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Phosphatidylinositol metabolism and polyoma-mediated transformation

(polyoma middle-sized T antigen/pp60$^{c\text{-}src}$/phosphatidylinositol kinase/tyrosine kinase)

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ABSTRACT **The effect of polyoma middle-sized tumor antigen (MTAg) on phosphatidylinositol metabolism has been characterized *in vivo* and *in vitro* using polyoma-transformed and polyoma-infected cells. Cells infected with transformation-competent polyoma virus exhibit increased levels of inositol phospholipids and the second messenger inositol trisphosphate. MTAg or pp60$^{c\text{-}src}$ immunoprecipitates from MTAg-transformed cells contain an activity that phosphorylates phosphatidylinositol and phosphatidylinositol 4-phosphate. This activity is induced in parallel with MTAg when the MTAg synthesis is regulated by hormonal or heavy metal inducers. Immunoprecipitates from one class of polyoma mutants defective in transformation have a reduced level of associated phosphatidylinositol kinase activity *in vitro* yet are capable of tyrosine phosphorylation on exogenous protein substrates at rates comparable to wild-type virus. Thus, for these mutants, phosphatidylinositol kinase activity is more tightly correlated with transformation than is protein kinase activity. These results suggest that alterations in phosphatidylinositol metabolism by MTAg play a role in transformation by polyoma virus.**

A possible mechanism for generating some of the phenotypic changes associated with transformation may involve altered phosphorylation and turnover of the membrane phospholipid phosphatidylinositol (PtdIns). Experiments with pp60$^{v\text{-}src}$ and pp68$^{v\text{-}ros}$, the transforming gene products of the Rous sarcoma virus and UR2 viruses, respectively, suggest that PtdIns phosphorylation is regulated by viral oncogene products. These studies have identified PtdIns kinase activities associated with pp60$^{v\text{-}src}$ and pp60$^{v\text{-}ros}$ proteins and increased PtdIns turnover *in vivo* in cells transformed with these oncogenes (1, 2). Increased turnover of PtdIns and its phosphorylated derivatives, phosphatidylinositol 4-phosphate (PtdIns*P*) and phosphatidylinositol 4,5-bis(phosphate) (PtdInsP_2), has been implicated in cellular responses to a wide variety of stimuli, including response to some growth factors (3, 4). PtdInsP_2 breakdown generates two second messengers, diacylglycerol and inositol tris(phosphate) (InsP_3). Diacylglycerol activates the calcium- and phospholipid-dependent protein kinase, which is the major cellular receptor for tumor promoting phorbol esters (5). InsP_3 mobilizes calcium from internal stores (6).

The roles of tyrosine and PtdIns phosphorylation in cellular growth regulation are relevant to the mechanism of transformation by the DNA tumor virus polyoma. Central to polyoma viral-mediated transformation is the activity of one of the polyoma virus early gene products, the middle-sized tumor antigen (MTAg) (7, 8). Mutations that alter MTAg but do not affect the other viral proteins can either abolish or drastically diminish the ability of the virus to transform (7, 9). In established lines, MTAg alone is sufficient to cause transformation (10). MTAg is a membrane-bound protein anchored by a stretch of hydrophobic amino acids at its carboxyl terminus (11, 12). A subpopulation of MTAg is found complexed to a membrane protein, pp60$^{c\text{-}src}$ (13, 14). MTAg is known to stimulate the protein kinase activity of pp60$^{c\text{-}src}$ *in vitro* (15), and it has been suggested that this interaction accounts for both the associated kinase activity and the mechanism of action of MTAg. While a simple model for polyoma transformation suggests that MTAg, in its interaction with pp60$^{c\text{-}src}$, causes that protein to act like its transforming viral homologue, pp60$^{v\text{-}src}$, MTAg-transformed cells show no increase in overall cellular phosphotyrosine and no increase in the phosphorylation of known pp60$^{v\text{-}src}$ substrates or other cellular proteins (16). However, since all polyoma mutants that fail to activate the pp60$^{c\text{-}src}$ tyrosine kinase *in vitro* fail to transform cells, the activation is thought to be essential (15).

Using polyoma-infected cells, we have demonstrated that MTAg/pp60$^{c\text{-}src}$ immunoprecipitates catalyze the phosphorylation of PtdIns (17). In this paper, we examine PtdIns metabolism and assay MTAg-associated PtdIns kinase activity in cells transformed or infected with wild-type or mutant MTAg.

