Studies of Partially Transforming Polyomavirus Mutants Establish a Role for Phosphatidylinositol 3-Kinase in Activation of pp70 S6 Kinase

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Infection of mouse fibroblasts by wild-type polyomavirus results in increased phosphorylation of ribosomal protein S6 (D. A. Talmage, J. Blenis, and T. L. Benjamin, *Mol. Cell. Biol.* 8:2309–2315, 1988). Here we identify pp70 S6 kinase (pp70^{S6K}) as a target for signal transduction events leading from polyomavirus middle tumor antigen (mT). Two partially transforming virus mutants altered in different mT signalling pathways have been studied to elucidate the pathway leading to S6 phosphorylation. An upstream role for mT–phosphatidylinositol 3-kinase (PI3K) complexes in pp70^{S6K} activation is implicated by the failure of 315YF, a mutant unable to promote PI3K binding, to elicit a response. This conclusion is supported by studies using wortmannin, a known inhibitor of PI3K. In contrast, stable interaction of mT with Shc, a protein thought to be involved upstream of Ras, is dispensable for pp70^{S6K} activation. 250YS, a mutant mT which retains a binding site for PI3K but lacks one for Shc, stimulates pp70^{S6K} to wild-type levels. Mutants 315YF and 250YS induce partial transformation of rat fibroblasts with distinct phenotypes, as judged from morphological and growth criteria. Neither mutant induces growth in soft agar, indicating that an increase in S6 phosphorylation, while necessary for cell cycle progression in normal mitogenesis, is not sufficient for anchorage-independent cell growth. In the polyomavirus system, the latter requires integration of signals from mT involving both Shc and PI3K.

The polyomavirus middle tumor antigen (mT) is required for induction of a broad spectrum of tumors in mice and for transformation of rat fibroblasts in culture (15, 35). mT is a membrane-bound nonenzymatic protein which acts by providing a core for the assembly of a number of cellular factors involved in intracellular signalling. The latter include pp60^{c-src} (and other members of the Src kinase family), phosphatidylinositol 3-kinase (PI3K), phospholipase C γ , the Shc proteins, members of the 14-3-3 family of proteins, and protein phosphatase 2A (11, 23, 27, 53, 55, 67, 75, 78). A crucial step in the assembly of mT complexes with some of these factors is the binding and activation of the protein tyrosine kinase pp60^{c-src} by mT, a step promoted by the action of a serine kinase(s) on mT (46). Phosphorylation of mT by pp60^{c-src} on specific tyrosines then generates binding sites for mT-associated proteins. Thus, phosphotyrosine at positions 250, 315, and 322 promote binding to Shc (11, 27), the p85 regulatory subunit of PI3K (2, 69), and phospholipase C γ (67), respectively.

The binding of these cellular factors to mT results in the generation of multiple intracellular signals that are presumably important for virus growth but which can also lead to neoplastic transformation in nonproductively infected cells. The same cellular factors are involved in a wide variety of signalling processes in normal cells. The Shc family of proteins contain an SH2 domain and bind to activated tyrosine kinase receptors (48, 56). Tyrosine phosphorylated Shc proteins are thought to be involved in mammalian Ras signalling pathways (47), serving as adapters to link receptors to Grb2 (62). Grb2, in turn, has been implicated in the binding of Sos, a Ras GDP/GTP exchange factor (47). PI3K is a dual-specificity phospholipid, protein (serine) kinase that is widely associated with tyrosine

kinase receptors (1, 12, 16). PI3K has been implicated in vesicular transport (36), insulin-activated glucose transport (17, 22, 52), internalization of the platelet-derived growth factor receptor (39), activation of pp70 S6 kinase (pp70^{S6K}) (17, 20, 24, 50, 57), and other signal transduction pathways (1). Phospholipase C γ is the critical enzyme in production of two important second messengers, inositol 1,4,5-triphosphate and diacylglycerol (6).

