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Transforming protein of avian sarcoma virus UR2 is associated with phosphatidylinositol kinase activity: Possible role in tumorigenesis

(tumor viruses/tyrosine kinases/phorbol esters)

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ABSTRACT **The transforming protein of avian sarcoma virus UR2, $p68^{v\text{-}ros}$, has an associated tyrosine-specific protein kinase activity similar to that of $p60^{v\text{-}src}$ and several other oncogene products. However, this activity has not been linked unequivocally to transformation, and the physiological action of these proteins remains in doubt. We now have found that immunoprecipitated $p68^{v\text{-}ros}$ also is associated with phosphatidylinositol (PtdIns) kinase (ATP:PtdIns 4-phosphotransferase, EC 2.7.1.67) activity. PtdIns 4,5-bisphosphate [PtdIns(4,5)P_2] specifically inhibits both this activity and the autophosphorylation of $p68^{v\text{-}ros}$. Moreover, cells transformed by UR2 showed significant increases in ^{32}P-labeling of PtdIns 4-phosphate (PtdIns4*P*) and PtdIns(4,5)P_2 and in the formation of their catabolites, inositol 1,4-bisphosphate and inositol 1,4,5-trisphosphate, as compared to uninfected cells. These results suggest that a physiologically relevant function of oncogene kinases might be the phosphorylation of PtdIns and that increased turnover of PtdIns4*P* and PtdIns(4,5)P_2 might play a role in transformation by increasing the formation of diacylglycerol, a catabolite of polyphosphoinositides that activates kinase C. This protein copurifies with the phorbol ester receptor, and its activation is likely to be intimately linked with mitogenesis. This hypothesis suggests a mechanism whereby certain oncogene proteins might cause the unrestricted growth typical of transformed cells and could explain why tumor promoters mimic many of the effects of transformation.**

Phosphoinositides (*P*-inositides) are quantitatively minor components of the plasma membrane that have been implicated in the responses of animal cells to a wide variety of stimuli [reviewed by Michell (1)]. An increased turnover of the *P*-inositides is amongst the earliest detectable events induced by occupancy of stimulus receptors (2, 3). One of the breakdown products of *P*-inositides, 1,2-diacylglycerol (1,2-Ac_2Gro), activates a recently described Ca^{2+}- and phospholipid-dependent protein kinase that phosphorylates serine residues, kinase C (4–8). Observations that polypeptide growth factors also activate *P*-inositide turnover (9–11) and that the receptor for phorbol esters copurifies with kinase C (12–14) suggest that *P*-inositide turnover also plays a key role in cell proliferation.

Transformation is a disorder of proliferation that can be induced by the expression of specific oncogenes. Some of the oncogene products phosphorylate specific tyrosine residues on proteins, an activity also possessed by growth factor receptors (15–18). It has been widely assumed that this kinase activity is directly related to transformation, and a great deal of effort has been expended to identify the substrates of the oncogene kinases (19–21). However, expression of the transformed phenotype does not correlate well with phosphorylation of putative substrates (22, 23), tyrosine kinase activity shows little substrate specificity *in vitro*, and the phosphorylation stoichiometries observed are often too low to be of obvious regulatory significance (21). The possibility that the physiological substrate of the oncogene kinases might not be tyrosine was raised by recent observations that $p60^{v\text{-}src}$ can phosphorylate glycerol (24, 25). Because of (*i*) the similarities between the activity of oncogene kinases and that of growth factor receptors (16–18) and (*ii*) the implication of *P*-inositide turnover in cell proliferation, it occurred to us to test the phosphorylating activity of one of these kinases, $p68^{v\text{-}ros}$, towards phosphatidylinositol (PtdIns). The protein $p68^{v\text{-}ros}$ is the oncogene product of avian sarcoma virus UR2 (26, 27); although unrelated to the $p60^{v\text{-}src}$ of Rous sarcoma virus (28), it will phosphorylate itself and exogenous protein substrates on tyrosine residues (27). We found that immunoprecipitated $p68^{v\text{-}ros}$ is also capable of phosphorylating PtdIns to PtdIns 4-phosphate (PtdIns4*P*). Both the synthesis of *P*-inositides *in vivo* and their breakdown were significantly enhanced by transformation of chicken cells with UR2.

