Phosphatidylinositol Kinase Activity in Virus-Transformed and Nontransformed Cells

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We assayed phosphatidylinositol (PI) kinase (EC 2.7.1.67) activity in detergent extracts of nontransformed or virus-transformed cells. Nontransformed chicken embryo fibroblasts (CEF) contain PI kinase activity with an apparent specific activity of 20 pmol/min per mg of protein. This activity sedimented as a single peak with a molecular weight of approximately 60,000 in a glycerol gradient, although immunoprecipitation with anti-p60^{src} sera showed that the PI kinase activity is distinct from p60^{c-src}. Extracts from CEF transformed by Rous sarcoma virus, Fujinami sarcoma virus, or avian sarcoma virus UR2 showed no elevation of PI kinase activity over nontransformed CEF. Removal of the oncogene products from extracts by immunoprecipitation did not change the level of PI kinase activity in extracts, suggesting that putative virus-coded PI kinases do not make a significant contribution to overall levels of PI kinase activity in transformed cells. Additionally, P140^{gag-fps} was separated from cellular PI kinase by phosphocellulose chromatography. This partially purified fraction contained low PI kinase activity distinct from P140^{gag-fps}, indicating that P140^{gag-fps} has no detectable PI kinase activity.

There has been a great deal of interest in an intracellular signaling system that utilizes inositol lipids and might be involved in regulating cell growth (1, 26, 29, 32). Various stimuli induce the rapid hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP₂) by phospholipase C to diacylglycerol and inositol trisphosphate which act as second messengers. Diacylglycerol activates protein kinase C, and inositol trisphosphate mobilizes intracellular calcium. This hydrolysis is followed by the phosphorylation of phosphatidylinositol (PI) and phosphatidylinositol-4-monophosphate (PIP). PIP₂ is formed from PI through stepwise phosphorylation by PI kinase (EC 2.7.1.67) and PIP kinase (EC 2.7.1.68). These kinases are known to exist in a variety of tissues (7, 8, 15, 17, 20-22, 30). There are also corresponding phosphomonoesterases (23, 33, 36), and the inositol lipids mentioned above are thought to be in equilibrium with each other through "futile cycles" constituted by these kinases and esterases. The close relation between this signaling system and cell growth regulation was suggested by the fact that several growth factors could induce PI turnover (10, 14, 34). In addition, tumor-promoting phorbol esters can stimulate protein kinase C directly (5).

The stimulation of this system in the transforming process of a tumor virus was first suggested by Diring and Friis (9), who showed that PI turnover is elevated in Rous sarcoma virus (RSV)-transformed quail cells. Direct involvement of oncogene products in this signaling system was suggested by Sugimoto et al. (37), who reported that the purified transforming protein of RSV (p60^{v-src}) has PI, PIP, and diacylglycerol kinase activity. Confirming the observation of Diring and Friis with a temperature-sensitive mutant of RSV, Sugimoto et al. suggested that the increased PI turnover is the direct effect of lipid kinase activity of p60^{v-src} and that the elevated PI turnover results in transformation through the overproduction of second messengers. Macara et al. (25) extended this hypothesis by showing that specifically immunoprecipitated avian sarcoma virus UR2 transforming gene product (P68^{gag-ros}) was able to phosphorylate

PI. They suggested that various oncogene products possessing tyrosine kinase activity might also have PI kinase activity and that PI kinase activity is responsible for transformation.

In this report, we asked two questions relevant to two important points of the above hypotheses. (i) If elevated PI turnover is a direct effect of the PI kinase activity of oncogene products, then what is the difference in the overall PI kinase activity between transformed and nontransformed cells, and in transformed cells how much of it is attributable to the PI kinase activity of the oncogene products, and (ii) is any PI kinase activity associated with the oncogene product of Fujinami sarcoma virus (FSV), P140^{gag-fps}, which has tyrosine kinase activity?

MATERIALS AND METHODS

Materials. [γ -³²P]ATP and [³H]leucine were from Amersham Corp. Lipids, AppppA [P¹,P⁴-di(adenosine-5') tetraphosphate], and quercetin were from Sigma Chemical Co., and precoated silica gel 60 glass plates for thin-layer chromatography were from EM Science.

