immunoprecipitates or in wheat-germ-lectin-agarose precipitates is increased 50-fold by stimulation of quiescent Balb/C 3T3 fibroblasts with PDGF. These results suggest that the Type I PtdIns kinase is regulated by agents which affect cell growth and transformation, whereas the Type II PtdIns kinase may be regulated by the local [ATP]/[ADP] ratio.

INTRODUCTION

The second messengers generated by the stimulated breakdown of the inositol phospholipids, inositol 1,4,5-trisphosphate [Ins(1,4,5) P_3] and diacylglycerol (DAG), have been implicated as mediators of a wide variety of hormonal signals [1–3]. Observations that mitogens rapidly stimulate phosphatidylinositol (PtdIns) turnover in many systems, and that PtdIns turnover may be constitutively activated in oncogenically transformed cells, have suggested that these second messengers may be involved in the regulation of mitogenesis [4–10]. Ins(1,4,5) P_3 and DAG are produced by hydrolysis of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5) P_2] by phospholipase C. PtdIns(4,5) P_2 is in turn generated by sequential phosphorylation of the 4- and 5-hydroxy groups of the inositol ring by PtdIns and PtdIns P kinases respectively. The extent to which DAG production depends on hydrolysis of PtdIns(4,5) P_2 rather than of PtdIns or PtdIns P remains uncertain [11,12]; however, it seems clear that Ins(1,4,5) P_3 can only be produced from breakdown of PtdIns P_2 , indicating that the degree of phosphorylation of the phosphoinositides is an important parameter in the regulation of second-messenger formation.

In spite of the recognition of the importance of stimulated phosphoinositide turnover as a signalling mechanism, the control points of this pathway remain poorly understood. The earliest observable event in most PtdIns turnover responses appears to be an increase in PtdIns P_2 hydrolysis by a PtdIns P_2 -specific phospholipase C [4,13,14]. However, in fibroblasts the magnitude of the Ins(1,4,5) P_3 and DAG signals produced appears to be quite sensitive to the extent of PtdIns and PtdIns P kinase activation [12].

Investigation of the possible importance of PtdIns kinase in the regulation of PtdIns turnover has been greatly hampered by a lack of information concerning the enzyme(s) responsible for this activity, which currently await purification. The subcellular distribution of this activity also remains unclear: it appears to be membrane-associated [15,16]; however, plasma membrane [17], Golgi [18], microsomes [19,20], mitochondria [21], nuclear envelope [22] and coated vesicles [23] have each been proposed as fractions in which PtdIns kinase may be enriched relative to other membrane fractions. The possible existence of more than one type of PtdIns kinase was first proposed almost 20 years ago by Harwood & Hawthorne [19]. They found that PtdIns kinase activity in endoplasmic reticulum was strongly

Abbreviations used: PtdIns, phosphatidylinositol; PtdIns(4,5) P_2 , phosphatidylinositol 4,5-bisphosphate; PtdIns P , phosphatidylinositol phosphate; Ins(1,4,5) P_3 , inositol 1,4,5-trisphosphate; DAG, diacylglycerol; PDGF, platelet-derived growth factor; NP-40, Nonidet P-40.

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stimulated by non-ionic detergent, whereas plasma-membrane PtdIns kinase activity appeared to be relatively detergent independent. Later workers, however, proposed that these observations were due to differences in the availability of PtdIns in the two subcellular fractions rather than to the existence of two distinct enzymes [18].

Our interest in PtdIns kinase stems from the observation that this activity specifically associates with the middle-T gene product of polyoma virus [7,8]. This gene is alone capable of inducing transformation of established cell lines [24]. A subpopulation of middle-T proteins associates with a cellular protein, pp60^{c-src}, at the plasma membrane [25] and activates it as a tyrosine kinase [26]. We have previously reported the co-precipitation of a PtdIns kinase activity with the middle-T/pp60^{c-src} complex in immunoprecipitates made from polyoma-infected or transformed cells [1]. PtdIns kinase activity has also been found to co-precipitate or co-purify with several other activated tyrosine kinases [5,27,28], raising the possibility that the association between these two activities *in vitro* reflects a regulatory interaction between them *in vivo*.

To investigate further the possible regulation of PtdIns kinase by tyrosine kinases, we have studied PtdIns kinase activity in non-transformed and transformed murine fibroblasts, and have found evidence for two biochemically distinct and physically separable types of PtdIns kinase. The distinctive properties of these two kinases that we have observed *in vitro* may shed some light on the possibilities for regulation of PtdIns kinase activity *in vivo*.

EXPERIMENTAL

Materials

Phospholipids were obtained from Avanti Polar Lipids. [γ -³²P]ATP was obtained from New England Nuclear. β -Octyl glucopyranoside (octyl glucoside), aprotinin, Sepharose CL-4B and Protein A-Sepharose CL-4B, adenosine, ADP and ATP were from Sigma. Wheat-germ-lectin-agarose was from Pharmacia. Polyoma-transformed 3T3 cells (Py-3T3) were provided by Van Cherington (Dana Farber Cancer Institute). A31 Balb-C 3T3 cells were from Charles Scher (Children's Hospital, Philadelphia, PA, U.S.A.). Highly purified PDGF was purchased from PDGF Inc. Polyclonal antibody prepared against the N-terminus of polyoma T antigen (anti-T) was provided by David Pallas (Dana Farber Cancer Institute), monoclonal anti-src antibody GD11 was provided by S. Parsons (University of Virginia Medical College), and affinity-purified polyclonal anti-phosphotyrosine antibody was provided by Morris White (Joslin Diabetes Research Centre).