MATERIALS AND METHODS

Cells and Viruses. NIH 3T3 cells (from C. D. Scher), 3T6 cells (from T. L. Benjamin), Schmidt–Rupin avian sarcoma virus-transformed normal rat kidney cells (from L. B. Chen), Fischer rat F111 cells (from T. L. Benjamin), and 14-2 cells (18) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) calf serum. The 45b cell line is a clonal line derived from NIH 3T3 cells cotransfected with the plasmid pSVMmt (19) and a neomycin resistance marker. The simian virus 40 (SV40) large tumor (T)- transformed NIH 3T3 cell line N883 was obtained from M. Brown and D. Livingston. NIH 3T3 cells transformed with a *HRAS* gene activated for transformation at codon 61 were provided by M. Corbley and C. Der (Dana Farber Cancer Institute, Boston).

Phospholipid and PtdIns Analysis *in Vivo*. NIH 3T3 cells were cultured in DMEM containing 10% (vol/vol) dialyzed calf serum at 4×10^5 cells per 35-mm tissue culture dish. After 24 hr, [^{3}H]*myo*-inositol (20 μCi/ml; 1 Ci = 37 GBq; NEN) was added, and the cells were labeled for 48–72 hr or to

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Abbreviations: MTAg, polyoma middle-sized tumor antigen; PtdIns, phosphatidylinositol; Ins, inositol; *P*, phosphate; SV40, simian virus 40; T, tumor.

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approximately one day after confluence. The labeling media was then removed, and the cells were mock-infected or infected with wild-type or mutant polyoma virus at a multiplicity of 10 plaque-forming units per cell for 1 hr in DMEM. The cells were reincubated in the original labeling media containing 2% (vol/vol) fresh dialyzed calf serum for 24 hr. To assay the affects of serum on PtdIns metabolism, 10% (vol/vol) fresh dialyzed serum was added. Cells were washed four times in cold phosphate-buffered saline, scraped off dishes in 0.5 ml MeOH/1 M HCl (1:1; vol/vol), and extracted once with 0.5 ml of $CHCl_3$. The organic phase was dried under nitrogen and redissolved in $CHCl_3$/MeOH/H_2O (2:1:1; vol/vol). Labeled PtdIns was separated, identified by TLC, and counted as described (17). The aqueous phase was dried and redissolved in 5 mM sodium borate, 60 mM ammonium formate. Labeled InsP was separated by anion-exchange chromatography (20) and quantitated by liquid scintillation counting.

Immunoprecipitations and Lipid and Protein Kinase Assays *in Vitro*. MTAg and pp60$^{c\text{-}src}$ immunoprecipitates were prepared as described (17). Lipid kinase assays were performed as described (17), except that kinase assays were for 10 min. Lipid kinase assays must be performed in the absence of non-ionic detergent. Rabbit anti-polyoma T serum was prepared using *Escherichia coli*-produced polyoma small tumor antigen as an immunogen (unpublished procedure).

To assay protein kinase activity, rabbit muscle enolase (5 μg) or yeast enolase (20 μg) was added to the immunoprecipitates. Kinase buffer (20 mM Tris·HCl, pH 7.4; 5 mM $MnCl_2$ or 5 mM $MgCl_2$; and 10 μM ATP) was added with 1–5 μCi [γ-^{32}P]ATP in a final volume of 50 μl. Incubation was continued for 15 min at 25°C with shaking. Reactions were terminated by boiling in sample buffer and analyzed by 10% $NaDodSO_4$/polyacrylamide electrophoresis (21).