Cells and viruses. Cultures of secondary chicken embryo fibroblasts (CEF) were maintained or infected as previously described (16). Rat 3Y1 cells and FSV-transformed 3Y1 cells (28) were maintained in Dulbecco modified Eagle medium (GIBCO Laboratories) containing 5% calf serum. The Schmidt-Ruppin strain of RSV subgroup A (SRA), FSV, and UR2 were prepared as previously described (16). NY501-infected CEF were prepared as previously described (19).

Labeling of cells, preparation of cell extracts and glycerol gradients. Labeling of cultures with [³H]leucine was done essentially as previously described (19). Cell extracts were prepared according to the method of Feldman et al. (11) in RIPA buffer containing 50 mM Tris hydrochloride (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM EDTA, and 100 Kallikrein inactivation units (KIU) of Trasylol per ml. Fractionation of cell extracts on glycerol gradients was performed as previously described (3). Each fraction was directly assayed for PI kinase activity.

PI kinase assay. PI kinase activities in RIPA extracts were

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assayed by adding 5 to 10 μ l (containing 0.1 to 0.5 μ g of protein) of extract to 40 μ l of the prewarmed assay buffer described by Macara et al. (25), consisting of 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-Tris (pH 7.4), 10 mM $MnCl_2$, 2 μ M [γ - ^{32}P]ATP (25 to 50 Ci/mmol), 200 μ g of PI per ml, and 0.1% Nonidet P-40. Occasionally, we used an assay buffer described by Sugimoto et al. (37), containing 10 mM KPO_4 (pH 7.1), 1 mM EDTA, 20 mM $MgCl_2$, 20 μ M [γ - ^{32}P]ATP (25 to 50 Ci/mmol), and 200 μ g of PI per ml. We included 1% Triton X-100 in the assay buffer to solubilize PI. After incubation at 25°C for 30 min, the reaction was stopped by adding 150 μ l of chloroform-methanol (1:2). Lipids were extracted by the method of Schacht (35). To analyze extracted lipids, dried lipids were dissolved in 30 μ l of chloroform-methanol-water (20:9:1), spotted on silica gel 60 plates and separated by 1-propanol-4 N NH_4OH (13:7). Plates were dried and autoradiographed to visualize the lipids that incorporated ^{32}P . Lipid standards were visualized by using iodine vapor. Radioactive spots were scraped off plates and quantitated by Cerenkov counting. Gel plates were used without activation.

Immunoprecipitation and protein kinase assay. For immunoprecipitation, RIPA extracts containing 10 to 50 μ g of protein were incubated with various antisera for 2 h on ice. Protein A-Sepharose (50% [vol/vol]) was added and mixed for 1 h to adsorb immune complexes. Protein A-Sepharose was pelleted, and the supernatant was assayed for PI kinase or subjected to a second cycle of immunoprecipitation. The Sepharose pellet was washed three times with RIPA buffer and processed for sodium dodecyl sulfate-polyacrylamide gel electrophoresis as previously described (12).

Protein kinase assays were performed in PI kinase assay buffers. For assaying protein kinase activity or PI kinase activity in the immunoprecipitate, the Sepharose pellet was further washed with the PI kinase assay buffer described above, without PI and ATP. The reaction was started by adding 40 μ l of prewarmed PI kinase assay buffer, and the incubation was continued for 30 min at 25°C. To examine protein kinase activity, the reaction was stopped by adding excess EDTA, and the Sepharose beads were washed three times with RIPA buffer and processed for polyacrylamide gel

FIG. 1. Extraction of PI kinase activity by RIPA buffer. A confluent monolayer of CEF in a 10-cm dish was either homogenized in 0.24 M sucrose or extracted by an equal volume of RIPA buffer. The unsolubilized portion of CEF after RIPA extraction was suspended by homogenization in an equal volume of RIPA buffer. A sample (5 μ l) of each was assayed by using the assay conditions of Macara et al. (25). Lanes: 1, RIPA extract; 2, unsolubilized portion; 3, whole-cell homogenate. F, Solvent front; O, origin; PA, phosphatidic acid; PIP₂, phosphatidylinositol-4,5-bisphosphate.