Precipitations of PtdIns kinase

For anti-T and anti-src immunoprecipitations, 1×10^7 normal or polyoma-transformed NIH 3T3 fibroblasts were lysed and immunoprecipitated as previously described [7], with the addition of 0.5 mM-NH₄VO₄ to the lysis buffer as a phosphatase inhibitor. For wheat-germ-lectin-agarose precipitates or anti-phosphotyrosine immunoprecipitates, 3×10^6 Balb 3T3 fibroblasts were lysed in 1% NP40/137 mM-NaCl/1 mM-CaCl₂/1 mM-MgCl₂/20 mM-Tris(pH 7.5)/1 mM-phenylmethanesulphonyl fluoride/aprotinin (1 μ g/ml)/1 mM-NH₄VO₄ for 15 min at

4 °C, spun for 10 min at 12000 g, and the supernatants were incubated with either 5 μ l of anti-phosphotyrosine antibody and 30 μ l of Protein A-Sepharose for 2 h or 100 μ l of wheat-germ-lectin-agarose for 1 h. The standard protocol for washing immunoprecipitates was: once with phosphate-buffered saline (138 mM-NaCl, 8 mM-Na₂HPO₄, 1 mM-KH₂PO₄), pH 7.4; once with 0.5 M-LiCl/0.1 mM-Tris/HCl, pH 7.6; once with distilled water; once with 0.1 mM-NaCl/0.02 M-Tris(pH 7.6)/1 mM-EDTA. As indicated in the text, some immunoprecipitates were washed three times with phosphate-buffered saline/1% NP40, followed by the standard wash procedure. For Sepharose CL-4B precipitations, cells were lysed in standard immunoprecipitate lysis buffer and incubated with 30 μ l of Sepharose CL-4B for 1 h and washed by the standard immunoprecipitation protocol.

For re-precipitation of wheat-germ-lectin eluates with anti-phosphotyrosine antibody, wheat-germ-lectin-agarose precipitates were incubated for 20 min at 4 °C in 1 mM-ATP/10 mM-MgCl₂/50 mM-Tris(pH 7.4)/1 mM-NH₄VO₄, eluted for 40 min at 10 °C in 0.2 M-N-acetylglucosamine/80 mM-Tris(pH 7.4)/1 mM-NH₄VO₄, and immunoprecipitated with anti-phosphotyrosine antibody as described above.

Anion-exchange chromatography

Polyoma-transformed 3T3 fibroblasts (1×10^7) were lysed in 1.8% octyl glucoside/80 mM-Tris/HCl(pH 7.4)/1 mM-NH₄VO₄/0.2 mM-phosphotyrosine/1 mM-phenylmethanesulphonyl fluoride/aprotinin (10 μ g/ml)/0.5 mM-EDTA and rocked at 4 °C for 15 min. Insoluble material was removed by centrifugation for 10 min at 12000 g. The cleared lysate was then diluted to (final concns.) 0.2% octylglucoside/5% glycerol/20 mM-Tris/HCl (pH 7.4)/0.4 mM-NH₄VO₄/0.1 mM-phenylmethanesulphonyl fluoride/aprotinin (1 μ g/ml), filtered twice through a Millex GV (0.2 μ m) filter, and loaded on to a Pharmacia FPLC Mono Q column; 0.25 mg of protein/ml was loaded in a total volume of 8 ml. Starting buffer was 0.2% octyl glucoside/5% glycerol/20 mM-Tris/HCl (pH 7.4)/0.4 mM-NH₄VO₄/aprotinin (1 μ g/ml). Proteins were eluted in this buffer with a linear NaCl gradient to a final concentration of 1 M-NaCl. The total elution volume was 100 ml.

PtdIns kinase assays

Washed Sepharose CL-4B precipitates or immunoprecipitates were assayed in a 50 μ l reaction mixture containing 20 mM-Tris/HCl, pH 7.4, 100 mM-NaCl, 10 mM-MgCl₂, 0.5 mM-EGTA, 100 μ M-PtdIns, 100 μ M-phosphatidylserine, 40 μ M-ATP and 10 μ Ci of [γ -³²P]ATP. PtdIns and phosphatidylserine were sonicated together at a total phospholipid concentration of 1 mg/ml as described previously [7] and added to the final reaction mixture. For assays of anion-exchange-chromatography fractions, 4 μ l of each fraction was assayed in the reaction mixture described above with 25 μ Ci of [γ -³²P]ATP/reaction. Assays were carried out for 90 s at 25 °C and stopped as described above. For all assays, the rate of production of PtdInsP was shown to be constant for the time period chosen. Reactions were stopped by addition of 80 μ l of 1 M-HCl, and reaction products were extracted and separated by t.l.c. as previously described [7]. Reaction products were detected by autoradiography, cut out, scraped, and quantified by liquid-scintillation

Table 1. PtdIns kinase activity in immunoprecipitates from non-transformed and polyoma-middle-T-transformed cells

Immunoprecipitates were prepared from 10^7 non-transformed (A–D) or middle-T-transformed (E–H) NIH 3T3 fibroblasts and assayed as described in the Experimental section. Immunoprecipitates designated 'NP40 in wash' (columns C,D,G,H) were washed three times with phosphate-buffered saline/1% NP40 before washing by the standard protocol (see the Experimental section). Immunoprecipitates were prepared with anti-T serum and Protein A–Sepharose CL-4B (row 1), with non-immune serum and Protein A–Sepharose CL-4B (row 2), or with Sepharose CL-4B alone (row 3), and were assayed in the presence (B,D,F,H) or absence (A,C,E,G) of 0.5% NP40. Formation of PtdInsP was determined from the incorporation of ^{32}P into PtdIns. Abbreviation: ND, not done.

	PtdIns kinase activity (fmol of PtdInsP formed)							
	3T3				PY-3T3			
	NP40 in wash... – NP40 in assay... – A	– + B	+ – C	+ + D	– – E	– + F	+ – G	+ + H
1. Anti-T + Protein A– Sepharose CL-4B	< 10	170	< 10	< 10	1100	160	980	30
2. Non-immune serum + Protein A–Sepharose CL-4B	< 10	175	ND	ND	< 10	190	ND	ND
3. Sepharose CL-4B alone	< 10	150	< 10	< 10	< 10	130	< 10	< 10

spectrometry. The [^{32}P]PtdInsP was identified by co-migration with unlabelled standard in both one- and two-dimensional t.l.c. [7].