MATERIAL AND METHODS

Materials. Protein A-Sepharose, Nonidet P-40 (NP-40), digitonin, phospholipids, and ATP were from Sigma. [γ-^{32}P]ATP (2000 Ci/mmol; 1 Ci = 37 GBq), ortho[^{32}P]phosphate and *myo*-[^{3}H]inositol were from Amersham. Catalytic subunit of cAMP-dependent kinase was kindly provided by F. Tang (Rochester). Dowex AG-1 and reagents for acrylamide gel electrophoresis were from Bio-Rad. Baker Si-250 silica gel plates for TLC were from VWR.

Viruses and Antisera. The origins of UR2 and some of the properties of its transforming protein have been described (26, 27). A temperature-sensitive (ts) mutant, UR2-R0200, has been isolated by one of us (P.B.). This mutant produces full expression of the transformed phenotype at 37°C but only partial expression at 42°C. Anti-*gag* antiserum was obtained from E. J. Smith.

Cell Cultures and Conditions. Chicken embryo fibroblast cultures were prepared and maintained as described (26). Infected cells were passaged 2 or 3 times until extensive morphological conversion was apparent.

Immunoprecipitation of $p68^{v\text{-}ros}$. Cell lysates were prepared with either RIPA buffer or a buffer containing 1% NP-40 (or Triton X-100), 1% aprotinin, 10 mM Tris, 100 mM NaCl, and 1 mM EDTA (pH 7.4). Plates (10 cm) containing about 10^7 cells were washed three times in Tris/glucose, and

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Abbreviations: *P*-inositides, phosphoinositides; PtdOH, phosphatidic acid; PtdEtn, phosphatidylethanolamine; PtdCho, phosphatidylcholine; 1,2-Ac_2Gro, 1,2-diacylglycerol; Ins(1,4)P_2, inositol 1,4-bisphosphate; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; PtdIns, phosphatidylinositol; PtdIns4*P* phosphaditidylinositol 4-phosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; RSV, Rous sarcoma virus; NP-40, Nonidet P-40; wt, wild type; ts, temperature sensitive.