RESULTS

Infection of Mouse Cells by Transformation Competent Polyoma Virus Results in Enhanced Levels of InsP_3 and Inositol Phospholipids. To determine if PtdIns metabolism is altered in cells infected with polyoma virus, we examined the levels of PtdIns$P$, PtdInsP_2, inositol 1,4-bis(phosphate) (InsP_2) and InsP_3 in NIH 3T3 cells infected with wild-type polyoma virus or with the middle T mutant py1387-T. py1387-T contains a nonsense mutation that eliminates the carboxyl terminal 37 amino acids of MTAg, rendering it completely defective for transformation and membrane association (22). Immunoprecipitates of py1387-T MTAg also lack the MTAg-associated tyrosine and PtdIns kinase activities (17, 22). The small and large T antigens are functionally unaffected by this mutation (22). NIH 3T3 cells were grown to confluence in the presence of [^{3}H]*myo*-Ins and then either infected with equal multiplicities of wild-type or mutant polyoma virus or mock infected. After 24 hr the cells were harvested, and the levels of PtdIns$P$, PtdInsP_2, InsP_2, and InsP_3 were determined. Fig. 1, lanes C, F, and I, compare the amount of label in InsP_3 in wild-type- and py1387-T-infected cells to the amount in mock-infected cells. Wild-type-infected cells show a 70 ± 17% increase in InsP_3 levels over py1387-T- or mock-infected cells. This increase in InsP_3 is similar to the enhancement of InsP_3 levels observed following stimulation of Swiss 3T3 cells with platelet-derived growth factor (23). The addition of fresh serum to confluent NIH 3T3 cells also resulted in a 70% increase in InsP_3 as compared to unstimulated cells (data not shown). The levels of InsP_2 were similarly elevated (data not shown). As shown in Fig. 1, lanes A, D, and G and lanes B, E, and H, the levels of PtdIns$P$ and PtdInsP_2 increased 40 ± 5% and 33 ± 4%, respectively, in wild-type polyoma, but not in py1387-T- or mock-infected NIH 3T3 cells.

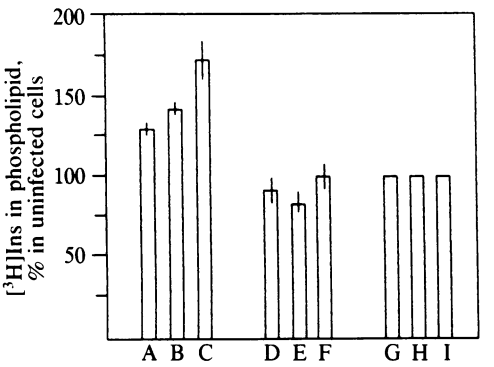

FIG. 1. PtdIns metabolite levels in NIH 3T3 cells 24 hr after polyoma virus infection. Cells were labeled to steady state with [^{3}H]*myo*-Ins prior to infection. Levels of PtdIns metabolites were standardized with respect to total labeled inositol containing phospholipid and are expressed as percentage of levels in uninfected cells. PtdIns$P$ (lanes A, D, and G), PtdInsP_2 (lanes B, E, and H), or InsP_3 (lanes C, F, and I) levels in cells infected with wild-type polyoma virus (lanes A, B, and C), transformation-defective mutant py1387-T (lanes D, E, and F), or mock-infected cells (lanes G, H, and I). In quiescent mock-infected cells, the molar ratio for PtdInsP_2:PtdIns was 0.028 ± 0.006 and for InsP_3:PtdIns was 0.03 ± 0.004.

It is difficult to evaluate whether these changes represent true mass changes or changes in isotope flux. Cells used in these studies were prelabeled for 3 days of exponential growth with [^{3}H]*myo*-Ins at which time the level of incorporation of label into cellular phospholipid is known to reach a steady state (24). However, the extent to which different pools of cellular phospholipid may utilize Ins synthesized from glucose rather than Ins added extracellularly is unknown. We have, therefore, expressed our results as a ratio of label in PtdIns$P$, PtdInsP_2, and phosphatidylinositol to label in total cellular PtdIns rather than to the absolute mass of these metabolites in the cell.

