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Evidence that the Rous sarcoma virus transforming gene product phosphorylates phosphatidylinositol and diacylglycerol

(cell transformation/transforming gene function)

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ABSTRACT **The ability of purified Rous sarcoma virus transforming gene product, pp60$^{v\text{-}src}$, to phosphorylate phosphatidylinositol and diacylglycerol was investigated. Phosphatidylinositol was phosphorylated to form both mono- and diphosphorylated derivatives. 1,2-Diacylglycerol was phosphorylated to form phosphatidic acid. These activities showed the same thermolability and the same sensitivity to inhibitors as shown by the casein kinase activity of pp60$^{v\text{-}src}$. In addition, when serum-starved chicken embryo fibroblasts transformed by a virus mutant temperature-sensitive for transformation were shifted from the nonpermissive to permissive temperature, an increase of 50–100% in the labeling of phosphatidylinositol 4-phosphate, phosphatidylinositol 4,5-bisphosphate, and phosphatidic acid was observed, as compared to uninfected cells.**

New information concerning the genes involved in malignant transformation is accumulating at an extraordinary rate. Retrovirus transforming genes and oncogenes from a variety of human tumors are now well characterized (see for example The Cancer Cell, Cold Spring Harbor Cell Proliferation Conference 1983), and in several cases the protein products of these genes have been identified by the use of immunological probes. Nevertheless, the biochemical events leading to malignancy after expression of these transforming proteins remain largely unknown, even in the case of what is, arguably, the best characterized one—the product of the Rous sarcoma virus (RSV) *src* gene, pp60$^{v\text{-}src}$. This protein has the capacity to phosphorylate protein substrates on tyrosine residues (1–3) and recently has been shown to phosphorylate low molecular weight alcohols such as glycerol (4, 5). Although a variety of proteins have been shown to become phosphorylated on tyrosine after expression of pp60$^{v\text{-}src}$ in infected cells (6–9), thus far no regulatory mechanism has been attributed to any of these phosphorylation reactions. Thus, the physiological significance of protein and glycerol phosphorylation by pp60$^{v\text{-}src}$ remains obscure.

Although tyrosine phosphorylation has been the focus of a great deal of attention, analysis of specific proteins, such as ribosomal protein S6, has revealed that protein phosphorylation on serine residues is also quantitatively altered by the expression of pp60$^{v\text{-}src}$ (10). These observations suggest that some effects of pp60$^{v\text{-}src}$ may be mediated by the direct or indirect regulation of a protein kinase and/or a phosphoprotein phosphatase specific for serine. One candidate enzyme is the cyclic AMP-dependent protein kinase, an enzyme known to be involved in the regulation of diverse pathways (11) and the subject of a recent communication from this laboratory (12). Another is the novel enzyme denoted C-kinase by Takai *et al.* (13) that requires Ca^{2+} and phospholipid but, at physiological concentrations of Ca^{2+}, depends on diacylglycerol for its activation. Recently it has been suggested that the tumor-promoting phorbol esters activate C-kinase by substituting for diacylglycerol; thus, this enzyme may play a role in the proliferative and tumor-promoting effects of these agents (14). It is also of interest that phorbol esters stimulate the phosphorylation of ribosomal protein S6 in serum-starved cells in a manner reminiscent of pp60$^{v\text{-}src}$ (unpublished data).

Little is known about the regulation of the enzymes involved in diacylglycerol synthesis and degradation; however, it seems clear that most diacylglycerol is produced by hydrolysis of phosphatidylinositol (PtdIns) by a phosphodiesterase and is removed by rephosphorylation to phosphatidic acid (15, 16). Regulation of the hydrolysis of PtdIns may involve a kinase that phosphorylates PtdIns in the inositol head group to form phosphatidylinositol 4-phosphate (PtdIns4*P*) and phosphatidylinositol 4,5-bisphosphate (PtdIns4,5P_2) (17). Because PtdIns4,5P_2 seems to be the best substrate for the phosphodiesterase, a plasma membrane kinase must be an important component in the regulation of PtdIns turnover and diacylglycerol production.

In view of the capacity of pp60$^{v\text{-}src}$ to phosphorylate glycerol and of the observation that PtdIns turnover is stimulated in RSV-transformed cells (18), we investigated the relevant lipids as pp60$^{v\text{-}src}$ substrates. In this manuscript we show that purified pp60$^{v\text{-}src}$ phosphorylated PtdIns to form mono- and diphosphorylated derivatives and, moreover, phosphorylated diacylglycerol to form phosphatidic acid, suggesting that it has the potential to regulate both the production and removal of diacylglycerol. Additional experiments also showed increased biosynthetic labeling of PtdIns4*P*, PtdIns4,5P_2, and phosphatidic acid in RSV-transformed cells. These data are discussed in regard to the activation of protein kinase C.