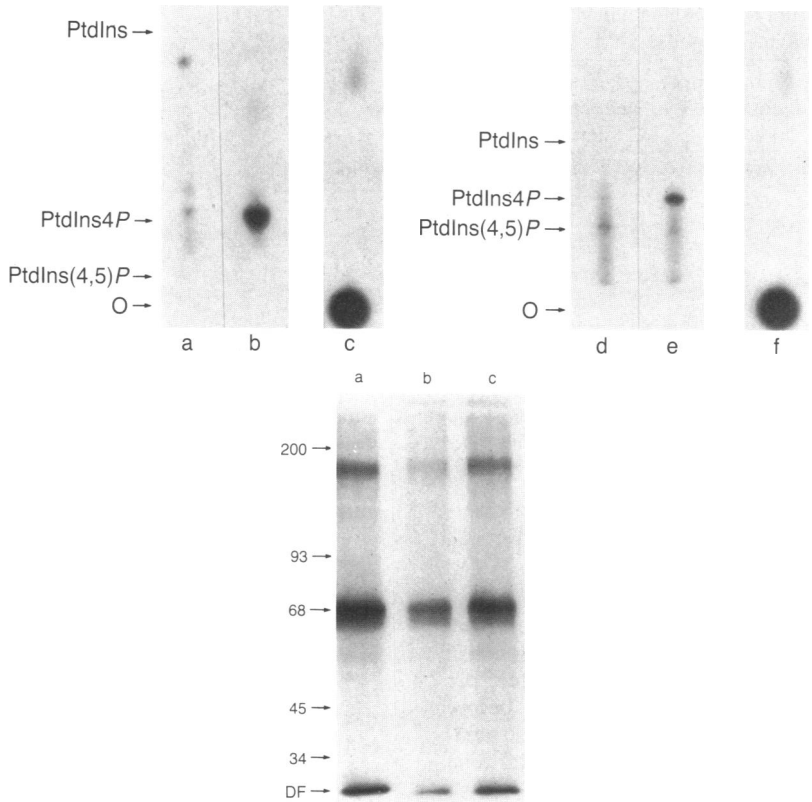

FIG. 1. (*Upper*) Phosphorylation of PtdIns by $p68^{v\text{-}ros}$. Autoradiographs of [^{32}P]phosphorylated products separated by TLC with either of two solvent systems, A (lanes a, b, and c) and B (lanes d, e, and f) as described. Lanes: a and d, no cell lysate; b and d, immunoprecipitated $p68^{v\text{-}ros}$; c and f, catalytic subunit of cAMP-dependent kinase. Exposures were for 24 hr, with a Cronex intensifer screen and Kodak 5BB film. In lanes a, b, d, and e, residual [γ-^{32}P]ATP at the origin was removed before autoradiography, to avoid obscuring any ^{32}P-labeled PtdIns(4,5)P_2, which runs close to the origin (O). (*Lower*) Effect of PtdIns(4,5)P_2 on autophosphorylation of $p68^{v\text{-}ros}$. Phosphorylations were performed and analyzed on 8% acrylamide gels as described by Feldman *et al.* (29) but with 2 μM [γ-^{32}P]ATP (0.50 μCi/nmol). Equal amounts of protein were applied to each lane. Lanes a, control; b, with 2 mg of PtdIns(4,5)P_2 per ml; c, with 2 mg of PtdIns(4,5)P_2 per ml and 1% NP-40. The bands at the dye front (DF) and at 180 kilodaltons may be viral proteins (28) or cell proteins nonspecifically precipitated with $p68^{v\text{-}ros}$. The K_m for phosphorylation of each band by ATP was similar to that for $p68^{v\text{-}ros}$ (data not shown). Numbers give molecular weights in kilodaltons.

the cells were solubilized in 1.0 ml of the lysis buffer. The lysates were centrifuged in a Microfuge (10 min at 4°C), and the supernatants were stored at −70°C. Lysate protein concentrations were 1.2–2.2 mg/ml. $p68^{v\text{-}ros}$ was immunoprecipitated by using anti-*gag* antiserum and protein A-Sepharose as described by Feldman *et al.* (29).

Phosphorylation of PtdIns. Phosphorylations were performed with immunoprecipitates from cell lysates in 50 mM Hepes/Tris (pH 7.4) containing 10 mM $MnCl_2$, 2 μM [γ-^{32}P]ATP (200 μCi/nmol), 2 mg of PtdIns per ml, and 0.1% NP-40 at 30°C except where otherwise indicated. In the absence of detergent, phospholipids were suspended by sonication. Phosphorylation was stopped by addition of 10 mM EDTA, and samples were stored at −20°C. After the addition of unlabeled carrier lipids, phospholipids were extracted by the procedure of Bligh and Dyer (30), with 1% acetic acid in the aqueous phase and in the 0.7% KCl washing solution to ensure complete extraction of the *P*-inositides. The washed chloroform phases were dried under nitrogen, and the lipid residues were dissolved in chloroform/methanol, 4:1 (vol/vol). Aliquots were spotted on oxalate-impregnated silica gel TLC plates and were separated by using either chloroform/methanol/ammonium hydroxide/water, 45:30:3:5 (vol/vol) (system A), or chloroform/methanol/acetic acid/water, 65:25:2:4 (vol/vol) (system B). Authentic lipids were run as standards on each plate.