FIG. 2. Dose-response curve of PI kinase activity for protein. A RIPA extract of CEF was diluted with RIPA buffer so that 5 μ l of it contained the indicated quantity of protein.

electrophoresis. For PI kinase activity, the reaction was stopped and lipids were extracted as described above. All sera were heated to 56°C for 30 min to inactivate PI kinase activity in them.

Phosphocellulose column chromatography. RIPA extracts of FSV-transformed 3Y1 cells (containing 8 mg of protein) were passed through a small column of phosphocellulose (0.3 ml) three times at a flow rate of 5 to 10 ml/h. The column was washed with 4 column volumes of RIPA buffer, and bound proteins were eluted with 3 column volumes of RIPA buffer containing 0.5 M NaCl. The eluted fraction was diluted with RIPA buffer without NaCl to reduce salt concentration.

RESULTS

PI kinase in CEF. We first characterized PI kinase activity in CEF. RIPA buffer extracted more than 90% of the cellular PI kinase activity (Fig. 1). This finding is consistent with the

TABLE 1. Effect of cations and ATP analogs on PI kinase activity in RIPA extracts^a

Modification	Incorporation of phosphates into PIP (%) in:	
	CEF	SRA-infected CEF
None	100 ^b	100 ^c
+10 mM $MgCl_2$ (– $MnCl_2$)	43	36
+10 mM $CaCl_2$ (– $MnCl_2$)	3	2
+10 mM $CoCl_2$ (– $MnCl_2$)	15	13
+10 mM $CaCl_2$	52	49
+0.25 mM AppppA ^d	24	24
+0.25 mM Quercetin	4	5

^a A 5- μ l sample of RIPA extracts from the indicated cells containing 0.1 μ g of protein was assayed for PI kinase activity by using the assay conditions of Macara et al. (25) with indicated modifications. The activity is expressed as a percentage of the activity assayed with no modification.

^b 100% represents 1.7 fmol/min incorporation of phosphates into PIP.

^c 100% represents 1.8 fmol/min incorporation of phosphates into PIP.

^d AppppA, P¹,P⁴-di(adenosine-5')tetraphosphate.

FIG. 3. Sedimentation of PI kinase activity through glycerol gradients. RIPA extracts (300 μ l) were sedimented through 5-ml 10 to 30% glycerol gradients in RIPA buffer. After sedimentation, 0.5-ml fractions were collected, and 5 μ l of each fraction was assayed for PI kinase activity. p60^{v-src} and P140^{src-ps} were used as molecular weight ($\times 10^3$) markers in parallel gradients. (A) CEF. (B) SRA-infected CEF.

results of Bostwick and Eichberg (2) showing that the PI kinase in rat brain microsomes was extracted with 1% Triton X-100. We used RIPA extract as the enzyme source for the subsequent experiments. We chose the PI kinase assay conditions described by Macara et al. (25). The dose dependence of the PI kinase reaction is shown in Fig. 2. Because dose dependence is not linear, all comparisons were made at 0.1 μ g of cellular protein per reaction. The specific activity of PI kinase in the crude extract was about 20 pmol/min per mg of protein, and the apparent K_m s for ATP and PI were about 8 μ M and 50 μ g/ml (this corresponds to approximately 57 μ M, assuming PI contains stearyl and arachidonyl groups as acyl groups), respectively. When the conditions of Sugimoto et al. (37) were used, but with 1% Triton X-100, the specific activity of the PI kinase increased about three- to fourfold (see Fig. 5), and the K_m for ATP also increased to about 130 μ M.