MATERIALS AND METHODS

Materials. $H_3{}^{32}PO_4$, carrier-free (285 Ci/mg; 1 Ci = 37 GBq), was purchased from New England Nuclear. [γ-^{32}P]ATP (5000–9000 Ci/mmol) was prepared as described (19). By using ion-exchange chromotography, pp60$^{v\text{-}src}$ was purified to homogeneity from European field vole cells transformed by the Schmidt–Ruppin strain of RSV (unpublished data). The catalytic subunit of cAMP-dependent protein kinase was purified from bovine heart as described by Beavo *et al.* (20) and was kindly provided by Yosef Graziani and James Maller. Both enzymes were kept in 10 mM potassium phosphate, pH 7.1/1 mM EDTA/5 mM dithiothreitol/50% glycerol at −20°C. The IgG fraction of serum containing antibody against pp60$^{v\text{-}src}$, taken from RSV-induced tumor bearing rabbits (TBR-IgG), was prepared as described (21). Silica gel 60 plates were obtained from Merck. PtdIns from bovine liver was obtained from Avanti Polar-Lipid (Bir-

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Abbreviations: RSV, Rous sarcoma virus; PtdIns, phosphatidylinositol; PtdIns4*P*, phosphatidylinositol 4-phosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; anti-pp60$^{v\text{-}src}$ IgG, TBR-IgG, IgG fraction of serum containing antibody against pp60$^{v\text{-}src}$, taken from RSV-induced tumor-bearing rabbits.

mingham, AL). Other lipids, α-casein, and quercetin were from Sigma.

Assay of Lipid Kinase Activity. The phosphorylation of PtdIns, PtdIns4*P*, and diacylglycerol was measured in 30 μl of 10 mM potassium phosphate, pH 7.1/1 mM EDTA/20 mM $MgCl_2$/20 μM [γ-^{32}P]ATP (<60 μCi) containing enzyme as indicated. Lipids were dried under a stream of nitrogen, evaporated *in vacuo* for 10 min, and suspended in 10 mM potassium phosphate, pH 7.1/1 mM EDTA. The suspension was sonicated for 1 hr at 40–50°C. Before the addition of $MgCl_2$ and [γ-^{32}P]ATP, the enzyme and lipids were incubated for 15 min at 4°C. The reactions were started by addition of $MgCl_2$ and [γ-^{32}P]ATP and, after 30 min at 30°C, were terminated by addition of 200 μl of 1 M HCl. Lipids were extracted with 300 μl of chloroform/methanol, 2:1 (vol/vol), and then with 200 μl of chloroform. The extracts were pooled, washed three times with 400 μl of methanol/1 M HCl, 1:1 (vol/vol), and evaporated under a stream of nitrogen. The phosphorylated lipids were separated on one-dimensional thin-layer plates (silica gel 60 impregnated with 1% potassium oxalate) with chloroform/methanol/4 M NH_4OH, 9:7:2 (vol/vol) (22). In two-dimensional thin-layer chromatography, the same chloroform/methanol/NH_4OH system was used in the first dimension, and 1-propanol/4 M NH_4OH, 13:7 (vol/vol) was used in the second dimension. Lipids were visualized by exposure to iodine or by autoradiography. The radioactive spots were cut out, and the radioactivity was quantitated by liquid scintillation spectrometry.

Assay of Protein Kinase Activity. Protein phosphorylation was measured as described above, except 20 μM [γ-^{32}P]ATP was added at <15 μCi. α-Casein was used at 1 mg/ml. The reactions were performed for 15 min at 30°C and terminated by the addition of 7.5 μl of 5 times concentrated electrophoresis sample buffer (23). The samples were then heated at 95°C for 2 min. Separation by $NaDodSO_4$/polyacrylamide gel electrophoresis and visualization of phosphoproteins were performed as described (24, 25).