Table 1. PtdIns kinase activity of immunoprecipitates from chick fibroblast lysates

Lysate	Additions	Pretreatment	Kinase activity, fmol/min/100 μg of protein
UR2	*α-gag*	—	1.33
UR2	*α-gag*	50°C, 30 min	0.09
UR2	None	—	0.21
Control	*α-gag*	—	0.20
Control	*α-gag*	50°C, 30 min	0.06
Control	None	—	0.12

Standard incubation was for 30 min at 30°C in 50 mM Hepes/Tris (pH 7.4) containing 10 mM $MnCl_2$, 2.0 mg of PtdIns per ml, 0.1% NP-40, 2 μM ATP, and immunoprecipitate from 100 μl of cell lysate. UR2 lysates are from cells transformed by UR2; control lysates are from uninfected cells; *α-gag* is anti-*gag* antiserum.

Table 2. Phosphorylation of PtdIns by immunoprecipitated $p68^{v\text{-}ros}$

Treatment	% of control activity
None	100*
Without $MnCl_2$	0
Without PtdIns	0
With 10 mM $MgCl_2$ (no $MnCl_2$)	59
With 150 mM KCl	62
With 0.05% digitonin (no NP-40)	120
Without NP-40	32
With 1 mg of PtdIns(4,5)P_2	
Without NP-40	9
With NP-40	70

*100% = 0.95 fmol/min per 100 μg of lysate protein. Standard assay conditions were as in Table 1.

Table 3. Effect of temperature on kinase activities of $p68^{v\text{-}ros}$ from UR2-wt and UR2-R0200-infected cells

Virus	Temperature	% of PtdIns kinase activity at 30°C	% of tyrosine autophosphorylation activity at 30°C
UR2-wt	30°C	100	100
	37°C	264	ND
	42°C	285	49
UR2-ts-R0200	30°C	100	100
	37°C	278	ND
	42°C	155	20

Conditions for measurement of PtdIns kinase activity were as described in Table 1. Autophosphorylation of $p68^{v\text{-}ros}$ was performed on duplicate samples as described in Fig. 1 *Lower*, for 10 min. Acrylamide gels of the reaction products were autoradiographed and scanned by using a densitometer with a peak integrator. Cell lysates were prepared from cells grown at the permissive temperature (37°C).

***In Vivo* Phosphorylation.** *In vivo* labeling and analysis of phospholipids was performed as described by Sawyer and Cohen (9) with confluent cell cultures. Cells were incubated in phosphate-free Dulbecco's modified Eagle's medium with 2% percent calf serum for 1 hr prior to labeling. To each dish was added 10 μCi of ortho[^{32}P]phosphate (carrier-free) per ml, and the cells were incubated for 1–2 hr at 37°C before extracts were prepared. A portion of the trichloroacetic acid-soluble fraction was removed from each dish to determine the uptake of [^{32}P]phosphate into the soluble phosphate pool of the cells.