While some aspects of the mechanisms involved in the formation of complexes between mT and these signalling molecules are recognized, the downstream effects of the complexes on transformation remain largely unknown. An important challenge at present is to link each of the proximal events of signalling from mT to specific downstream biochemical events and pathways that collectively lead to the emergence of a fully transformed phenotype. The binding of both PI3K and Shc to mT is essential for transformation, as revealed by early work on mT mutants (13, 28, 29, 45). Mutant 315YF, in which tyrosine 315 is replaced by phenylalanine (originally designated mutant 1178T [13]), encodes an mT that activates pp60^{c-src} but fails to bind PI3K (69). 315YF induces incomplete transformation of rat fibroblasts (13) and is compromised in its ability to induce tumors in mice (34). Similarly, mT mutants in which tyrosine 250 is replaced by phenylalanine (250YF mutants) fail to bind Shc (11, 27) and are also defective for transformation (28, 29, 45).

Here we utilize mutants 250YS and 315YF to discriminate between the downstream effects of mT acting through PI3K or Shc at discrete molecular and cell biological levels. Activation of pp70^{S6K} was chosen as a target for several reasons. Previous work has shown that two mT-dependent mechanisms contribute to increased S6 phosphorylation. One involves activation of an S6 kinase(s) and is dependent on mT activation of pp60^{c-src}; the other is independent of mT–pp60^{c-src} interaction and may reflect negative regulation of an S6 phosphatase (68). How mT exerts these effects, and specifically whether pp70^{S6K} is in-

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volved, is unknown. S6 phosphorylation is principally controlled by pp70^{S6K} (8, 21), and the function of this enzyme is essential for serum-induced G₁ progression in rat embryo fibroblasts (42, 60). Ribosomes that contain phosphorylated S6 selectively translate a family of mRNA transcripts containing a polypyrimidine tract at the 5' end (37, 38). The pathways by which pp70^{S6K} are regulated are complex and yet to be fully defined (77), but they are distinct from the well-characterized mitogen-activated protein kinase pathway which functions in the activation of pp90^{rsk} (7, 66). Thus, the normal regulation of this enzyme, as well as its role in translational control of cell cycle progression (37, 42, 60), raises interesting questions in relation to neoplastic transformation. The abilities of the mutants to induce morphological transformation and loss of cell growth control have been studied and compared. Results have shown that pp70^{S6K} is activated through mT-PI3K interaction alone, that both Shc and PI3K pathways independently give rise to decreased serum dependence of growth but to a lesser extent than with wild-type virus, and that both pathways are essential for induction of anchorage-independent growth.

MATERIALS AND METHODS

Viruses and cells. Polyomavirus stocks were prepared on primary baby mouse kidney cells (73). Polyomavirus mutants of NG-59 (5) and 1178T (13) have been described previously. Mutant 1178T, in which tyrosine 315 in mT is replaced by phenylalanine, is referred to henceforth as 315YF. Polyomavirus mutant 250YS encodes an mT with serine in place of tyrosine at residue 250. Mutant 250YS was generated by site-directed mutagenesis using the oligomer 5'-CCCCACCTCTTCTGTATG and a single-stranded template from a subclone containing the wild-type viral mT coding sequence from *Pst*I (nucleotide 484) to *Eco*RI (nucleotide 1560) as previously described (33). The mutated base is underlined. This mutation does not alter the amino acid sequence in the overlapping large-T-antigen reading frame. The DNA fragment containing the mutation was ligated to the appropriate fragment of the cloned viral genome and transfected into NIH 3T3 cells. The viral lysate was plaque purified and expanded on baby mouse kidney cells.

NIH 3T3 cells were grown at 37°C on Dulbecco modified Eagle medium (DMEM) supplemented with 5% calf serum. Nearly confluent monolayers were infected at a multiplicity of 10 to 50 PFU per cell. Following a 90-min adsorption period, the monolayer was washed once with phosphate-buffered saline (PBS), fresh medium containing 0.1% bovine serum albumin (BSA) was added, and cells were incubated for 35 to 40 h.

Rat F111 cell lines PyF-WT, PyF-315YF, and PyF-250YS, stably expressing wild-type, 315YF, or 250YS mT, were generated by infecting F111 cells with the respective polyomaviruses and cloning the resulting foci.