Labeling of Cells with $H_3{}^{32}PO_4$. Chicken embryo fibroblasts infected by NY68 strain of RSV were grown at the nonpermissive temperature, 41°C, as described (26). Uninfected chicken embryo fibroblasts were used as control. The confluent cultures were incubated for 18–20 hr in the absence of serum. The serum-starved cells were incubated in phosphate-free medium at 41°C, and carrier-free $H_3{}^{32}PO_4$ was added to 0.1 mCi/ml at the indicated time. During the labeling experiments, the incubation temperature was shifted to the permissive temperature, 35°C, at various times. The total time of exposure to radioactive phosphate was 75 min for all experiments. After the labeling, the cells were washed twice with cold Krebs–Ringer buffer, then 1 ml of 0.5 N HCl was added, and the cells were scraped with a rubber policeman and transferred onto 2 ml of cold chloroform. The dishes were rinsed with 1 ml of 1 M HCl and then with 2 ml of methanol. The rinses were combined with the chloroform suspension, mixed, and allowed to stand for 30 min. The lower organic phase was saved, and the aqueous phase was extracted again with 1 ml of chloroform. The combined organic phases were washed three times with 2 ml of methanol/1 M HCl, 1:1 (vol/vol), and evaporated under a stream of nitrogen at 50°C. The lipids were separated and visualized as described above.

RESULTS

Phosphorylation of Lipids. When purified pp60$^{v\text{-}src}$ was incubated with [γ-^{32}P]ATP in the presence of PtdIns, phosphorylated compounds were produced. The major phosphorylated product migrated with PtdIns4*P* upon thin-layer chromatography (Fig. 1). In addition, a slight amount of radioactivity, <10% of the major product, migrated with PtdIns4,5P_2. When

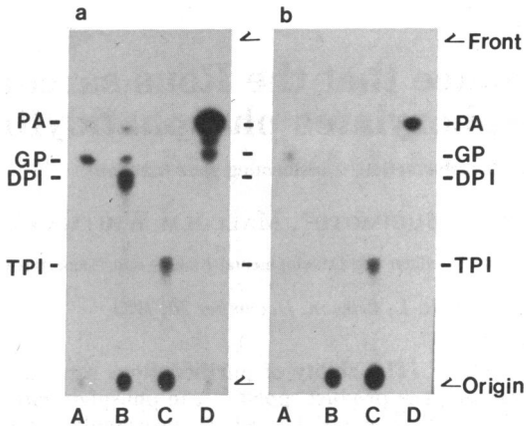

FIG. 1. Thin-layer chromatographic analysis of lipids radiolabeled with [γ-^{32}P]ATP. pp60$^{v\text{-}src}$ (*a*) or the catalytic subunit of cAMP-dependent protein kinase (*b*) in 10 mM potassium phosphate, pH 7.1/1 mM EDTA was preincubated with lipid substrate for 15 min at 4°C. The reaction was then started by addition of [γ-^{32}P]ATP and $MgCl_2$ to a final concentration of 20 μM and 20 mM, respectively. After incubation for 30 min at 30°C, the reaction was terminated and lipid was extracted. Lanes: A, no substrate; B, PtdIns (200 μg/ml); C, PtdIns4*P* (200 μg/ml); D, 1,2-dioleoylglycerol (200 μM). PA, phosphatidic acid; GP, glycerol phosphate; DPI, PtdIns4*P*; TPI, PtdIns4,5P_2.

PtdIns4*P* was used as a substrate, the major phosphorylated product migrated with PtdIns4,5P_2. This activity of pp60$^{v\text{-}src}$ was about 50% of its PtdIns-phosphorylating activity. We note in lanes B and C that a highly phosphorylated product(s) remains at the origin, but it has not been characterized. Furthermore, 1,2-dioleoylglycerol and 1,3-dioleoylglycerol were both phosphorylated by pp60$^{v\text{-}src}$ to yield products that migrated with phosphatidic acid. 1,2-Dioleoylglycerol, a natural substrate for phosphatidic acid production, was phosphorylated at 3 times the rate of 1,3-dioleoylglycerol. Although 1,3-dioleoylglycerol is likely to be contaminated with 1,2-dioleoylglycerol, this amount of phosphorylation suggests that 1,3-dioleoylglycerol is also a substrate of pp60$^{v\text{-}src}$. We have not succeeded in separating β-phosphatidic acid from α-phosphatidic acid by thin-layer chromatography. The phosphorylated products were further characterized by two-dimensional thin-layer chromatography supporting the identification (data not shown). Thus, pp60$^{v\text{-}src}$ is capable of phosphorylating a variety of diacylated glycerol substrates.