MTAg Immunoprecipitates from Polyoma-Transformed Cells Catalyze Phosphorylation of PtdIns. We also examined polyoma-transformed cells for activities that phosphorylate PtdIns. We used 14-2 cells, an NIH 3T3 cell line transformed with a MTAg cDNA expressed from a metallothionein promoter (18). 14-2 cells produce approximately the same amount of MTAg as do polyoma virus-infected cells and are morphologically transformed, as assayed by their ability to form foci and grow in soft agar (18). Immunoprecipitates of 14-2 cells using rabbit anti-T serum had 20 to 50-fold higher PtdIns kinase activity than parallel immunoprecipitates from nontransformed NIH 3T3 cells (Fig. 2*A*, lanes 1 and 2). These results were obtained under conditions where direct assay of whole-cell lysates results in less than a 2-fold increase in PtdIns kinase activity in 14-2 cells compared to NIH 3T3 cells (data not shown). Immunoprecipitates prepared from 14-2 cells with rat anti-polyoma ascites fluid (14) or a monoclonal antibody against MTAg, PAB815 (25), contained less PtdIns kinase activity than rabbit anti-T immunoprecipitates by a factor of 3 to 4. However, ascites fluid and PAB815 immunoprecipitated less MTAg by a factor of 3 to 4 as assayed in a tyrosine kinase assay. Treatment of 14-2 cells with 1 μM cadmium sulfate for 7 hr to induce the metallothionein promoter resulted in a 3-fold stimulation of immunoprecipitable PtdIns kinase activity (Fig. 2*B*, lanes 1 and 2), tyrosine kinase activity (data not shown), and MTAg (18).

MTAg-transformed cell immunoprecipitates prepared with a monoclonal antibody to pp60$^{c\text{-}src}$, GD11 (26), also show 20-fold increases in PtdIns kinase activity as compared to NIH 3T3 cells (Fig. 3). Anti-pp60$^{c\text{-}src}$ immunoprecipitates from cells transformed with pp60$^{v\text{-}src}$ show 20-fold increases as well (Fig. 3). Anti-pp60$^{c\text{-}src}$ immunoprecipitates from 3T3 cells transformed with SV40 large T antigen or an activated

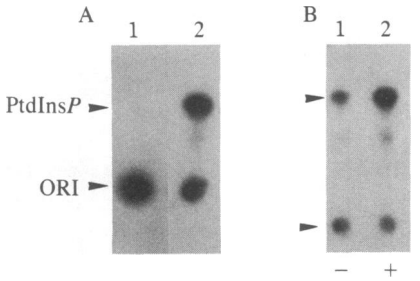

FIG. 2. PtdIns activity in polyoma immunoprecipitates from the middle T-transformed line 14-2. Autoradiograph of a TLC separation of the reaction products from PtdIns kinase assays performed on polyoma immunoprecipitates from 14-2 cells. The arrow indicates the PtdIns kinase reaction product PtdIns*P*. The identity of products was confirmed by chromatography with unlabeled standards. (*A*) Lane 1, control NIH 3T3 cells. Lane 2, 14-2 cells. (*B*) Induction of PtdIns kinase activity with cadmium. Lane 1, untreated 14-2 cells. Lane 2, 14-2 cells treated with 1 μM cadmium sulfate 7 hr prior to immunoprecipitation. Ori, origin.

c-*ras* gene showed no increase in PtdIns kinase activity above nontransformed cells (Fig. 3). Thus, the increase in PtdIns kinase activity in polyoma immunoprecipitates is not a consequence of transformation in general but is specific for cells transformed by MTAg or $pp60^{v\text{-}src}$.

To determine if MTAg/$pp60^{c\text{-}src}$ immunoprecipitates could also catalyze the phosphorylation of PtdIns*P*, immunoprecipitates from 14-2 cells were incubated with PtdIns*P* in a kinase assay. Fig. 4 (lanes 1–4) compares the phosphorylated products obtained using PtdIns or PtdIns*P* as substrates. An increase in PtdIns*P* kinase activity is evident in MTAg-transformed cells. Under the assay conditions used, the phosphorylation of PtdIns*P* is approximately 20% as efficient as that of PtdIns. No significant increase in diacylglycerol kinase activity is evident in MTAg/$pp60^{c\text{-}src}$ immunoprecipitates from 14-2 cells compared to control immunoprecipitates (lanes 5 and 6).

We also investigated PtdIns kinase activities in the cell line 45b, an NIH 3T3 derived line in which the middle and small MTAg is expressed under control of the dexamethasone-regulated murine mammary tumor virus promoter. Addition of dexamethasone to the medium of 45b cells results in the appearance of at least two transformation parameters, focus formation and anchorage-independent growth (data not shown). Dexamethasone did not induce anchorage-independent growth in control NIH 3T3 cells (data not shown). 45b cells were grown in the presence or absence of dexamethasone, and the cell lysates were immunoprecipitated with rabbit anti-T serum. The immunoprecipitates were assayed