PDGF stimulation of cells

For anti-phosphotyrosine immunoprecipitates and wheat-germ-lectin-agarose precipitates, Balb-3T3 fibroblasts were split 1:10 and grown to quiescence for 5 days. Quiescent cells were stimulated for 10 min with 10 units of purified PDGF/ml. Cells were then lysed and immunoprecipitated as described above.

RESULTS

Association of PtdIns kinases activities with immunoprecipitates

The identification of two distinct PtdIns kinase activities in fibroblast lysates was initially made in anti-middle-T immunoprecipitates prepared from non-transformed and polyoma-transformed 3T3 fibroblasts. As we have reported in detail previously [7], and as shown in Table 1, approx. 100-fold more PtdIns kinase activity is present in anti-T immunoprecipitates from polyoma-transformed cells than in immunoprecipitates from non-transformed 3T3 cells (Table 1, column E versus column A). This PtdIns kinase activity cannot be removed by washing the immunoprecipitates with phosphate-buffered saline/1% NP40 (column G). In addition, it utilizes sonicated detergent-free phospholipid as substrate, and is inhibited by 0.5% NP40 (column H). This NP40-inhibitable, middle-T associated, PtdIns kinase activity cannot be detected with non-immune serum (Table 1, row 2) or with Sepharose CL-4B alone (row 3, column E). However, if the non-immune-serum control immunoprecipitates or Sepharose CL-4B control precipitates were not washed with phosphate-buffered saline/1% NP40, and were assayed in the presence of 0.5% NP40, a significant PtdIns kinase activity was observed. This NP40-dependent activity was precipitated

from both control and polyoma-transformed cells in similar quantities, and was precipitated equally well by Sepharose CL-4B alone as by anti-T antibody + Protein A–Sepharose (columns B, F). This activity could be completely removed from the Sepharose or Protein A–Sepharose beads by extensive washing with phosphate-buffered saline/1% NP40 (column D).

Inhibition of PtdIns kinase by adenosine and ADP

The possibility that the PtdIns kinase activity which specifically associates with middle-T immunoprecipitates is distinct from that which is non-specifically carried along with Sepharose CL-4B beads was further investigated by comparing the sensitivity of these two activities to inhibition by adenosine. Adenosine has previously been shown to be a potent inhibitor of PtdIns kinase from several tissues [29,30]. Fig. 1 shows the adenosine-inhibition curves for middle-T/pp60^{c-src}-associated PtdIns kinase and for the NP40-dependent Sepharose-associated PtdIns kinase. The two activities show strikingly different sensitivities to inhibition by adenosine; the middle-T/pp60^{c-src}-associated kinase is inhibited less than 50% by 200 μM -adenosine at 40 μM -ATP, whereas the Sepharose-associated activity from either mouse or rat fibroblast lysates is inhibited with an apparent K_i of 20 μM at 40 μM -ATP. PtdIns kinase activity specifically associated with anti-pp60^{v-src} immunoprecipitates prepared from v-src-transformed rat fibroblasts showed adenosine-inhibition kinetics identical with those of the PtdIns kinase present in middle-T/pp60^{c-src} immunoprecipitates (Fig. 1). That the observed differences in adenosine sensitivity of these two activities was not due to differences in standard assay conditions for middle-T/pp60^{c-src}-associated PtdIns kinase and Sepharose-associated PtdIns kinase was verified by assaying these activities under identical detergent conditions (Fig. 1). These results suggest that the PtdIns kinase which co-precipitates with middle T/pp60^{c-src} or pp60^{v-src} is functionally distinct from the activity which associates with Sepharose CL-4B.

Fig. 1. Adenosine inhibition of immunoprecipitated PtdIns kinase activities

Immunoprecipitates from 10^7 non-transformed or transformed cells were assayed for PtdIns kinase activity in the presence of increasing concentrations of adenosine at $40 \mu\text{M}$ -ATP. Anti-src immunoprecipitates from middle-T-transformed mouse NIH 3T3 cells (■) or v-src-transformed rat F111 cells (●) were prepared as in Table 1 (with NP40 wash) and assayed as described in the Experimental section, in the absence of detergent. Sepharose CL-4B precipitates prepared from 10^7 mouse NIH 3T3 cells (□) or rat F111 cells (○) were prepared as in Table 1 (no NP40 wash) and assayed in the presence of 0.5% NP40. ♦, Anti-T immunoprecipitates from Py-3T3 cells washed three times in phosphate-buffered saline/1% NP40 in addition to standard wash protocol, assayed in 0.02% NP40 and various concentrations of adenosine; ◇, Sepharose CL-4B precipitation from Py-3T3 cells, assayed in 0.02% NP40. All assays were normalized to 100% in the absence of adenosine. Total product formed during a 10 min assay in the absence of adenosine was 980 fmol of PtdInsP for middle-T/pp60^{c-src} precipitates, 280 fmol of PtdIns for pp60^{v-src} precipitates, 180 fmol of PtdInsP for Sepharose CL-4B precipitates from NIH 3T3 cells, and 140 fmol of PtdInsP for Sepharose CL-4B precipitates from rat F111 cells. Data represent the averages of three separate experiments.