When the purified catalytic subunit of cAMP-dependent protein kinase was similarly tested at a concentration that had α-casein-phosphorylating activity comparable to that of pp60$^{v\text{-}src}$, the phosphorylation of PtdIns and diacylglycerols occurred at 1–3% of the level seen with pp60$^{v\text{-}src}$. On the other hand, PtdIns4*P* was phosphorylated by the catalytic subunit to 20% of the level obtained with pp60$^{v\text{-}src}$. Thus, pp60$^{v\text{-}src}$ shows relative specificity for phosphorylation of hydroxyl groups on PtdIns and on diacylglycerols.

Kinetic Parameters of the Lipid-Phosphorylating Activities of pp60$^{v\text{-}src}$. The formation of these phosphorylated products by pp60$^{v\text{-}src}$ in the presence of 20 μM [γ-^{32}P]ATP was linear for at least 30 min. The apparent K_m values for ATP were similar for PtdIns, 1,2-dioleoylglycerol, and α-casein phosphorylation (80–90 μM for all three; Table 1). In addition, the apparent K_m values for $MgCl_2$ also were similar for these three reactions (4–6 mM); 50 mM $MgCl_2$ was slightly inhibitory in the three phosphorylation reactions, and the optimum concentration was 20 mM. Both PtdIns and 1,2-dioleoylglycerol exhibited a high affinity for pp60$^{v\text{-}src}$, as judged by their apparent K_m values as substrates (65 μg/ml and 50 μM, respectively), in comparison with glycerol or the synthetic peptide [Val^5]angiotensin II (K_m = 100 mM and 1 mM, re-

Table 1. Kinetic parameters for the phosphorylation of various substrates by pp60$^{v\text{-}src}$

Substrate	K_m ATP*	K_m $MgCl_2$†	K_m Substrate‡	V_{max}, μmol/mg/min
Phosphatidylinositol	84 μM	6.4 mM	65 μg/ml§	0.0119
1,2-Diacylglycerol	90 μM	4.0 mM	50 μM	0.0630
α-Casein	90 μM	6.0 mM	105 μg/ml	0.433

*The purified pp60$^{v\text{-}src}$ was incubated with PtdIns (200 μg/ml), 200 μM 1,2-dioleoylglycerol, or α-casein (1 mg/ml) in the presence of 20 mM $MgCl_2$ and various concentrations (10–1000 μM) of [γ-^{32}P]ATP.

†Substrates at these same concentrations as in above footnote and [γ-^{32}P]ATP at 20 μM were used with various concentrations (2–50 mM) of $MgCl_2$.

‡The pp60$^{v\text{-}src}$ was incubated with 20 μM [γ-^{32}P]ATP, 20 mM $MgCl_2$, and the following concentrations of substrates: PtdIns, 10–500 μg/ml; 1,2-dioleoylglycerol, 10–500 μM; and α-casein, 50–2000 μg/ml. K_m and V_{max} values were estimated by double reciprocal curves.

§If one assumes that PtdIns contains stearoyl and arachidonyl groups as acyl groups, 65 μg/ml corresponds approximately to 74 μM.

spectively) (5, 27). In intact membrane systems, the apparent K_m values for the phosphorylation of PtdIns were found to be 90 μM and 2.6 mM, for ATP and $MgCl_2$, respectively (28). These values are similar to those presented here for pp60$^{v\text{-}src}$. Kanoh *et al.* have purified diacylglycerol kinase from pig brain cytosol and found the K_m values for ATP and diacylglycerol to be 300 μM and 60 μM, respectively (29). The diacylglycerol kinase activity is dependent on the presence of either deoxycholate or phospholipids, in particular phosphatidylcholine, but PtdIns is strongly inhibitory. In the case of the phosphorylation of 1,2-dioleoylglycerol by pp60$^{v\text{-}src}$, neither deoxycholate nor phosphatidylcholine significantly affected the activity, and PtdIns was not inhibitory at 0.1 mg/ml. The V_{max} for α-casein phosphorylation by pp60$^{v\text{-}src}$ (in 20 mM $MgCl_2$ with 1 mg of α-casein per ml and saturating ATP) was similar to those reported previously (5, 23). The V_{max} for PtdIns and 1,2-dioleoylglycerol phosphorylation were relatively low (in 20 mM $MgCl_2$ with 200 μg of PtdIns per ml or 200 μM 1,2-dioleoylglycerol). The turnover numbers by pp60$^{v\text{-}src}$ were calculated to be about 1 min^{-1} and 5 min^{-1} for the phosphorylation of PtdIns and 1,2-dioleoylglycerol, respectively. On the other hand, the V_{max} for glycerol phosphorylation has been reported to be in the range of 0.35 μmol/mg per min (5).