Table 1 shows the effects of various divalent cations and ATP analogs on the PI kinase activity of CEF. Substitution of Mn^{2+} by Mg^{2+} reduced the activity about 50%. Ca^{2+} hardly supported the PI kinase activity, and the addition of Ca^{2+} to 10 mM was inhibitory. The same inhibitory effect of Ca^{2+} has been demonstrated for PI kinase of various tissues (7, 17, 22). Quercetin and AppppA, known to inhibit the tyrosine kinase activity of the RSV oncogene product p60^{v-src} (13, 27), could also inhibit this PI kinase activity. Inhibition was more than 90% for quercetin and 76% for AppppA at a concentration of 250 μ M (in the assay conditions of Macara et al. [25]). Inhibition of PI kinase activity by ATP analogs was also observed by Buckley with human erythrocyte PI kinase (4).

FIG. 4. PI kinase activity in CEF and NY501-infected CEF. (A) Comparison of PI kinase activity in extracts of CEF and NY501-infected CEF. Each reaction contained 0.1 μ g of cellular protein. (B) Sequential immunoprecipitation of p60^{v-src}. Extracts from CEF or NY501-infected CEF labeled with [³H]leucine were subjected to three cycles of immunoprecipitation with anti-p60^{v-src} (18). Precipitated proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorographed. Numbers indicate each cycle. Bar indicates the position of p60^{v-src}. (C) PI kinase activity remaining in the supernatant after each cycle of immunoprecipitation. Each reaction should contain 0.1 μ g of cellular protein if there were no loss of cellular protein by immunoprecipitation.

FIG. 5. PI kinase activity in CEF and SRA-infected CEF. Each reaction contained 0.5 μ g of cellular protein. (A) Assay conditions of Macara et al. (25). (B) Assay conditions of Sugimoto et al. (37) with 1% Triton X-100.

When the CEF extract was sedimented through a 10 to 30% glycerol gradient, PI kinase activity formed a single peak at around 60,000 molecular weight (Fig. 3A). The PI kinase activity in rat 3Y1 cells also formed a single peak around 60,000 molecular weight (data not shown).

p60^{c-src} and PI kinase in CEF. The sedimentation behavior of PI kinase activity prompted us to test the hypothesis that the enzyme activity is due to p60^{c-src}. First, we assayed PI kinase level in CEF infected with NY501, a virus that overproduces p60^{c-src} instead of p60^{v-src} (19). No elevation in PI kinase activity was detected compared with uninfected CEF (Fig. 4A). Next, we immunoprecipitated p60^{c-src} with antiserum raised against denatured p60^{v-src} synthesized in *Escherichia coli* (18) and asked whether PI kinase activity coprecipitates with p60^{c-src} or remains in the supernatant. More than 90% of the PI kinase activity remained in the supernatant, even after three cycles of immunoprecipitation (Fig. 4C). In contrast, most of the p60^{c-src} in CEF precipitated from the extract at the first cycle (Fig. 4B). The monoclonal antibody 327 that recognizes both p60^{c-src} and p60^{v-src} (24) was also unable to immunoprecipitate cellular PI kinase activity (data not shown). These results strongly suggest that the PI kinase in CEF is associated with a molecule distinct from p60^{c-src}. There remains, however, the possibility that this cellular PI kinase is a specially modified form of p60^{c-src}. This is unlikely because the overexpressed p60^{c-src} coded for by NY501 has the same amino acid sequence as chicken p60^{c-src} and is known to undergo the same modifications (such as myristylation and phosphorylation) in CEF (18).

TABLE 3. Separation of P140^{gag-fps} and PI kinase activity by phosphocellulose

Step	Total protein (mg)	Total PI kinase (pmol/min)	Total P140 ^{gag-fps} protein kinase (fmol/min) ^a
Crude extract	8.0	140	650
Flowthrough	6.5	150	ND ^b
Eluate with 0.5 M NaCl RIPA	ND	0.8	330

^a The activity was determined by the amount of phosphates incorporated into P140^{gag-fps}.

^b ND, Not determined.

PI kinase in transformed cells. Overall PI kinase activity in SRA-infected CEF was at the same level as that in uninfected CEF (Fig. 5). The levels were also the same in UR2- and FSV-infected cells (Table 2). Moreover, the effects of divalent cations and ATP analogs on PI kinase activity (Table 1) and the sedimentation profile of enzyme activity (Fig. 3B) were essentially the same as those in CEF. This indicates that the major PI kinase in SRA-infected CEF is the cellular PI kinase already present in CEF before infection.