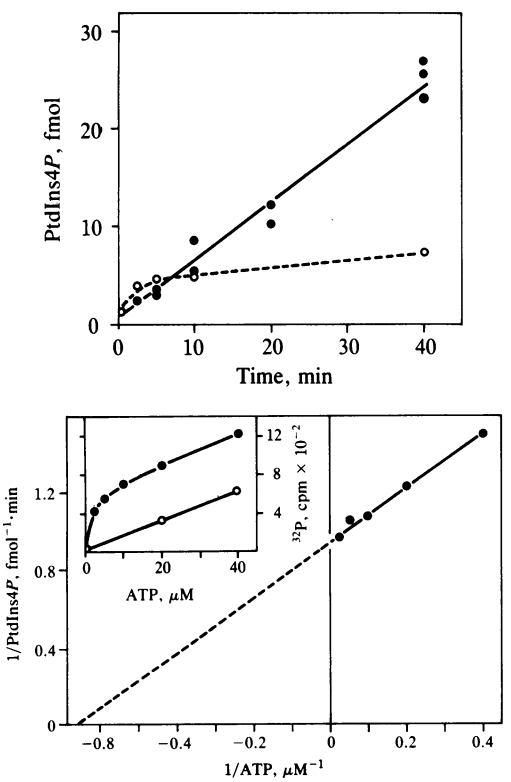

FIG. 2. (*Upper*) Time course of phosphorylation of PtdIns by $p68^{v\text{-}ros}$. Phosphorylations were performed as described at 30°C (2 μM [γ-^{32}P]ATP; 100 μCi/nmol). Radioactive spots were scraped from the TLC plates and assayed for ^{32}P. Phosphorylations were performed either in the presence (●) or absence (○) of 0.1% NP-40. (*Lower*) Phosphorylation of PtdIns as a function of ATP concentration (●). Phosphorylation was performed as in *A*, with 0.1% NP-40 at various ATP concentrations. Samples were run also in the absence of $p68^{v\text{-}ros}$ (○) to correct for background ^{32}P due to residual ATP in the applied samples (*Inset*). Data on the double reciprocal plot is corrected for background. Points represent means of duplicate samples. $K_d \approx 1 \times 10^{-6}$ M.

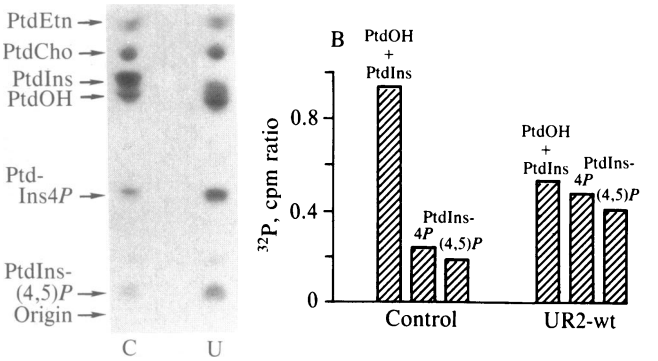

FIG. 3. Effect of transformation by UR2 on *in vivo* phosphorylation of *P*-inositides. Uninfected [control (C)] and UR2-transformed cells [UR2-wt (U)] were grown in phosphate-free medium 1 hr prior to addition of ortho[^{32}P]phosphate (25 μCi/ml). They were incubated for a further 1.5 hr before addition of 5% trichloroacetic acid (0°C). Lipid extracts were analyzed by TLC with system A. Ratios given are of *P*-inositides/(PtdCho + PtdEtn). Arrows show the positions of authentic standards run on the same plate. The differences from the respective control values ± the calculated standard deviation from duplicate samples of two separate experiments were: 74 ± 30% for PtdIns(4,5)P_2, 52 ± 27% for PtdIns4*P*, and −38 ± 11% for PtdIns + PtdOH.

***In Vivo* Labeling of Inositol Phosphates.** Cells were incubated for 2–4 hr with 10 μCi of *myo*-[^{3}H]inositol per ml and then with fresh medium for 1 hr. They were then washed with Hepes-buffered saline containing 10 mM inositol (4°C) and extracted with 5% trichloroacetic acid, which was removed