In the absence of detergent and in low salt, pp60$^{v\text{-}src}$ forms large aggregates (unpublished data). In the experiments reported here, the lipid phosphorylation by pp60$^{v\text{-}src}$ was performed in the absence of detergent and at low salt. Nonidet P-40 (0.05%) and KCl (150 mM) stimulated the phosphorylation of PtdIns and 1,2-dioleoylglycerol at least 2-fold under these conditions (data not shown); however, additional studies are necessary in order to assess the significance of these results. It is likely that under the conditions we used, only a small fraction of the active sites of pp60$^{v\text{-}src}$ can interact with lipid molecules in the absence of detergent and high salt. It is difficult to judge what may happen *in vivo*. *In vivo* pp60$^{v\text{-}src}$ is associated with the plasma membrane and presumably is in close contact with surrounding lipids (30–33). If the plasma membrane is considered as a solution of lipid 4–5 nm in width, PtdIns would be present at a concentration greater than 10 mM and, thus, should have a high probability of associating with pp60^{src}.

Inhibition of the Phosphorylation of PtdIns and 1,2-Dioleoylglycerol. Evidence that the lipid kinase activities described above are indeed due to pp60$^{v\text{-}src}$ and not to an impurity in the preparation is provided by inhibition experiments with antibodies raised against pp60$^{v\text{-}src}$. Preincubation of pp60$^{v\text{-}src}$ with TBR-IgG specifically decreased the α-casein phosphorylation to 16% of that with normal IgG as control. Similarly the phosphorylation of PtdIns and 1,2-dioleoylglycerol was decreased to 19% and 9% of the control, respectively. TBR-IgG did not inhibit the casein kinase activity of the catalytic subunit of cAMP-dependent protein kinase, indicating that the inhibition by TBR-IgG is not due to a nonspecific kinase inhibitor in the IgG preparation (data not shown).

Quercetin, an inhibitor of pp60$^{v\text{-}src}$ (34), similarly inhibited the phosphorylation of PtdIns, 1,2-dioleoylglycerol, and α-casein with K_i values of approximately 10 μM (Fig. 2). In addition, all three kinase activities were inactivated in parallel when pp60$^{v\text{-}src}$ was preincubated at 41°C (Fig. 3). These data support the contention that the phosphorylation of PtdIns and of 1,2-dioleoylglycerol is due to pp60$^{v\text{-}src}$ itself.

PtdIns4,5P_2, a phosphorylation product of PtdIns4P, strongly inhibited pp60$^{v\text{-}src}$ activities, including the phosphorylation of PtdIns, 1,2-dioleoylglycerol, α-casein, and autophosphorylation. The inhibitory effect of PtdIns4,5P_2 was not specific to pp60$^{v\text{-}src}$, as it also inhibited α-casein phosphorylation by the catalytic subunit of cAMP-dependent protein kinase (data not shown).

$^{32}P_i$ Incorporation into Lipid in Chicken Embryo Fibroblasts Infected by a Temperature-Sensitive Transformation Mutant of RSV. In order to determine the effect of the activity of pp60$^{v\text{-}src}$ on the phosphorylation of PtdIns, PtdIns4P, and diacylglycerol *in vivo*, these compounds were analyzed from radiolabeled chicken embryo fibroblasts infected with a temperature-sensitive transformation mutant of RSV, NY68. At 41°C these cells exhibit a nontransformed phenotype because the pp60$^{v\text{-}src}$ is inactivated, but as early as 1 hr after shift to the permissive temperature (35°C), several transformation-specific parameters become evident (26). As a control, uninfected cells were used in parallel. After the temperature was shifted from the nonpermissive temperature, 41°C, to the permissive temperature, 35°C, the total incorporation of $^{32}P_i$ into cells was not significantly affected. However, a significant increase in $^{32}P_i$ incorporation into