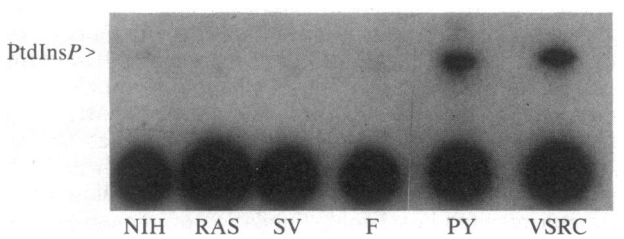

FIG. 3. PtdIns kinase activities in $pp60^{c\text{-}src}$ immunoprecipitates from cell lines transformed with various oncogenes. Autoradiograph of a TLC separation of the reaction products from PtdIns kinase assays. Immunoprecipitates were from 1×10^6 cells using the $pp60^{c\text{-}src}$ monoclonal GD11. Cell lines are nontransformed NIH 3T3 (NIH), activated *HRAS*-transformed NIH 3T3 (RAS), SV40 large T-transformed NIH 3T3 (SV), nontransformed F111 rat cells (F), MTAg-transformed line 14-2 (PY), and v-*src*-transformed rat (VSRC).

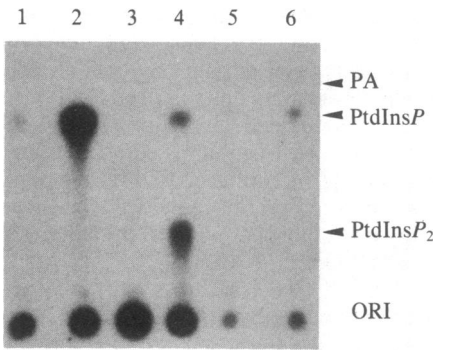

FIG. 4. Lipid substrate specificity of immunoprecipitated lipid kinase activity. Anti-polyoma immunoprecipitates were made from control NIH 3T3 (lanes 1, 3, and 5) or MTAg-transformed 14-2 cells (lanes 2, 4, and 6) and assayed for lipid kinase activity using PtdIns (lanes 1 and 2), PtdIns*P* (lanes 3 and 4), or diacylglycerol (lanes 5 and 6) in phosphatidylserine as lipid substrates. Lipid substrate at 0.4 mg/ml was sonicated with phosphatidylserine at 2 mg/ml and added to the reaction mixture to a final concentration of 0.2 mg of total lipid per ml. 1,2 Diolein (Sigma) was used as diacylglycerol substrate. Reaction products were separated by TLC, and products were identified by comigration with unlabeled standards. Arrows indicate kinase reaction products, PtdIns*P*, PtdInsP_2, and phosphatidic acid (PA). Ori, origin.

for both PtdIns kinase and tyrosine kinase activity. As shown in Fig. 5, both tyrosine kinase (lanes 1–3) and PtdIns kinase (lanes 4–6) activities were stimulated 10-fold and 5-fold, respectively, when 45b cells were treated with 1 μM dexamethasone for 16 hr. Immunoprecipitates made with rabbit anti-T serum or $pp60^{c\text{-}src}$ antiserum prepared from NIH 3T3 cells treated with dexamethasone showed no increase in PI kinase or tyrosine kinase activity compared to untreated NIH 3T3 cells (data not shown). Dexamethasone treatment of 45b cells also resulted in significant increases in *in vivo* levels of PtdIns*P*, PtdInsP_2, and InsP_3 (data not shown).

Immunoprecipitates of Some MTAg Mutants Are Wild Type for Protein Phosphorylation *in Vitro* but Impaired in Their Ability to Phosphorylate PtdIns. Using mutants of polyoma virus defective in both associated tyrosine kinase activity and transformation, we have previously demonstrated a close correlation between PtdIns kinase activity and transformation (17). Here, we extend these observations to polyoma-transformation-defective mutants whose MTAgs retain their associated tyrosine kinase activity (B. Bockus and B.S., unpublished data). dl23, a representative of this class, is a deletion mutant that is extremely defective in its ability to transform cells (27). dl23-infected rat cells fail to form