Separation of two distinct PtdIns kinase activities by ion-exchange chromatography

To investigate more directly the possibility that murine fibroblasts have two functionally distinct PtdIns kinase activities, we attempted to resolve these putative activities by ion-exchange chromatography of detergent-solubilized fibroblast membranes. Eluate fractions from FPLC anion-exchange chromatography (Mono Q) were assayed under two different sets of assay conditions, one of which was chosen to optimize activity of the middle-T/pp60^{c-src}-associated PtdIns kinase (0.02% octyl glucoside, $100 \mu\text{M}$ -adenosine) relative to the Sepharose-associated PtdIns kinase, the other to optimize activity of the Sepharose-associated PtdIns kinase (0.5% NP40). Assays of column fractions under these conditions revealed two distinct peaks of PtdIns kinase: when assayed in 0.02% octyl glucoside/ $100 \mu\text{M}$ -adenosine, the peak of activity was eluted at 200 mM -NaCl (arbitrarily

Fig. 2. Resolution of two PtdIns kinases by anion-exchange chromatography

(a) Octyl glucoside-solubilized Py-NIH 3T3 fibroblasts were chromatographed on a Mono Q anion-exchange column. The right-hand ordinate indicates the percentage of progress through a linear gradient from 0 to 1 M -NaCl, with the buffer system described in the Experimental section. Fractions were assayed either under Type I PtdIns kinase assay conditions ($120 \mu\text{M}$ -adenosine, $40 \mu\text{M}$ -ATP) (●) or Type II PtdIns kinase assay conditions (0.5% NP40, $100 \mu\text{M}$ -ATP) (○). Protein (2 mg) was loaded on the column, and the total activity of the starting material was 240 pmol/min under Type II assay conditions and 40 pmol/min under Type I assay conditions. Total activity recovered in peak 2 was 80 pmol/min (Type II assay conditions) and the total activity in peak 1 was 11 pmol/min (Type I assay conditions). (a) PtdIns kinase activities in column fractions of solubilized Py NIH-3T3 fibroblasts. (b) Rechromatography of fractions 10–14 from (a); fractions were assayed under both Type I (●) and Type II (○) assay conditions. (c) Rechromatography of fractions 18–23 from (a); fractions were assayed under Type I (●) and Type II (○) assay conditions as in (b). Specific activity of Type I PtdIns kinase in peak fraction of column B was $40 \text{ pmol/min per mg}$; that of Type II PtdIns kinase in peak fraction of column C was $150 \text{ pmol/min per mg}$.

Table 2. Comparison of immunoprecipitated and anion-exchange-purified kinase activities

Data comparing immunoprecipitated PI kinase and Type I and Type II PI kinases purified by anion-exchange chromatography are summarized below: ND, not done. Arrows indicate relative stimulation or inhibition by 0.5% concentration of the indicated detergent. Three arrows indicates stimulation/inhibition by a factor of 10 or greater; two arrows indicates stimulation/inhibition by a factor of 4–6; one arrow indicates stimulation/inhibition by a factor of 2.

	Type I	Middle-T-associated	Type II	Sephacrose CL-4B-associated
K_m for ATP	10 μM	12 μM	34 μM	38 μM
K_i for adenosine	150 μM	180 μM	20 μM	18 μM
K_i for ADP	ND	ND	40 μM	38 μM
pH optimum	7.6	7.5	8.0	7.9
NP40 effect	↓↓↓	↓↓↓	↑↑↑	↑↑↑
Triton effect	↓↓↓	↓↓↓	↑↑↑	↑↑↑
Octyl glucoside	↓↓	↓↓	↑	↑

designated as Type I PtdIns kinase; Fig. 2a, ●). When the same column fractions were assayed in the presence of 0.5% NP40 and no adenosine, the peak of PtdIns kinase activity was eluted at 400 mM-NaCl (Type II PtdIns kinase; Fig. 2a, ○). Peak fractions of the Type I PtdIns kinase were then dialysed into loading buffer, rechromatographed under conditions identical with those for the initial column, and eluate fractions again assayed for PtdIns kinase activity under both sets of conditions. The Type I PtdIns kinase was again eluted at 200 mM-NaCl, and very little activity was detected at the position where Type II PtdIns kinase is eluted (400 mM-NaCl) under either assay condition (Fig. 2b). Similarly, when the peak fractions of the Type II PtdIns kinase were rechromatographed, only Type II PtdIns kinase could be detected, and its elution position was the same as from the first column (Fig. 2c). After a third cycle of chromatography of peak fractions under identical conditions, Type I and Type II kinase were each found again to be eluted at their respective characteristic positions as single kinase types (results not shown). Column separations shown were done in detergent below its critical micelle concentration (0.02% octyl glucoside); when the octyl glucoside concentration was raised above the critical micelle concentration, Type II PtdIns kinase was eluted somewhat earlier in the salt gradient (200–300 mM-NaCl), decreasing resolution of this kinase from Type I PtdIns kinase (results not shown). Thus the two types of PtdIns kinase activities can be physically separated and do not interconvert upon subsequent chromatography.

Biochemical characterization of Type I and Type II PtdIns kinase, and comparison with immunoprecipitate-associated PtdIns kinases

Comparison of the Type I and Type II PtdIns kinases obtained by ion-exchange chromatography with the two distinct activities observed in immunoprecipitate assays was made by examination of several biochemical properties of these enzymes (Table 2). The middle-T-associated PtdIns kinase was similar to the Type I PtdIns kinase with respect to K_m for ATP (10 μM). In contrast, the Sepharose CL-4B-associated PtdIns kinase behaved like Type II kinase in that it had a higher K_m for ATP (38 μM). Fig. 3 shows that the Type I PtdIns kinase strongly resembles the middle-T/pp60^{c-src}- and pp60^{v-src}-associated PtdIns kinases (Fig. 1) with respect to inhibition

by adenosine, whereas Type II PtdIns kinase resembles the activity which non-specifically associates with Sepharose CL-4B. A detailed analysis of inhibition by adenosine of Type I and Type II kinase is shown in Fig. 4. Adenosine inhibition of Type I PtdIns kinase appears to be mixed competitive and non-competitive inhibition; the apparent K_i for adenosine, extrapolated from the effect of adenosine on K_m for ATP, was 180 μM (Fig. 4a). Adenosine inhibition of Type II kinase was simple competition with ATP, with a K_i of 20 μM , corresponding closely to the value obtained for the Sepharose-associated PtdIns kinase (Table 2). The same distinction between K_i for adenosine for Type I and Type II kinase was obtained when the two activities were assayed under identical detergent conditions (0.02% NP40), indicating that the difference in this parameter was not due to a difference in standard assay conditions between the two enzymes (results not shown). When [³²P]PtdInsP was

Fig. 3. Adenosine inhibition of Type I and Type II PtdIns kinases

Fraction 12 from the column shown in Fig. 2(b) was assayed in the absence of detergent at 40 μM -ATP for 1 min in the presence of increasing concentrations of adenosine (●). Fraction 20 from the column shown in Fig. 2(c) was assayed in the presence of 0.5% NP40 + 40 μM -ATP for 1 min in the presence of increasing concentrations of adenosine (▲). Wheat-germ-lectin-agarose precipitate from PDGF-stimulated fibroblasts was assayed in 40 μM -ATP and increasing concentrations of adenosine (△).