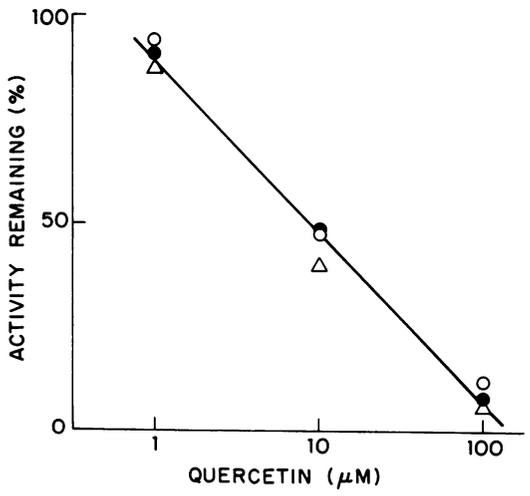

FIG. 2. Effect of quercetin on the purified pp60$^{v\text{-}src}$ activities. The pp60$^{v\text{-}src}$ preparation was preincubated with substrate for 10 min at 4°C, quercetin solubilized in dimethylsulfoxide was added, and this mixture was incubated for 5 min at 4°C. Then the reaction was started by addition of [γ-^{32}P]ATP and $MgCl_2$ to a final concentration of 20 μM and 20 mM, respectively. The final concentration of dimethylsulfoxide was adjusted to 3% as described (34). The final concentration of PtdIns (○), 2-dioleoylglycerol (△), and α-casein (●), respectively, was 200 μg/ml, 200 μM, and 1 mg/ml. The phosphorylating activities were measured as described in the text.

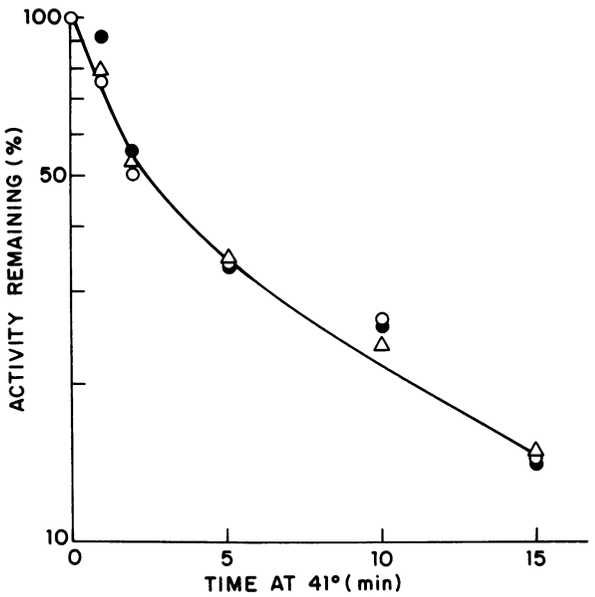

FIG. 3. Thermolability of the purified pp60$^{v\text{-}src}$ activities. Four microliters of the pp60$^{v\text{-}src}$ preparation was incubated at 41°C in 10 mM potassium phosphate, pH 7.1/1 mM EDTA/25% (vol/vol) glycerol. Samples were cooled on ice at the indicated time. The final concentrations of PtdIns (○), 1,2-dioleoylglycerol (△), α-casein (●), [γ-^{32}P]ATP, and $MgCl_2$ were the same as those in Fig. 2. The remaining activities were measured as described in the text.

PtdIns4,5P_2 and phosphatidic acid was observed in the NY68-infected cells within 20 min (Fig. 4). After 2 hr, the radioactivity in phosphatidic acid had more than doubled, while that in PtdIns4P and PtdIns4,5P_2 had increased by 50%. A 20% increase in $^{32}P_i$ incorporation into PtdIns was observed after 2 hr. No significant increase in $^{32}P_i$ incorporation into any of these lipids was observed in the uninfected cells following the temperature shift.

DISCUSSION

Several investigators recently have suggested that polyphosphoinositides are the primary substrates of agonist-dependent hydrolysis of inositol phospholipids (15, 17, 35). In addition it was proposed that (*i*) the inositol phosphates thus produced may function as second messengers because they are generated rapidly in response to the agonist and are destroyed rapidly and (*ii*) they may function to mobilize calcium from internal reservoirs (36). The data presented here showing that pp60$^{v\text{-}src}$ has the capacity *in vitro* to generate polyphosphoinositides is of interest because in cells these compounds may contribute to the phenotype of the transformed cell. It should be noted, however, that the phosphorylated products observed here have merely been shown to comigrate with known compounds, and the sites of phosphorylation have not been independently confirmed.

Another product of the hydrolysis of polyphosphoinositides is diacylglycerol, a compound that is able apparently to activate protein kinase C (13), raising the possibility that activation of this kinase may be responsible for the transformation-specific phosphorylation of ribosomal protein S6 on serine. However, our observation that pp60$^{v\text{-}src}$ also phosphorylates diacylglycerol to produce phosphatidic acid suggests no simple model for the activation of C-kinase by pp60$^{v\text{-}src}$. Based on these data and the models of others mentioned above, pp60$^{v\text{-}src}$ could be involved in both the generation and removal of the diacylglycerol. In this regard an analogy may be drawn with the regulation of isocitrate dehydrogenase by phosphorylation-dephosphorylation. The responsible kinase and phosphatase activities apparently reside in a single polypeptide chain, and the ratio of the two activities is regulated by 3-phosphoglycerate (37). Similarly, regulation of pp60$^{v\text{-}src}$ or pp60$^{c\text{-}src}$ by an as yet unidentified component may result in changes in the steady-state levels within the cell of the substrates under study here.