Cell extracts, immunoprecipitations, and immunoblot analysis. Confluent 90-mm-diameter dishes of cells were washed once in STE (150 mM NaCl, 50 mM Tris-HCl [pH 7.2], 1 mM EDTA) and extracted for 20 min at 4°C with 0.35 ml of lysis buffer [10 mM potassium phosphate (pH 7.2), 1 mM EDTA, 5 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 10 mM MgCl₂, 50 mM β-glycerophosphate, 1 mM Na₂VO₄, 2 mM dithiothreitol, 40 μg of phenylmethylsulfonyl fluoride per ml, 0.5% Nonidet P-40]. Lysates were cleared at 13,000 × g, heated in sample buffer for 5 min at 95°C, and resolved by electrophoresis on a sodium dodecyl sulfate (SDS)-7.5 or 10% polyacrylamide gel (40). mT and pp70^{S6K} were detected by enhanced chemiluminescence (ECL; Amersham), using the mouse monoclonal antibody F4, which recognizes the common amino-terminal region of large T antigen, mT, and small T antigen (54), or a rabbit antiserum prepared against pp70^{S6K} (from J. Blenis, Harvard Medical School). Anti-Shc immunoblots of mT immunoprecipitated with mouse monoclonal antibody PAb762 (27) (from S. Dilworth, Hammersmith Hospital, London, England) were prepared with ECL and a polyclonal rabbit antiserum to Shc (Transduction Laboratories). Anti-p85 immunoblots of mT immunoprecipitated with anti-T ascites fluid (64) were prepared with anti-rat PI3K (Upstate Biotechnology) and ¹²⁵I-labeled second antibody. Quantitation of immunoblots was performed with either ECL phosphor imaging analysis or scintillation counting of bands labeled with ¹²⁵I-labeled second antibody and found to be linear over a 5- to 10-fold range.

Kinase assays. mT-associated PI3K was assayed in immunoprecipitates made with anti-T ascites fluid as described previously (26). To measure the effect of wortmannin on PI3K activity in vitro, extracts prepared from wortmannin-treated cells were immunoprecipitated with anti-T ascites fluid, washed, and assayed for PI3K activity in the presence of wortmannin at various concentrations. Wortmannin was added to the enzyme assay mixture because PI3K inhibition by wortmannin can be somewhat reversed during the process of immunoprecipitation (52). pp70^{S6K} activity was measured essentially as described by Terada et al. (70) in washed immunoprecipitates prepared from 0.3 mg of total

protein with a polyclonal rabbit antiserum against pp70^{S6K} (from J. Blenis). The immune complexes were collected on protein A/G plus agarose (Oncogene Science) and washed twice with PBS and once with S6 kinase buffer (20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid [HEPES; pH 7.4], 10 mM MgCl₂, 1 mg of BSA per ml, 0.4 mM dithiothreitol). To start the reaction, the beads were mixed with 40 μl of S6 kinase buffer containing 6 μg of S6 peptide (RRRLSSLRA; Upstate Biotechnology), 4 nmol of ATP, 10 μCi of [^γ-³²P]ATP, and 50 ng of IP-20 (protein kinase inhibitor; Sigma). The reaction proceeded for 15 min at 30°C and was terminated by adding 0.1 M EDTA and heating at 95°C for 5 min. Following a brief centrifugation, aliquots (20 μl in duplicate) were applied to phosphocellulose discs and washed twice in 0.18 M phosphoric acid. Radioactivity was measured by scintillation counting.

PI3K products in [³H]myoinositol-labeled cells. F111 cell lines stably expressing T antigens were cultured at 37°C on inositol-free DMEM supplemented with 0.5% calf serum and 10 μCi of [³H]myoinositol (DuPont NEN) per ml for 48 h. Cells were washed two times with ice-cold PBS and lysed with 1 N HCl-methanol (1:1, vol/vol). Cells were scraped from the plate, and lipids were extracted with CHCl₃. The CHCl₃ layer was reextracted with 1 N HCl-methanol (1:1) and dried under N₂. Phospholipids were deacylated with 25% methylamine-methanol-*n*-butanol (4:4:1, by volume) for 50 min at 53°C and dried in vacuo. After extraction with *n*-butanol-petroleum ether-ethyl formate (20:4:1, by volume), the radiolabeled glycerolipids were analyzed by high-pressure liquid chromatography as previously described (3). Lipid standards were generated in PI3K assays using phosphatidylinositol (PI) and PI 4-phosphate as substrates.