Fig. 4. Inhibition of PtdIns kinase activities by adenosine and ADP

Anion-exchange-purified Type I or Type II PtdIns kinases were assayed under linear conditions at several concentrations of ATP for several concentrations of inhibitors. A Lineweaver-Burk plot of data from a representative experiment is shown for each case: (a) adenosine inhibition of Type I PtdIns kinase; (b) adenosine inhibition of Type II PtdIns kinase; (c) ADP inhibition of Type II PtdIns kinase. Insets: data from the corresponding Lineweaver-Burk plots were pooled and used to estimate the K_i values for adenosine and ADP.

Fig. 5. Effect of NP40 on PtdIns kinase activities

Immunoprecipitated or anion-exchange-purified PtdIns kinase activities were assayed under standard reaction conditions in the presence of increasing concentrations of NP40. ●, anti-T immunoprecipitate from middle-T-transformed NIH 3T3 cells, washed three times in phosphate-buffered saline/1% NP40 before standard wash procedure. ■, Sephadex CL-4B precipitate from middle-T-transformed NIH 3T3 cells, washed only by the standard wash procedure. ○, Fraction 12 from column shown in Fig. 2(b). □, Fraction 20 from column shown in Fig. 2(c). Activities were normalized to maximal activity obtained for each kinase type. Maximal activities were 100 fmol/min (●), 35 fmol/min (□), 15 fmol/min (○), 20 fmol/min (■).

added to peak kinase fractions and incubated under Type I or Type II assay conditions for 90 s, less than 20% of the PtdInsP was degraded, indicating that PtdInsP phosphatase cannot account for the kinetic differences observed between different column fractions under Type I and Type II assay conditions (results not shown).

The high affinity of the Type II PtdIns kinase for adenosine relative to its affinity for ATP suggested the possibility that this kinase might be competitively

inhibited by adenine nucleotides under physiological conditions. We therefore investigated the kinetics on inhibition of this kinase by ADP. Inhibition by ADP appears to be competitive with ATP, with a K_i of 40 μ M (Fig. 4c). Identical ADP-inhibition kinetics were obtained for the Sephadex-associated PtdIns kinase (results not shown). Competitive inhibition by AMP was also tested; however, AMP inhibits Type II kinase relatively poorly, with an apparent K_i of 95 μ M at 40 μ M-ATP (results not shown).

The distinction between the middle-T/pp60^{c-src}-associated PtdIns kinase and the Sephadex-associated kinase was initially observed as a difference in the effect of the non-ionic detergent NP-40 on these activities (Table 1). The effect of NP40 on the PtdIns kinase activities obtained by column chromatography of solubilized cell membranes was also investigated (Fig. 5). Middle-T/pp60^{c-src}-associated PtdIns kinase and Type I PtdIns kinase are both inhibited at least 90% by 0.5% NP40, whereas Sephadex-associated PtdIns kinase and Type II PtdIns kinase are both stimulated by the addition of 0.5% NP40. Concentrations of NP40 above 1% are inhibitory to both PtdIns kinases at the phospholipid concentration tested (results not shown). The detergents Triton X-100 and octyl glucoside also inhibit Type I PtdIns kinase and stimulate Type II PtdIns kinase, at concentrations above their critical micelle concentrations. A summary of the properties distinguishing Type I and Type II kinase activities, and a comparison with the two activities identified in immunoprecipitates, is provided in Table 2.

Affinity precipitation of Type I PtdIns kinase from PDGF-stimulated cells

The possibility that Type I PtdIns kinase is either phosphorylated by certain tyrosine kinases or is regulated by association with a tyrosine kinase substrate is suggested by the specific association of a fraction of this enzyme with the tyrosine kinases middle-T/pp60^{c-src} and pp60^{v-src}. We further investigated this possibility in non-transformed cells by looking at cells stimulated by PDGF. PDGF causes a rapid stimulation of PtdIns turnover in fibroblasts [31–34], and its receptor is a protein tyrosine kinase [35,36]. This receptor can be partially purified by wheat germ lectin affinity chroma-

Table 3. PtdIns kinase association with wheat-germ-agarose or anti-phosphotyrosine immunoprecipitates from PDGF-stimulated fibroblasts

Wheat-germ-lectin-agarose precipitates or immunoprecipitates were prepared as described in the Experimental section from 3×10^6 Balb C/3T3 fibroblasts grown to quiescence and stimulated for 5 min with 4 units of purified PDGF/ml (+PDGF) or control buffer (–PDGF). Reactions were carried out for 5 min at 25 °C.

	PtdIns kinase activity (pmol of PtdInsP formed)	
	–PDGF	+PDGF
Wheat-germ-lectin-agarose	0.015	1.8
Anti-phosphotyrosine immunoprecipitate	0.01	0.8
Anti c-src immunoprecipitate	0.05	0.07
Anti-phosphotyrosine-immunoprecipitate + 0.5 mM free phosphotyrosine	0.01	0.025
Anti-phosphotyrosine-immunoprecipitated wheat-germ eluate	0.01	0.45

tography of detergent-solubilized cell extracts [37,38]. We found that very little PtdIns kinase bound to wheat-germ-lectin-agarose when quiescent cells were used; however, if the cells were treated with PDGF for 5 min before lysis, a 50–100-fold increase in wheat-germ-lectin-associated PtdIns kinase activity was observed (Table 3). The wheat-germ-lectin-associated PtdIns kinase had properties indicating that it is exclusively Type I PtdIns kinase (Fig. 3). This result indicates that a fraction of the Type I PtdIns kinase associates with a glycoprotein after stimulation of intact cells with PDGF.