Little is known about the levels of these compounds in normal as compared to transformed cells, but our preliminary results are consistent with those obtained *in vitro*. When pp60$^{v\text{-}src}$ is activated by shifting NY68-transformed chicken embryo fibroblasts from the nonpermissive to permissive temperature, we observed enhanced incorporation of ^{32}P into PtdIns4P, PtdIns4,5P_2, and phosphatidic acid. Obviously, additional experiments are necessary to assess the significance of these data. However, Macara *et al.* (38) have obtained results independently with another avian sarcoma virus that they suggest indicate a role for its transforming protein in phospholipid metabolism.

The possibility that pp60$^{v\text{-}src}$ or pp60$^{c\text{-}src}$ may activate pathways leading to the malignant phenotype through the generation of second messengers and not by the covalent modification of various proteins indicates that approaches other than those used to date are necessary. Previous efforts have focused on the search for proteins modified by tyrosine phosphorylation; however, if the substrates described in this communication are of physiological significance and result in activation of, for example, protein kinase C, the enzyme

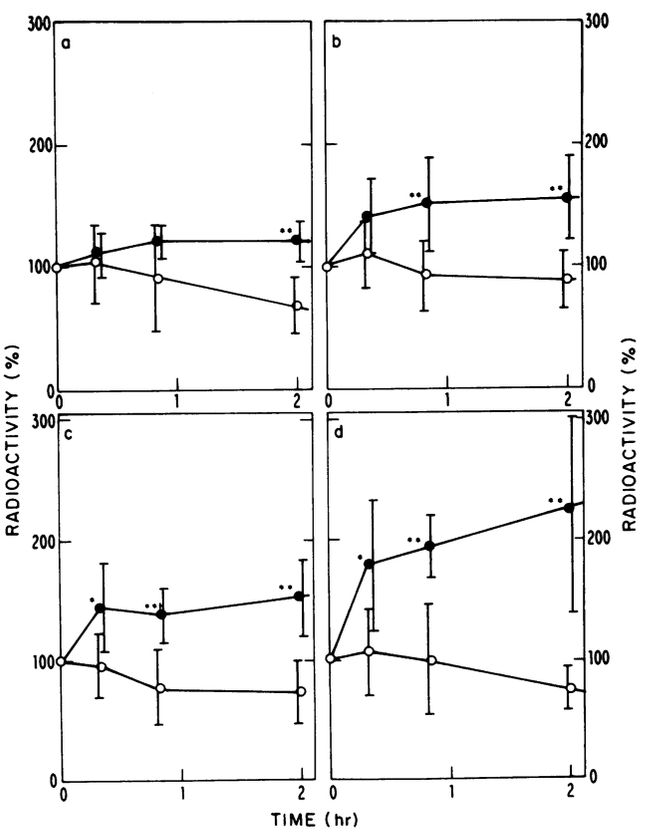

FIG. 4. $^{32}P_i$ incorporation into lipid in chicken embryo fibroblasts. Chicken fibroblasts uninfected (○) or infected by a temperature-sensitive transformation mutant of RSV, NY68 (●), were grown at 41°C. Under each condition, the cells were labeled with carrier-free $H_3{}^{32}PO_4$ for 75 min. During the incubation of the cells with $H_3{}^{32}PO_4$, the temperature was shifted from the nonpermissive temperature, 41°C, to the permissive temperature, 35°C. The abscissa indicates the labeling time at 35°C. (*a*) PtdIns. (*b*) PtdIns4P. (*c*) PtdIns4,5P_2. (*d*) Phosphatidic acid. Error bars represent the standard deviation in four (uninfected) and six (infected) experiments. P values (versus uninfected) were determined by the Student t test. *, $P < 0.05$; **, $P < 0.01$.

itself need not be modified on tyrosine, nor covalently modified in any manner. The difficulties in obtaining a biochemical description of such pathways are obvious, but because of their importance, these questions demand attention.