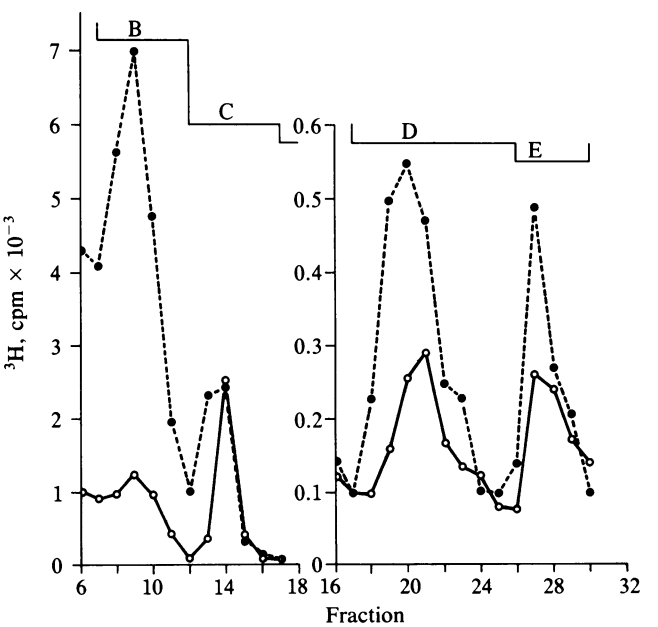

FIG. 4. Analysis of ^{3}H-labeled inositol phosphates by anion-exchange chromatography. Control (○) and UR2-transformed (●) cells were labeled with *myo*-[^{3}H]inositol as described, and extracts were separated on 1.0-ml Dowex columns (31, 32). Elution buffers were: A (not shown), water; B, 5 mM disodium tetraborate/60 mM sodium formate; C, 0.1 M formic acid/0.2 M ammonium formate; D, 0.1 M formic acid/0.4 M ammonium formate; and E, 0.1 M formic acid/1.0 M ammonium formate. Eluted peaks correspond to *myo*-inositol (A; not shown), glycerophosphoinositol (B), inositol 1-phosphate (C), inositol 1,4-bisphosphate [Ins(1,4)P_2] (D), and inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] (E). All counts (cpm) are normalized to the total *myo*-[^{3}H]inositol added in each experiment. The ratio of total ^{3}H to total cell protein was the same in each case.

by washing five times with 3 vol of dipropyl ether, and the extracts were processed and analyzed on Dowex formate columns as described by Berridge *et al.* (31). Inositol [^{32}P]phosphate standards were prepared from human erythrocytes by the procedure of Downes and Michell (32). Protein was determined by the method of Lowry (33) after removal of trichloroacetic acid, sonication, and solubilization in boiling 5% sodium dodecyl sulfate.

RESULTS

p68$^{v\text{-}ros}$ Phosphorylates PtdIns. Incubation of immunoprecipitated p68$^{v\text{-}ros}$ with [γ-^{32}P]ATP and PtdIns resulted in the formation of a ^{32}P-labeled species that comigrates on two TLC systems with authentic PtdIns4*P*; virtually no labeled PtdIns 4,5-bisphosphate [PtdIns(4,5)P_2] was detectable in either the presence or absence of detergent (Fig. 1 *Upper*). No PtdIns4^{32}P was detectable in assays lacking cell lysate (Fig. 1 *Upper*) or in the absence of Mn^{2+} or PtdIns. Preincubation of immunoprecipitated p68$^{v\text{-}ros}$ at 50°C for 30 min abolished this activity (Table 1). When lysates from control cells were used, or when anti-*gag* antiserum was omitted, only low levels of kinase activity were detectable (Table 1). Phosphorylation of PtdIns was enhanced by addition of detergent and reduced somewhat by replacement of Mn^{2+} by Mg^{2+} or by addition of physiological concentrations of KCl (Table 2). In the absence of detergent, PtdIns(4,5)P_2 inhibited phosphorylation of PtdIns by p68$^{v\text{-}ros}$. The inhibition was relieved by addition of 0.1% NP-40 (Table 2). Moreover, PtdIns(4,5)P_2 also inhibited the autophosphorylation of p68$^{v\text{-}ros}$ (Fig. 1 *Lower*) in the absence of detergent. Other phospholipids (PtdIns, PtdIns4*P*, phosphatidic acid (PtdOH), 1,2-Ac_2Gro, and phosphatidylserine) had no effect on either kinase activity of p68$^{v\text{-}ros}$ (data not shown). Other, variable faint phosphorylated spots were sometimes observed on the TLC, both in the presence and absence of lysate (Fig. 1 *Upper*, lane a). The identities of these spots are not known. Catalytic subunit of cAMP-dependent kinase from beef heart at 1.4 μg/ml produced no detectable PtdIns4*P* under similar conditions to those used for the p68$^{v\text{-}ros}$ kinase (Fig. 1 *Upper*). The faint spot running near PtdIns is of unknown composition.