To test the possibility that Type I PtdIns kinase is either phosphorylated on tyrosine or associates with a tyrosine-phosphorylated protein in non-transformed cells, immunoprecipitates were made from quiescent or PDGF-stimulated cells by using an anti-phosphotyrosine antibody. Anti-phosphotyrosine antibodies have been shown to immunoprecipitate PDGF receptor and other less abundant tyrosine-phosphorylated proteins [37,38]. As with wheat-germ-lectin-agarose, this antibody precipitated 80-fold more PtdIns kinase from PDGF-stimulated cells than from quiescent cells. A non-immune serum and anti-pp60^{c-src} antibody failed to precipitate significant PtdIns kinase from either quiescent or PDGF-stimulated cells (Table 3). When 0.5 mM free phosphotyrosine was added with the anti-phosphotyrosine antibody, it prevented immunoprecipitation of PtdIns kinase activity (Table 3). Phosphoserine and phosphothreonine did not have this effect (results not shown), verifying that the antibody was specifically precipitating tyrosine-phosphorylated protein. These data suggest that the PDGF receptor either directly interacts with the PtdIns kinase or phosphorylates a protein which associates with the PtdIns kinase. When wheat-germ-lectin precipitates were phosphorylated *in vitro*, eluted with *N*-acetylglucosamine, and re-precipitated with anti-phosphotyrosine antibodies, 40% of the Type I PtdIns kinase activity present in the wheat-germ-lectin precipitate could be re-precipitated with anti-phosphotyrosine antibodies.

DISCUSSION

The present study demonstrates the presence of two functionally distinct PtdIns kinase activities in 3T3 fibroblasts. These two activities are physically separable by two methods, immunoprecipitation (Table 1) and anion-exchange chromatography (Fig. 2), and are distinguishable by examination of several biochemical parameters (summarized in Table 2). Both kinases appear to be present in both normal and transformed cells. One of these kinases, arbitrarily designated as Type I, is responsible for the PtdIns kinase activity that we have previously reported to be associated with middle-T and pp60^{v-src} immunoprecipitates. We have now found that this same kinase is associated with wheat-germ-agarose precipitates and anti-phosphotyrosine immunoprecipitates from non-transformed fibroblasts stimulated with PDGF.

The possibility of the existence of two distinct PtdIns kinases has previously been raised by Harwood & Hawthorne [19] in a study of subcellular fractions from hepatocytes. In that study, a detergent-stimulated PtdIns kinase activity was observed to predominate in the microsomal fraction, whereas plasma-membrane PtdIns kinase activity was found to be relatively detergent-

independent. Several biochemical criteria distinguishing between these two activities were examined; however, because these characterizations were done under conditions which entirely inhibit our Type I activity (in the presence of 2% Cutscum), a direct comparison of the two activities reported here with those previously reported activities is difficult. The mere fact that neither activity observed by Harwood & Hawthorne [19] is inhibited by 2% non-ionic detergent suggest that both are quite distinct from the Type I PtdIns kinase reported here. Our Type II PtdIns kinase appears similar to the endoplasmic-reticulum-localized activity of Harwood & Hawthorne [19], as well as to an adenosine-sensitive PtdIns kinase observed in erythrocyte ghosts [30]. A PtdIns kinase with properties very similar to the Type II PtdIns kinase reported here has also been observed in bovine brain (G. Endemann, unpublished work). Bovine brain also has an adenosine-resistant PtdIns kinase with kinetic properties distinguishing it from either the Type I or the Type II PtdIns kinases reported here (G. Endemann, unpublished work).

An important technical consideration brought to light in the present study involves the methodologies used in immunoprecipitate studies of PtdIns kinase activity. Several recent studies investigating the co-precipitation of PtdIns kinase activity with various oncogene products have assayed immunoprecipitates in the presence of NP40 [9,39,40]. Under these conditions, PtdIns kinase was observed in immunoprecipitates; however, no difference in this activity was found between immunoprecipitates of the oncogene products and control immunoprecipitates. In each case the authors concluded that no PtdIns kinase was specifically associated with the oncogene product investigated. However, the present study demonstrates that a 100-fold difference in Type I PtdIns kinase activity between a middle-T/pp60^{c-src} immunoprecipitate and control immunoprecipitates is almost completely masked when assays are done in the presence of NP40 (Table 1). Furthermore, the binding of Type II PtdIns kinase to Sepharose CL-4B indicates that the presence of Type II PtdIns kinase activity in an immunoprecipitate does not necessarily imply an interaction between this activity and any protein component of the immunoprecipitate complex.

Although the present study demonstrates the existence of separable and distinguishable PtdIns kinase activities in fibroblasts, it does not directly address the question of whether these two activities reflect two different enzymes or functionally modified forms of the same enzyme. The many physical and kinetic differences between the Type I and Type II kinase activities seem difficult to account for by a simple covalent modification (e.g. phosphorylation): these enzymes retain their distinguishing characteristics through repeated chromatography steps (Fig. 2). The possibility that these two activities can be inter-converted through covalent modification or interaction with a regulatory subunit cannot, however, be ruled out.

No direct evidence is available concerning the regulation of either Type I or Type II PtdIns kinase *in vivo*; however, several of the properties of these activities reported here do suggest possible modes of regulation for each of these activities. The observation that the K_i for adenosine of Type II kinase is considerably lower than its K_m for ATP raises the possibility that this activity may be regulated, at least in part, by competitive inhibition by

adenine nucleotides or nucleosides. Competitive inhibition of PtdIns kinase by adenosine in muscle cells (and erythrocytes) has been proposed [29,30]; however, it seems unlikely that adenosine concentrations in the cytosol or at the plasma membrane are ever sufficiently high to compete effectively with ATP. A more plausible possibility may be that ADP acts as a competitive inhibitor of Type II PtdIns kinase, coupling its activity to the energy charge of the cell. Tight coupling of polyphosphoinositide concentrations to cellular energy charge has been reported in yeast [41]. In mammalian cells, such regulation is only likely under conditions of energy starvation or in cases where local fluctuations in [ATP]/[ADP] ratios occur, owing to rapid utilization of ATP.