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1. Collett, M. S. & Erikson, R. L. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 2021–2024.
2. Levinson, A. D., Oppermann, H., Levintow, L., Varmus, H. E. & Bishop, J. M. (1978) *Cell* **15,** 561–572.
3. Hunter, T. & Sefton, B. M. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 1311–1314.
4. Richert, N. D., Blithe, D. L. & Pastan, I. (1982) *J. Biol. Chem.* **257,** 7143–7150.
5. Graziani, Y., Erikson, E. & Erikson, R. L. (1983) *J. Biol. Chem.* **258,** 2126–2129.
6. Radke, K. & Martin, G. S. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 5212–5216.
7. Sefton, B. M., Hunter, T., Beemon, K. & Eckhart, W. (1980) *Cell* **20,** 807–816.
8. Erikson, E. & Erikson, R. L. (1980) *Cell* **21,** 829–836.
9. Cooper, J. A., Reiss, N. A., Schwartz, R. J. & Hunter, T. (1983) *Nature (London)* **302,** 218–223.
10. Decker, S. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 4112–4115.
11. Krebs, E. G. & Beavo, J. A. (1979) *Annu. Rev. Biochem.* **48,** 923–959.
12. Graziani, Y., Maller, J. L., Sugimoto, Y. & Erikson, R. L. (1984) in *Eleventh Cold Spring Harbor Conferences on Cell Proliferation and Cancer* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), in press.
13. Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T. & Nishizuka, Y. (1979) *J. Biol. Chem.* **254,** 3692–3695.
14. Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kiddawa, U. & Nishizuka, Y. (1982) *J. Biol. Chem.* **257,** 7847–7851.
15. Kirk, C. J., Creba, J. A., Downes, C. P. & Michell, R. H. (1981) *Biochem. Soc. Trans.* **9,** 377–379.
16. Rittenhouse-Simmons, S. (1979) *J. Clin. Invest.* **63,** 580–587.
17. Berridge, M. J. (1983) *Biochem. J.* **212,** 849–858.
18. Diringer, H. & Friis, R. R. (1977) *Cancer Res.* **37,** 2979–2984.
19. Johnson, R. A. & Walseth, T. F. (1979) *Adv. Cyclic Nucleotide Res.* **10,** 135–167.
20. Beavo, J. A., Bechtel, P. J. & Krebs, E. G. (1974) *Methods Enzymol.* **38,** 299–308.
21. Brugge, J. S. & Erikson, R. L. (1977) *Nature (London)* **269,** 346–348.
22. Gonzales-Sastre, F. & Folch-Pi, J. (1968) *J. Lipid Res.* **9,** 532–533.
23. Graziani, Y., Erikson, E. & Erikson, R. L. (1983) *J. Biol. Chem.* **258,** 6344–6351.
24. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
25. Erikson, R. L., Collett, M. S., Erikson, E., Purchio, A. F. & Brugge, J. S. (1980) *Cold Spring Harbor Symp. Quant. Biol.* **44,** 907–917.
26. Kawai, S. & Hanafusa, H. (1971) *Virology* **46,** 470–479.
27. Wong, T. W. & Goldberg, A. R. (1983) *J. Biol. Chem.* **258,** 1022–1025.
28. Jergil, B. & Sundler, R. (1983) *J. Biol. Chem.* **258,** 7968–7973.
29. Kanoh, H., Kondoh, H. & Ono, T. (1983) *J. Biol. Chem.* **258,** 1767–1774.
30. Rohrshneider, L. R. (1979) *Cell* **16,** 11–24.
31. Willingham, M. C., Jay, G. & Pastan, I. (1979) *Cell* **18,** 125–134.
32. Krueger, J. G., Wang, E. & Goldberg, A. R. (1980) *Virology* **101,** 25–40.
33. Courtneidge, S. A., Levinson, A. D. & Bishop, J. M. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 3783–3787.
34. Graziani, Y., Erikson, E. & Erikson, R. L. (1983) *Eur. J. Biochem.* **135,** 583–589.
35. Agranoff, B. W., Murthy, P. & Seguin, E. B. (1983) *J. Biol. Chem.* **258,** 2076–2078.
36. Streb, H., Irvine, R. F., Berridge, M. J. & Schulz, F. (1983) *Nature (London)* **306,** 67–69.
37. LaPorte, D. C. & Koshland, D. E., Jr. (1983) *Nature (London)* **305,** 286–290.
38. Macara, I. G., Marinetti, G. V. & Balduzzi, P. C. (1984) *Proc. Natl. Acad. Sci. USA* **81,** in press.