At 42°C the PtdIns kinase activity of immunoprecipitated lysate from cells infected by UR2-R0200, a ts mutant of UR2, was inhibited ≈2-fold as compared to the activity of immunoprecipitates from UR2 wild-type (UR2-wt)-infected cells (Table 3). Autophosphorylation of p68$^{v\text{-}ros}$ from UR2R0200 was also significantly decreased relative to that of p68$^{v\text{-}ros}$ from UR2-wt, at 42°C. Therefore, the kinase activity appears to be an authentic activity of p68$^{v\text{-}ros}$ rather than of some other enzyme immunoprecipitated by the anti-*gag* antiserum.

Kinetic Characteristics of the Phosphorylation of PtdIns. In the presence of detergent (0.1% NP-40), phosphorylation of PtdIns was found to have proceeded linearly over at least 40 min at 30°C. When using sonicated substrate in the absence of detergent, phosphorylation occurred more slowly and appeared to stop after about 10 min (Fig. 2 *Upper*).

Phosphorylation was proportional to the amount of lysate used (data not shown) and displayed saturation kinetics with increasing ATP concentration, with a K_m of about 1×10^{-6} M (Fig. 2 *Lower*). This value is similar to the K_m for autophosphorylation of p68$^{v\text{-}ros}$ (2×10^{-6} M; data not shown). The optimum concentration of Mg^{2+} appeared to be about 5 mM.

Increased Phosphorylation of PtdIns *In Vivo*. To determine if the observed PtdIns kinase (ATP:PtdIns 4-phosphotransferase, EC 2.7.1.67) activity of p68$^{v\text{-}ros}$ is exhibited in UR2-infected cells, control and transformed chick fibroblasts were incubated with ortho[^{32}P]phosphate, and the incorporation into phospholipids was estimated after analysis by TLC. Cells infected with UR2-wt were grown to confluence at 37°C and were phenotypically transformed at the start of the experiment. A clear increase in the [^{32}P]phosphorylation of both PtdIns(4,5)P_2 and PtdIns4*P* was detected, relative to that of PtdIns, phosphatidylcholine (PtdCho), and phosphatidylethanolamine (PtdEtn) (Fig. 3). The observed increases could not be ascribed to differences in the soluble ^{32}P pool in the cells or to differences in the total lipid phosphate between extracts from the cells. Similar results were obtained in several replicate experiments.

Increased Production of Inositol Polyphosphates *In Vivo*. *P*-inositides are hydrolyzed by phospholipase C to 1,2-Ac_2Gro and inositol phosphates. However, 1,2-Ac_2Gro also can be produced by hydrolysis of other phospholipids, and an increase in its total cell concentration does not necessarily reflect an increase in *P*-inositide turnover. Therefore, to compare rates of *P*-inositide hydrolysis, we measured the levels of the inositol phosphates in control and UR2-transformed cells after labeling with *myo*-[^{3}H]inositol. Results of these experiments are shown in Fig. 4. The peaks were coeluted with ^{32}P-labeled inositol phosphate standards prepared from erythrocytes (32), and the elution pattern appeared very similar to that obtained by others (33). It is apparent that peaks B, D, and E, which correspond to inositol glycerophosphate, Ins(1,4)P_2, and Ins(1,4,5)P_3 contained more ^{3}H in extracts from UR2-transformed cells than in uninfected cells. These results indicate that transformation by UR2 causes a significant increase in the rate of turnover of polyphosphatidylinositols, and therefore an increase in production of 1,2-Ac_2Gro.

DISCUSSION

We have presented evidence that the transforming protein of avian sarcoma virus UR2 is associated with PtdIns kinase activity *in vitro* and that *in vivo* transformation of chicken