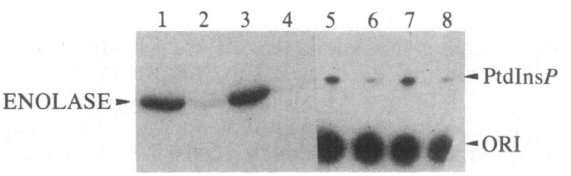

FIG. 5. Comparison of tyrosine protein kinase and PtdIns kinase activities in immunoprecipitates from the long terminal repeat from murine mammary tumor virus/polyoma-transfected cell line 45b. Cells with or without dexamethasone treatment (5 μM for 16 hr) were immunoprecipitated with rabbit anti-T serum, and immunoprecipitates were assayed for the ability to phosphorylate enolase or PtdIns. Lanes 1–4, autoradiogram of *in vitro* protein kinase assay. Lanes 5–8, autoradiogram of *in vitro* PtdIns kinase assay. Lanes 1 and 5, 45b cells treated with dexamethasone. Lanes 2 and 6, untreated 45b cells. Lanes 3 and 7, polyoma virus-infected NIH 3T3 cells. Lanes 4 and 8, mock-infected NIH 3T3 cells treated with dexamethasone. Ori, origin.

colonies in soft agar (27). Rare foci are induced by dl23 only after long latency periods (28). T antigens were immunoprecipitated from dl23-infected 3T6 cells with rabbit anti-T serum, and the immunoprecipitates were divided in half and assayed for PtdIns or enolase kinase activity. The PtdIns kinase activity associated with dl23 immunoprecipitates was reduced by a factor of 8 to 10 relative to wild-type immunoprecipitates (Fig. 6, lanes 4–6). In contrast, both dl23 and wild-type immunoprecipitates were equally efficient in catalyzing the phosphorylation of enolase on tyrosine residues (Fig. 6, lanes 1–3). The identical experiment was performed with three additional polyoma mutants defective in transformation, dl1015 (28), dl1014/py1178T (29), and py1178T (30). Immunoprecipitates of these mutants also had reduced PtdIns kinase activity compared with wild type. However, as with dl23, these mutant MTAgs were as effective as wild-type MTAg in phosphorylating enolase (Table 1; B. Bockus and B.S., unpublished data). In these experiments, wild-type- and mutant-infected cells contained equal amounts of MTAg and $pp60^{c\text{-}src}$ by [^{35}S]methionine labeling (data not shown). PtdIns kinase assays performed using immunoprecipitates from SV40 large T-transformed 3T3 cells infected with py1178T, NG59, or wild-type polyoma virus gave identical results as immunoprecipitates from mutant- or wild-type-infected 3T3 or 3T6 cells (data not shown). Thus the increase in PtdIns kinase activity associated with transformation competent MTAg is not merely a consequence of the transformed state of the cells.

DISCUSSION

We have examined the effects of MTAg on PtdIns metabolism *in vivo* and *in vitro*. Cells infected with polyoma virus exhibit increased levels of PtdIns*P* and PtdInsP_2 and a second messenger derived from their breakdown, InsP_3 (Fig. 1). MTAg immunoprecipitates from cells transformed by the MTAg gene contain an activity that phosphorylates PtdIns and PtdIns*P* *in vitro* (Figs. 2 and 4). This activity is induced in parallel with MTAg when the MTAg synthesis is regulated by heavy metal (Fig. 2) or hormonal (Fig. 5) inducers. We have extended our previous observations of the close correlation between MTAg-associated PtdIns kinase activity and transformation (17). Several MTAg mutants that are defective in transformation have reduced associated PtdIns kinase activity yet have levels of tyrosine kinase activity comparable to wild-type MTAg (Fig. 6, Table 1).