We also compared PI kinase activity in extracts of virus-transformed CEF before and after immunoprecipitation of the oncogene products (Table 2). PI kinase levels remained unchanged after immunoprecipitation, and less than 1% of the total PI kinase activity was coprecipitated with the oncogene products. However, this quantitative comparison between activity in the extract and in immunoprecipitates may be misleading, because the enzyme is in the solid phase in the precipitate. Qualitatively, these immunoprecipitates do allow tyrosine phosphorylation of exogenous substrates.

PI kinase activity of P140^{gag-fps}. The contribution of the putative PI kinase activity of P140^{gag-fps} to the total PI kinase activity in FSV-transformed CEF could not be greater than 10% even if one allowed a generous margin for experimental error (Table 2). To measure the PI kinase activity of P140^{gag-fps} more directly, we used P140^{gag-fps} from FSV-transformed 3Y1 cells partially purified on phosphocellulose. About 50% of the protein kinase activity of P140^{gag-fps} bound to phosphocellulose, whereas less than 1% of the PI kinase did (Table 3). More than 99% of the residual PI kinase activity eluted with P140^{gag-fps} by RIPA buffer containing 0.5 M NaCl was subsequently separable from P140^{gag-fps} by immunoprecipitation (Table 4). Furthermore, the level

TABLE 2. PI kinase activity before and after immunoprecipitation

Virus (serum) ^a	Sp act of PI kinase in extracts before IP ^b (pmol/min per mg)	Total protein in extracts (μ g)	Total PI kinase activity in extracts before IP (fmol/min)	Total PI kinase activity remaining in supernatant after IP (fmol/min [%]) ^c	Total PI kinase activity immunoprecipitated (fmol/min [%]) ^c
SRA (TBR)	18	30	530	530 (100)	0.3 (0.06)
SRA (NIS)	18	30	530	510 (96)	0.2 (0.04)
UR2 (anti-G)	18	13	240	250 (104)	0.3 (0.1)
FSV (anti-G)	19	18	340	330 (97)	0.2 (0.06)

^a TBR, Tumor-bearing rabbit serum; NIS, nonimmune serum; anti-G, anti-gag serum.

^b IP, Immunoprecipitation.

^c The activity expressed as a percentage of total PI kinase activity before immunoprecipitation is shown in parentheses.

TABLE 4. PI kinase activity before and after immunoprecipitation of fraction eluted with 0.5 M NaCl RIPA buffer

Serum used for IP ^a	Total PI kinase activity (fmol/min)		
	Before IP	In supernatant after IP	Precipitated by IP
Anti-G ^b	33	33	0.06
NIS ^c	33	34	0.06

^a IP, Immunoprecipitation.

^b Anti-G, Anti-*gag* serum.

^c NIS, Nonimmune serum.

of immunoprecipitable PI kinase activity was the same with anti-*gag* serum compared with nonimmune serum. P140^{*gag-fps*} from FSV-transformed CEF also gave essentially the same results (data not shown). These results show that P140^{*gag-fps*} has no detectable PI kinase activity. The limit of detection was about 10⁻⁵ of the total PI kinase activity in the cell.

DISCUSSION

PI kinase in CEF. We found that RIPA extracts of CEF contain most of the cellular PI kinase activity. This finding is consistent with the results of Bostwick and Eichberg (2). Therefore, we used whole-cell extracts prepared in RIPA buffer as an enzyme source, although there was a possibility that RIPA buffer could limit the PI kinase activity detectable in cell extracts. The apparent specific activity of PI kinase is about 20 pmol/min per mg of protein in crude detergent extracts. The specific activity of PI kinase observed here was low compared with those previously reported for various tissues (0.1 to 10 nmol/min per mg of protein). This may be a reflection of the assay conditions or the cells we used. Sedimentation of detergent extracts of various tissues through a glycerol gradient provides a convenient method to distinguish different forms of PI kinases. The sedimentation profile of the endogenous CEF PI kinase through a 10 to 30% glycerol gradient showed a single peak at around 60,000 molecular weight. PI kinase activities in both CEF and 3Y1 cells showed the same sedimentation profile, suggesting that the structures of PI kinases are conserved in different species.