The PtdIns kinase activity found to co-precipitate with the activated tyrosine kinases middle T/pp60^{c-src} and pp60^{v-src} has here been identified as Type I PtdIns kinase. pp60^{c-src} in eukaryotic or prokaryotic systems has no intrinsic PtdIns kinase activity (H. Piwnicka-Worms, D. Kaplan & M. Whitman, unpublished work; [42]), and Type I PtdIns kinase is physically separable from the middle-T/pp60^{c-src} and pp60^{v-src} immunoprecipitate complexes (M. Whitman & D. Kaplan, unpublished work), indicating that Type I PtdIns kinase activity is not an intrinsic activity of the tyrosine kinases themselves, but is rather a co-precipitating cellular enzyme. The total Type I kinase activity co-precipitating with middle T/pp60^{c-src} (220 fmol/min in Table 1, row 1, column E) is only a very small percentage of the Type I kinase in the starting lysate (40 pmol/min). As we have discussed elsewhere [43], this co-precipitation clearly does not represent a stoichiometric association between middle-T/pp60^{c-src} and Type I PtdIns, but may rather reflect the trapping of a catalytic tyrosine kinase/substrate complex which remains associated during membrane solubilization and subsequent immunoprecipitation. Sugimoto & Erickson [44] have reported an increase in total membrane PtdIns kinase activity in membranes from cells transformed with a temperature-sensitive mutant of pp60^{v-src} upon shift to the permissive temperature. This observation is consistent with PtdIns kinase regulation by pp60^{v-src}; however, it does not address the question of whether this regulation is direct or indirect. The observation that the addition of the phosphotyrosine phosphatase inhibitor NH₄VO₄ during cell lysis and immunoprecipitates strongly enhances the amount of Type I PtdIns kinase activity recovered in middle T/pp60^{c-src} [43] provides additional indirect support for the hypothesis that Type I PtdIns kinase is regulated by tyrosine phosphorylation.

A link between tyrosine phosphorylation and Type I PtdIns kinase in non-transformed cells has been implicated by the ability of wheat-germ lectin and anti-phosphotyrosine antibodies to precipitate this enzyme from PDGF-stimulated cells (Table 3). PDGF has previously been shown to cause both a rapid increase in PtdIns turnover and increases in the second messengers DAG and IP₃ in stimulated cells [31–34]. PDGF rapidly stimulates phosphorylation of several cellular proteins on tyrosine [35], and the PDGF receptor itself has been demonstrated to possess a PDGF-dependent tyrosine kinase activity [36]. We have reported here that PDGF stimulation of quiescent Balb 3T3 cells rapidly increases 50–100-fold the amount of anti-phosphotyrosine-immunoprecipitable Type I PtdIns kinase activity. Wheat-

germ lectin and anti-phosphotyrosine precipitation, alone or in series, have previously been used for partial purification of the PDGF receptor [37]. Although these procedures are not sufficient to purify this receptor to homogeneity, the PDGF-dependence of PtdIns kinase with these precipitates indicates that this may be a PDGF-receptor-linked phenomenon. Epidermal growth factor, which has little effect on PtdIns turnover in these cells [45], does not significantly increase wheat-germ-lectin or anti-phosphotyrosine-precipitable PtdIns kinase (D. Kaplan, unpublished work). As with middle-T/pp60^{c-src} immunoprecipitates, only a small portion of the total Type I PtdIns kinase present in a cell lysate co-precipitates with PDGF receptor. As discussed above, this may reflect a catalytic rather than stoichiometric interaction between these two enzymes.

The role of PtdIns kinase activation in the regulation of the hormone-stimulated PtdIns turnover pathway remains to be elucidated. Evidence that both growth-factor stimulation and oncogenic transformation involve activation of PtdIns (4,5)P₂-specific phospholipase C [8,34] is consistent with a model in which both the enzymes catalysing PtdIns(4,5)P₂ synthesis (PtdIns and PtdInsP kinases) and the enzyme(s) catalysing PtdIns(4,5)P₂ breakdown (phospholipase C) are common targets for growth factors and oncogene products. In a number of systems, an increase in PtdIns(4,5)P₂ and PtdInsP appears to coincide with increased inositol polyphosphate production, suggesting concomitant activation of phospholipase C and the phosphoinositide kinases [46,47,33]. Increased PtdInsP and PtdIns(4,5)P₂ also coincide with increased Ins(1,4,5)P₃ upon cellular expression of polyoma middle-T and pp68^{v-ros} [5,48]. Recent reports that treatment of neutrophils with cyclic AMP, or of murine erythroleukaemia cells with dimethyl sulphoxide, simultaneously decreases PtdInsP, PtdIns(4,5)P₂ and Ins(1,4,5)P₃ raises the intriguing possibility that in these systems PtdIns turnover may be inhibited, at least in part, through inhibition of the phosphoinositide kinases [47,49].

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REFERENCES

1. Hokin, L. E. (1985) *Annu. Rev. Biochem.* **54**, 205–235
2. Berridge, M. J. & Irvine, R. F. (1984) *Nature (London)* **312**, 315–321
3. Nishizuka, Y. (1984) *Nature (London)* **308**, 693–698
4. Michell, R. H., Kirk, C. J., Jones, L. M., Downes, C. P. & Creba, J. A. (1981) *Philos. Trans. R. Soc. London B* **296**, 123–137
5. Berridge, M. (1984) *Biochem. J.* **220**, 345–360
6. Macara, I. G. (1985) *Am. J. Physiol.* **248**, C3–C9
7. Whitman, M., Kaplan, D. R., Schaffhausen, B., Cantley, L. & Roberts, T. M. (1985) *Nature (London)* **315**, 239–241
8. Kaplan, D. K., Whitman, M. R., Schaffhausen, B., Raptis, L., Garcea, R. L., Pallas, D., Roberts, T. M. & Cantley, L. C. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 3624–3628