Transformation and plaque assays. Transformation assays on established F111 rat fibroblasts and plaque assays using UCI-B cells have been described previously (30). To measure anchorage-independent growth of F111 cell lines stably expressing T antigens, cells were seeded in 0.4% agar medium at a density of 10⁴ cells per 60-mm-diameter dish. Fifty randomly selected colonies were scored for cell number after 6 days at 37°C.

RESULTS

mT-dependent activation of pp70^{S6K} requires binding of PI3K but not of Shc. By using specifically altered mutants of polyomavirus, it should be possible to discern the individual contributions of discrete signalling pathways involving mT to the overall process of neoplastic transformation at the molecular and cell biological levels. Two mutants previously described were chosen for this study on the basis of their known properties; a third mutant was constructed and analyzed in this study. NG-59, a prototype of the hr-t mutant group altered in both mT and small T antigen, is totally null in all aspects of cell transformation and mT-associated functions (14, 63). 315YF, a partially transforming mutant, encodes an mT specifically blocked in binding of PI3K but is fully able to activate pp60^{c-src} (13). 250YS has an alteration in mT predicted to result in an inability to bind the Shc family of proteins. The point mutations in 350YF and 250YS are both silent in the overlapping reading frame encoding large T antigen. Both large and small T antigens are therefore normal in these mutants. Both mutants can be grown in culture as well, or nearly as well, as wild-type virus.

In cells infected by polyomavirus, induction of S6 kinase activity depends on activation of pp60^{c-src} by mT (68); thus, either mutant 315YF acting through Shc or mutant 250YS acting through PI3K, or possibly both, might be expected to retain this function. NIH 3T3 cells were infected by wild-type virus or various mutants and were serum starved. Cell extracts were then prepared and used to monitor mT interactions with PI3K and Shc and the level of pp70^{S6K} activation. By infecting with various dilutions of virus, cells expressing similar levels of mT could be compared (Fig. 1A). Binding of mT to PI3K was evaluated by in vitro PI3K assays in T-antigen immunoprecipitates, while Shc binding to mT was determined by immunoblot analysis of mT immunoprecipitates. NG-59 fails to bind both PI3K and Shc, as expected for a mutant that fails to associate with pp60^{c-src}. 315YF binds Shc but not PI3K, and 250YS binds PI3K but not Shc (Fig. 1B and C). The abilities of mutant and wild-type mTs to promote phosphorylation and activation of pp70^{S6K} were determined by immunoblot analysis (Fig. 1D) and direct enzyme assay of anti-pp70^{S6K} immunoprecipitates

FIG. 1. Activation of pp70^{S6K}. NIH 3T3 cells were either mock infected or infected with mutant or wild-type (WT) polyomaviruses and serum starved for 36 h. Mock-infected, serum-stimulated cells were treated with 10% calf serum for the final 2 h of the starvation period. (A) Immunoblot of mT in cell lysates. (B) In vitro PI3K activity in T-antigen immunoprecipitates made with anti-T ascites fluid. PIP, PI phosphate. (C) Immunoblot of Shc in mT immunoprecipitates made with PAb762. (D) Immunoblot of pp70^{S6K} in cell lysates. (E) In vitro S6 kinase activity in pp70^{S6K} immunoprecipitates made with a polyclonal rabbit antiserum to pp70^{S6K}. Numbers following the virus types indicate viral dilutions used for infections. Data in all panels were from extracts generated in the same experiment. Similar results were found in two separate experiments.