We showed that the PI kinase activity in CEF was not immunoprecipitated by an antibody that recognizes p60^{*c-src*}. Moreover, overproduction of p60^{*c-src*} did not increase the PI kinase level in the cells. These data indicate that the PI kinase activity in CEF is not encoded by the *c-src* gene. It is unlikely that this PI kinase activity is encoded by *c-ros*, because the expression of *c-ros* is undetectable in CEF (W. S. Neckameyer and L.-H. Wang, manuscript in preparation). We do not now know whether p60^{*c-src*} has any PI kinase activity at all. The immunoprecipitate containing overproduced p60^{*c-src*} showed no elevated PI kinase activity over that of a control precipitate with nonimmune serum (data not shown).

PI kinase in transformed cells. We found that virus-transformed CEF contain the same level of PI kinase activity as untransformed CEF. Moreover, depletion of viral oncogene products by immunoprecipitation did not change the overall PI kinase activity in the remaining cell extract supernatants. These data suggest that the contribution of virus-coded PI kinases to the overall PI kinase activity is less than our experimental error, which was about 10%. These

results stand in contrast to that of tyrosine kinase activity in RSV-transformed cells. Clinton and Roskoski (6) showed that the overall tyrosine kinase activity in RSV-transformed BHK cells is fivefold higher than that in nontransformed BHK cells. Removing p60^{*v-src*} from extracts by immunoprecipitation reduced the kinase activity to the same level as in the extracts from nontransformed cells.

Our observations suggest that elevated PI turnover in RSV- or UR2-transformed cells is not the direct effect of PI kinase activity of oncogene products. Furthermore, our results indicate that the viral transformation does not change the overall level of PI kinase activity. This suggests that the activity(s) other than PI kinase activity is altered in transformed cells and is responsible for the elevation of PI turnover in RSV- or UR2-transformed cells. One possible enzyme might be the phospholipase C that hydrolyzes PIP₂.

Macara et al. (25) reported a PI kinase activity of 1 fmol/min for P68^{*gag-ros*} that immunoprecipitated from extracts of UR2-transformed cells containing 100 µg of protein. Our results indicate the presence of at least a thousandfold higher cellular PI kinase activity in extracts of UR2-transformed cells than that reported for P68^{*gag-ros*}. The presence of cellular PI kinase limits the value of assaying immunoprecipitates for estimating the PI kinase activity of oncogene products. We observed that the apparent PI kinase activity in immunoprecipitates correlates with immunoglobulin content and that some sera, if not heat inactivated, contained PI kinase activity (data not shown). Also, the variation among experiments was much higher than 10%. We were unable to detect any reproducible difference between PI kinase activity in immunoprecipitates that contained P68^{*gag-ros*} and those that did not (data not shown). It should be noted that all the sera used in this study, except tumor-bearing rabbit serum, allow the tyrosine phosphorylation of exogenous substrates by the oncogene products in immunoprecipitates.

The phosphocellulose-binding property of P140^{*gag-fps*} enabled us to separate more than 99% of cellular PI kinase from P140^{*gag-fps*}. Our preliminary attempts (data not shown) to separate p60^{*v-src*} from cellular PI kinase by using DEAE-cellulose, phosphocellulose, Affi-Gel Blue, and octyl-Sepharose were unsuccessful. We tried to estimate the PI kinase activity of P140^{*gag-fps*} by immunoprecipitation by using the phosphocellulose purified fraction. We could not find any indication that P140^{*gag-fps*} has PI kinase activity (Table 4). Our findings are consistent with that of Moelling et al. (31) that P140^{*gag-fps*} retained on Sepharose beads by monoclonal anti-p19 antibody fails to show elevated PI kinase activity over control beads. Although we cannot rule out the possibility that a low level of PI kinase activity is associated with P140^{*gag-fps*}, it appears unlikely that the level is sufficient to provide the major mechanism of cell transformation by FSV.

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