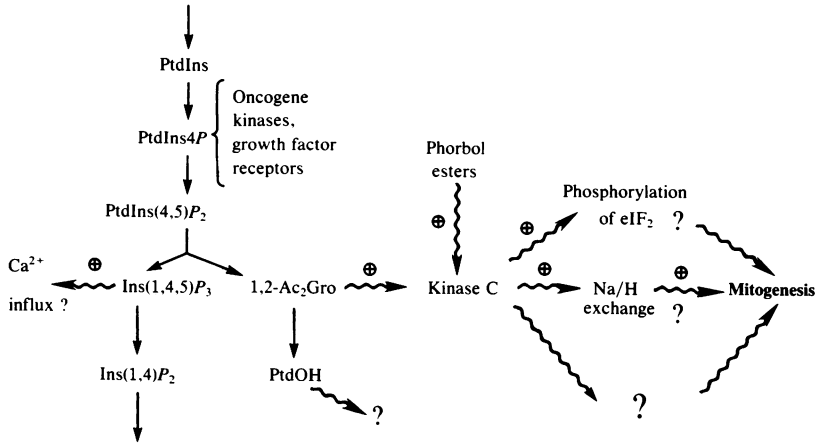

FIG. 5. Hypothetical mechanism of transformation by oncogene kinases. Straight arrows represent metabolic conversions; wavy arrows represent modulations of activity, for instance, by covalent modification. References concerning postulated events in the scheme are provided in the text. eIF_2, eukaryotic initiation factor.

embryo fibroblasts by UR2 is accompanied by an increase in *P*-inositide turnover. The *in vitro* activity was specific to anti-*gag* antiserum immunoprecipitates from UR2-infected cells and was abolished by preincubation of the immunoprecipitates at 50°C, indicating that the activity is due neither to an endogenous PtdIns kinase nor to a contaminating kinase in the PtdIns itself. The association of the activity with $p68^{v\text{-}ros}$ was supported by the temperature-dependent behavior of the kinase immunoprecipitated from a ts mutant of UR2, R0200. The inability of the cyclic AMP-dependent kinase to phosphorylate PtdIns demonstrates that the observed activity is not an artefact common to all kinases under the conditions of the assay. A small and variable amount of PtdIns kinase activity, detected in the absence of anti-*gag* antiserum or when lysates from control cells were used, probably represents nonspecific binding of an endogenous enzyme to the Sepharose. PtdIns kinases are membrane-bound enzymes that readily aggregate and bind to a variety of chromatographic materials (34, 35).

The possibility that the PtdIns kinase activity associated with $p68^{v\text{-}ros}$ *in vitro* might be of physiological significance is supported by our observation of an increase in *P*-inositide turnover *in vivo* in UR2-transformed cells. These results also are supported by the observations of Diringer and Friis (36) on PtdIns breakdown in Rous sarcoma virus-transformed quail cells. Such a function would explain the high "tyrosine kinase" activities detected in brain (37) and in platelets and erythrocytes (38), which possess unusually high concentrations of polyphosphoinositides and high PtdIns kinase activities (1). The results also raise the interesting possibility that other "tyrosine kinases," particularly those associated with growth factor receptors (16–18), might phosphorylate PtdIns.

We now suggest a mechanism by which an increased turnover of *P*-inositides induced by $p68^{v\text{-}ros}$, other similar viral oncogene kinases, or receptor kinases might result in the enhanced growth of susceptible cells. This mechanism is outlined in Fig. 5. We suggest that an increase in the rate of formation of PtdIns4*P* and PtdIns(4,5)P_2, catalyzed directly by the viral or receptor kinases, causes an increase in the rate of hydrolysis of these components to 1,2-Ac_2Gro and inositol phosphates. 1,2-Ac_2Gro is a potent activator of kinase C (5, 7), an enzyme that copurifies with the phorbol ester receptor (12, 13). Phorbol esters are tumor promoters and potent mitogens. One of the targets of activated kinase C may be the Na^+/H^+ exchanger (39); another may be initiation factor eIF_2 which is phosphorylated by kinase C (40). Other PtdIns metabolites might act on systems unrelated to kinase C. Ins(1,4,5)P_3 has recently been implicated in the regulation of Ca^{2+} gates, for instance (41).

The proposed mechanism provides an explanation for the activation of proliferation in transformation, for the location of oncogene kinases in membranes, for the high "tyrosine kinase" activity present in certain nonproliferating cell-types, and for the strong correlation between the changes induced by tumor promoters and tumor viruses.

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