(Fig. 1E). Maximal or nearly maximal levels of phosphorylation and activation were achieved by adding 10% serum to quiescent cells as a positive control. Infection by wild-type virus and mutant 250YS activated pp70^{S6K} to levels comparable to that achieved with serum. Phosphorylation which accompanies activation led to a reduced mobility on SDS-polyacrylamide gel electrophoresis of both the 70-kDa α II form of pp70^{S6K} (Fig. 1D) and the 85-kDa α I form (not shown). Mutants NG-59 and 315YF were without effect on phosphorylation and activation of pp70^{S6K}. Activation of pp70^{S6K} thus correlates with the ability of mT to bind PI3K. mT activation of pp60^{c-src} and binding of Shc without binding of PI3K are clearly not sufficient to activate pp70^{S6K}.

We have observed that PI3K activity coimmunoprecipitating with 250YS mT is somewhat variable and usually slightly lower than that coimmunoprecipitating with wild-type mT. To further verify the binding of PI3K to 250YS mT, levels of the p85

regulatory subunit of PI3K were measured along with PI3K activity in T-antigen immunoprecipitates prepared from extracts containing equal levels of mT protein. The results in Fig. 2 show that the level of p85 bound to 250YS mT complexes and the amount of associated PI3K activity were between 50 and 70% of those seen in wild-type immunoprecipitates. Similar results have been obtained in several independent experiments. Tyrosine at residue 250 in mT may therefore contribute somewhat to the stability of binding and of PI3K activity in the in vitro assay. The data nevertheless support the conclusion that activation of pp70^{S6K} by 250YS and wild-type mT correlates with binding of PI3K.

PI3K products in cell lines expressing wild-type and mutant mTs. Although it is generally accepted that in vitro binding of PI3K to activated growth factor receptors or mT correlates with in vivo activation of the enzyme, two exceptions have been reported. mT mutants in which proline 248 is replaced with leucine (248m) or amino acids 338 to 347 are deleted (*dl1015*) are reported to bind PI3K in vitro but fail to activate the enzyme in vivo (44). Also, a mutant of the nerve growth factor receptor Trk-A which lacks tyrosine at residue 490, the purported binding site for Shc, fails to activate PI3K both in vitro and in vivo, whereas a Trk-A receptor lacking tyrosine 751, the purported binding site for p85, retains ability to activate PI3K in vitro and in vivo (4). To confirm that in vitro binding of PI3K to mT correlates with in vivo activation, rat fibroblast cell lines expressing wild-type, 315YF, and 250YS mTs were assessed for steady-state levels of PI 3,4-bisphosphate [PI(3,4)P₂]. The data in Fig. 3 show little increase in PI(3,4)P₂ formation in 315YF mT-expressing cells, whereas PI(3,4)P₂ levels were increased threefold in 250YS and wild-type mT-expressing cells. The finding for 315YF supports the report by Ulug et al. (74) that mutant *dl23*, which also fails to bind and activate PI3K in vitro, shows no increase in levels of 3-phosphorylated inositol lipids in vivo. Although mutants 250YS and 248m have similar in vitro biochemical phenotypes, i.e., both are negative for mT-Shc binding (Fig. 1) (27) but positive for mT-PI3K binding in vitro (Fig. 1) (2, 28), unlike 250YS (discussed below), mutant 248m is completely transformation defective (28) and fails to

FIG. 2. Binding of p85 and PI3K to 250YS mT. Extracts containing equal levels of mT were prepared from NIH 3T3 cells infected as described in the legend to Fig. 1. p85 immunoprecipitating with mT was subjected to immunoblot analysis using an anti-PI3K antibody (Upstate Biotechnology) and ¹²⁵I-labeled second antibody. Labeled bands were excised and quantitated by liquid scintillation counting. PI3K activity in T-antigen immunoprecipitates was assayed as described in Materials and Methods and quantitated by phosphor imaging analysis. The data were from duplicate determinations. WT, wild type.

FIG. 3. Steady-state levels of PI(3,4)P₂ in [³H]myoinositol-labeled F111 cells expressing different mTs. F111 cell lines stably expressing T antigens were labeled with [³H]myoinositol for 48 h. Lipids were extracted and analyzed as described in Materials and Methods. The results are averaged from three datum points in a single experiment. Similar results were obtained in further experiments.

raise the level of the 3-phosphorylated inositol lipids in vivo (44).