9. Jackowski, S., Rettenmeier, C. W., Sher, C. J. & Rock, C. O. (1986) *J. Biol. Chem.* **261**, 4978–4985
10. Whitman, M., Fleischmann, L. F., Chahwala, S. B., Rosoff, P. & Cantley, L. C. (1986) in *Receptors and Phosphoinositides* (Putney, J., ed.), pp. 197–217, Alan R. Liss, New York
11. Majerus, P. W., Wilson, D. B. & Connolly, T. M. (1985) *Trends Biochem. Sci.* **10**, 168–171
12. Chahwala, S., Fleischman, L. & Cantley, L. (1987) *Biochemistry*, **26**, 612–622
13. Berridge, M. J. (1983) *Biochem. J.* **212**, 849–858
14. Creba, J. A., Downes, C. P., Hawkins, P. T., Brewster, G., Michell, R. H. & Kirk, C. J. (1983) *Biochem. J.* **212**, 733–747
15. Hokin, L. E. & Hokin, M. R. (1964) *Biochim. Biophys. Acta* **84**, 563–567
16. Michell, R. H. & Hawthorne, J. N. (1965) *Biochem. Biophys. Res. Commun.* **21**, 333–339
17. Kai, M., White, G. L. & Hawthorne, J. N. (1966) *Biochem. J.* **101**, 328–337
18. Jergil, B. & Sundler, R. (1983) *J. Biol. Chem.* **258**, 7968–7973
19. Harwood, J. L. & Hawthorne, J. N. (1969) *Biochim. Biophys. Acta* **171**, 75–87
20. Colodzin, M. & Kennedy, E. P. (1965) *J. Biol. Chem.* **250**, 3771–3780
21. Hajra, A. K., Seguin, E. B. & Agranoff, B. W. (1968) *J. Biol. Chem.* **243**, 1609–1616
22. Smith, C. D. & Wells, W. W. (1983) *J. Biol. Chem.* **258**, 2130–2134
23. Campbell, C. R., Fishman, J. B. & Fine, R. E. (1985) *J. Biol. Chem.* **260**, 10948–10951
24. Treisman, R., Novak, U., Favalaro, J. & Kamen, R. (1981) *Nature (London)* **292**, 595–600
25. Courtneidge, S. A. & Smith, A. E. (1983) *Nature (London)* **303**, 435–439
26. Bolen, J. B., Thiele, C. J., Israel, M. A., Yonemoto, W., Lipsich, L. A. & Brugge, J. S. (1984) *Cell* **38**, 767–777
27. Macara, I. G., Marinetti, G. V. & Balduzzi, P. C. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 2728–2732
28. Fry, M. J., Gebhardt, A., Parker, P. J. & Foulkes, G. (1985) *EMBO J.* **4**, 3173–3178
29. Doctrow, S. R. & Lowenstein, J. (1985) *J. Biol. Chem.* **260**, 3469–3476
30. Buckley, J. T. (1977) *Biochim. Biophys. Acta* **498**, 1–9
31. Habenicht, A. J., Glomset, J. A., King, W. C., Nist, C., Mitchell, C. D. & Ross, R. (1981) *J. Biol. Chem.* **256**, 12329–12335
32. Hagewa-Sasaki, H. (1985) *Biochem. J.* **232**, 99–109
33. Berridge, M., Heslop, J., Irvine, R. F. & Brown, K. D. (1984) *Biochem. J.* **222**, 195–201
34. Chu, S. H. W., Hoban, C. J., Owen, A. J. & Creyer, R. P. (1985) *J. Cell. Physiol.* **124**, 391–396
35. Cooper, J. A., Bowen-Pope, E., Raines, E., Ross, R. & Hunter, T. (1982) *Cell* **31**, 263–268
36. Ek, B. & Heldin, C. H. (1982) *J. Biol. Chem.* **257**, 10486–10491
37. Williams, L. T., Tremble, P. M., Daniel, T. O., Coughlin, S. R., Giels, G. & Wang, J. Y. J. (1985) in *Growth Factors and Transformation* (Feramisco, J., ed.), pp. 175–182, Cold Spring Harbor Laboratory, Cold Spring Harbor
38. Daniel, T. O., Tremble, P. M., Frackleton, A. R. & Williams, L. T. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 2684–2689
39. Sugano, S. & Hanafusa, H. (1985) *Mol. Cell. Biol.* **5**, 2399–2404
40. Koch, W., Carbone, A. & Walter, G. (1986) *Mol. Cell. Biol.* **6**, 1866–1874
41. Talwalkar, R. T. & Lester, R. L. (1973) *Biochim. Biophys. Acta* **306**, 412–421
42. Piwnicka-Worms, H., Kaplan, D., Whitman, M. & Roberts, T. (1986) *Mol. Cell. Biol.* **6**, 2033–2040
43. Whitman, M., Kaplan, D., Roberts, T. M., Cantley, L. C. & Schaffhausen, B. S. (1986) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **45**, 2647–2651
44. Sugimoto, Y. & Erikson, R. (1985) *Mol. Cell. Biol.* **5**, 3194–3198
45. Besterman, J. M., Watson, S. P. & Cuatrecasas, P. (1986) *J. Biol. Chem.* **262**, 723–727
46. Taylor, M. V., Metcalfe, J. C., Hesketh, T. R., Smith, G. A. & Moore, J. P. (1984) *Nature (London)* **312**, 463–466
47. Della Bianca, V., De Togni, P., Grzeskowiak, M., Vicentini, L. M. & DiVirgilio, F. (1986) *Biochim. Biophys. Acta* **886**, 441–447
48. Macara, I. G., Marinetti, G. V., Livingston, J. N. & Balduzzi, P. C. (1985) in *Growth Factors and Transformation* (Feramisco, J., ed.), pp. 365–368, Cold Spring Harbor Laboratory, Cold Spring Harbor
49. Faletto, D. L., Arrow, A. S. & Macara, I. G. (1985) *Cell* **43**, 315–325

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