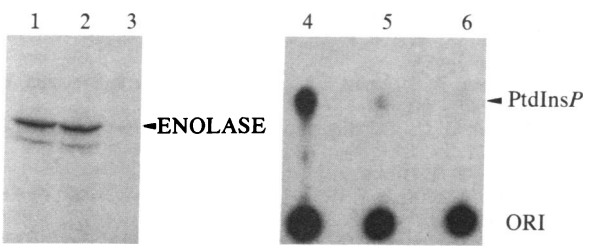

FIG. 6. Comparison of protein and PtdIns kinase activities in polyoma immunoprecipitates from 3T6 cells infected with wild-type and dl23 mutant polyoma. Lanes 1–3, *in vitro* protein kinase assay. Lanes 4–6, *in vitro* PtdIns kinase assay. Lanes 1 and 4, wild-type-infected 3T6 cells. Lanes 2 and 5, dl23-infected 3T6 cells. Lanes 3 and 6, mock-infected 3T6 cells. The bands labeled enolase (lanes 1–3) and PtdIns*P* (lanes 4–6) were cut out of the gels and quantitated by scintillation counting. The cpm obtained were as follows: lane 1, 490 cpm; lane 2, 473 cpm; lane 3, 23 cpm; lane 4, 605 cpm; lane 5, 90 cpm; lane 6, 17 cpm. Greater than 90% of the label incorporated into enolase is tyrosine phosphate, as determined by phosphoamino acid analysis. Lipid and protein kinase assays were linear over the time of the assay and were dependent upon the amount of immunoprecipitated cell lysate added. Ori, origin.

Table 1. Relative transformation abilities and kinase activities associated with polyoma mutants

Polyoma strain	PtdIns kinase activity	Tyrosine kinase activity	Transformation competence
RA (wild type)	100	+	+
Mock-infected	7	−	−
NG59	10	−	−
py1387-T	8	−	−
dl23	14	+	±
dl1015	23	+	±
py1178T	23	+	±
dl1014/py1178T	19	+	±

NIH 3T3 or 3T6 cells were infected with wild-type or mutant polyoma virus and *in vitro* tyrosine and PtdIns kinase activities assayed on polyoma immunoprecipitates. Values are relative to wild-type-infected cells. Results are the average of at least three separate experiments performed with parallel samples. Wild-type- and mutant-infected cells made comparable levels of middle T as measured by incorporation of [^{35}S]methionine. Transformation abilities are from Carmichael and Benjamin (36) for RA and NG59; Carmichael *et al.* (22) for py1387-T; Ito *et al.* (27) for dl23; Magnusson *et al.* (28) for dl1015; Carmichael *et al.* (30) for py1178T; and Schaffhausen *et al.* (29) for dl1014/py1178T. PtdIns and tyrosine kinase activity values were obtained as in Fig. 6. For tyrosine kinase activity: wild-type activity (+), no activity (−). Transformation competence is the percent foci induced compared to wild-type (+). No foci (−). Less than 1% (±).

Several lines of evidence support the notion that we are measuring a MTAg-specific PtdIns kinase activity present in the immunoprecipitates. While PtdIns kinase activity in immunoprecipitates from polyoma-transformed cells and from nontransformed cells differs by a factor of 20, we see differences of less than a factor of 2 in total PtdIns kinase activity from whole-cell lysates. Immunoprecipitates made with $pp60^{c\text{-}src}$ antiserum show enhanced PtdIns kinase activity only in cells transformed by MTAg or $pp60^{v\text{-}src}$ and not in cells transformed by SV40 large T antigen or activated *ras* (Fig. 3). It is important to note that immunoprecipitates from cells infected with either wild-type or dl23 mutant virus contain the same amount of $pp60^{c\text{-}src}$ and MTAg as assayed by [^{35}S]methionine metabolic labeling, yet dl23 immunoprecipitates contain only 15% of the PtdIns kinase activity of wild-type immunoprecipitates. Also, diacylglycerol kinase activity is present in cell membranes in greater specific activity than PtdIns kinase activity (unpublished observations), yet we see little or no diacylglycerol kinase activity in our immunoprecipitates (Fig. 4, lanes 5 and 6).

The observation that polyoma virus infection alters PtdIns metabolism *in vivo* and that stable transformation of cells by polyoma induces a PtdIns kinase that can be measured *in vitro* does not prove a causal relationship between alteration of PtdIns metabolism and MTAg-mediated transformation. However, our measurements on mutants such as dl23, dl1015, py1178T, and dl1014/py1178T suggest such a relationship. These viruses fail to transform at wild-type levels (7, 14, 28–30) yet show no obvious defect in the MTAg-associated tyrosine kinase activity (Table 1 and Fig. 6). It is still possible that the MTAg/$pp60^{c\text{-}src}$ complex of these mutants is defective in the tyrosine phosphorylation of specific protein substrates *in vivo*, though such substrates have yet to be identified (see below). Our measurements show that the PtdIns kinase activity associated with the MTAg/$pp60^{c\text{-}src}$ complex of the mutants is clearly reduced (it is only 15% of wild-type activity, Fig. 6). Hence, the associated PtdIns kinase activity more accurately predicts the transforming ability of the mutants than tyrosine kinase activity.

We have also found significant increases in the levels of the PtdIns metabolites PtdIns*P*, PtdInsP_2 and InsP_3 following

infection of 3T3 cells with wild-type polyoma virus. The observation that both InsP_3 and its immediate precursor PtdInsP_2 increase upon infection with transformation-competent polyoma virus is consistent with the hypothesis that InsP_3 production is enhanced by middle T through increased production of PtdInsP_2 rather than through direct activation of phospholipase C. Our results do not, however, rule out the possibility that phospholipase C is directly activated. The changes we have observed are quantitatively comparable to changes in PtdIns metabolites associated with mitogenic stimulation and retroviral transformation in other systems (3, 31). Increased turnover of PtdIns has also been found upon DNA tumor virus transformation of fibroblasts (32). Transformation of NIH 3T3 cells by the *ras* oncogene also results in an increase in the breakdown products of PtdInsP_2. However, unlike polyoma virus-infected or polyoma-transformed NIH 3T3 cells, the PtdInsP_2 level is decreased in *ras*-transformed cells (24). Thus *ras* transformation and MTAg appear to affect different steps in the PtdIns turnover pathway.

The changes we observe *in vivo* are consistent with the MTAg-associated phospholipid kinase activities found *in vitro*. Activities phosphorylating PtdIns to PtdIns*P* and PtdIns*P* to PtdInsP_2 are associated with MTAg immunoprecipitates. Increases in these activities *in vivo* might be expected to increase levels of PtdIns*P* and PtdInsP_2, as well as increasing InsP_3 production by increasing PtdInsP_2 available for hydrolysis by phospholipase C (33). However, the immunoprecipitate contains less than 1% of the PtdIns kinase activity present in whole-cell lysates. Since our protocol quantitatively immunoprecipitates MTAg, the bulk of cellular PtdIns kinase activity is not associated with immunoprecipitated MTAg. Similarly, McDonald *et al.* (34) and Sugimoto and Erikson (35) have concluded that the pp60$^{v\text{-}src}$ and pp56 tyrosine kinases contribute little to the total PtdIns phosphotransferase activity in cells transformed by Rous sarcoma or Moloney leukemia virus, respectively. This suggests the possibility that the immunoprecipitates bring down a small fraction of a PtdIns kinase activity that nevertheless plays a significant role in transformation.

The protein(s) responsible for the PtdIns kinase activity in polyoma immunoprecipitates and the relevance of this activity to the observed changes in PtdIns*P* and PtdInsP_2 *in vivo* remain to be determined. There are several possible explanations linking our observations of increased levels of phosphatidylinositol *in vivo* and the coimmunoprecipitation of PtdIns kinase activity and tyrosine kinase activity *in vitro*. It is unlikely that MTAg itself is responsible for the immunoprecipitable activity, since 100-fold overproduction of MTAg via adenovirus vectors results in only slightly more immunoprecipitable PtdIns kinase activity than is seen in polyoma-transformed cells (unpublished data). A second possibility is that pp60$^{c\text{-}src}$ possesses both the tyrosine and PtdIns kinase activity observed in the immunoprecipitates. We know from overproduction studies that pp60$^{c\text{-}src}$ by itself has little or no PtdIns kinase activity (unpublished data). In addition, we can separate the bulk of the immunoprecipitable PtdIns kinase activity from MTAg/pp60$^{c\text{-}src}$. Thus, the PtdIns kinase activity observed in immunoprecipitates is likely to be derived from a cellular enzyme other than pp60$^{c\text{-}src}$. The properties of this enzyme may be altered by specific association with the MTAg/pp60$^{c\text{-}src}$. The MTAg/pp60$^{c\text{-}src}$ complex may phosphorylate and activate cellular PtdIns kinase(s) leading to the overall increase in PtdIns turnover *in vivo*. The specific association of PtdIns kinase activity with MTAg/pp60$^{c\text{-}src}$ complexes would reflect the tendency of kinases and substrates to associate during purification. Thus a fraction of the bulk cellular PtdIns kinase would remain associated with MTAg during the immunoprecipitation procedure. In the case of mutants such as dl23 the ability of the MTAg/pp60$^{c\text{-}src}$ complex to associate with or activate the cellular PtdIns kinase may be impaired.

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