Wortmannin inhibits activation of pp70^{S6K} by wild-type virus and mutant 250YS virus. Wortmannin at >10 nM is a potent inhibitor of PI3K both in vivo and in vitro (58, 71), although it may have additional effects (25). To support the finding that PI3K is on the pathway of mT-dependent pp70^{S6K} activation, further evidence was sought by using wortmannin as a PI3K inhibitor. Serum-starved NIH 3T3 cells infected with wild-type or mutant 250YS virus were treated for 1 h prior to lysis with increasing concentrations of wortmannin. In cells expressing equivalent levels of mT (Fig. 4A), the in vivo inhibitory concentration curves for pp70^{S6K} phosphorylation (Fig. 4B) and activation (Fig. 4C) and for PI3K in vitro (Fig. 4D) were similar for both wild-type- and 250YS-infected cells. These results support the involvement of PI3K in mT-dependent activation of pp70^{S6K}.

Polyomavirus mutants 315YF and 250YS induce partial and phenotypically distinct forms of transformation. Neoplastic transformation of cells in culture has been assessed by different parameters, including morphological changes, growth as foci on normal cell monolayers, and anchorage-independent growth. In F111 rat fibroblasts, these phenotypic changes show graded responses to increasing levels of mT expression, with morphological changes requiring the lowest levels and anchorage-independent growth requiring the highest levels (59). To analyze the contributions of the mT-Shc and mT-PI3K pathways to various parameters of transformation, mutants 250YS and 315YF were used along with wild-type virus to infect F111 cells. 315YF and 250YS induced weak foci with distinct focal morphologies that made them readily distinguishable from each other as well as from wild-type foci (Fig. 5). Cells comprising foci induced by 315YF were of normal size, fusiform, loosely packed, and moderately adherent to the plastic substratum (Fig. 5C). Cells transformed by 250YS appeared to be smaller and more polygonal, closely packed, and tightly adherent to the dish (Fig. 5D). By comparison, cells comprising wild-type foci tended to be more round and refractile in appearance, densely packed, and weakly adherent (Fig. 5B).

The efficiencies of transformation (transforming units per PFU) were compared by focus formation and by growth in soft

agar. Scored by focus-forming ability, the mutants were lower by a factor of 10 to 30 compared with the wild type and by 1,000-fold or more when compared by induction of growth in soft agar (Table 1). These results show that signalling through either PI3K or Shc alone can give rise to morphological transformation, but with reduced efficiency and notable qualitative differences from wild-type virus, and that neither pathway by itself is sufficient for induction of anchorage-independent growth.

Serum and anchorage dependence of growth in cells stably transformed by mutants 250YS and 315YF. F111 cells stably expressing 250YS or 315YF mutant mT were cloned from primary foci, confirmed by immunoblotting to express comparable levels of mT, and tested for their abilities to grow in low

FIG. 4. Inhibition of pp70^{S6K} and PI3K activity by wortmannin. NIH 3T3 cells were infected with either wild-type (WT) or mutant 250YS polyomavirus and serum starved for 36 h. The indicated concentrations of wortmannin diluted in dimethyl sulfoxide were added for the final hour of the starvation period. (A) Immunoblot of mT in cell lysates. M, mock infection. (B) Immunoblot of pp70^{S6K} in cell lysates. (C) In vitro S6 kinase activity in pp70^{S6K} immunoprecipitates prepared as described in the legend to Fig. 1E. (D) In vitro PI3K activity in T-antigen immunoprecipitates prepared as described in the legend to Fig. 1B. Wortmannin was added to in vitro PI3K assay mixtures 2 min before the reaction was started by addition of [³²P]ATP. The data in all panels were from extracts generated in the same experiment. Similar results were found in two separate experiments.

FIG. 5. Morphologies of F111 rat fibroblasts infected by wild-type and mutant polyomavirus. (A) Mock, uninfected control; (B) wild-type focus; (C) 315YF focus; (D) 250YS focus.

serum and in soft agar. The results in Fig. 6 show that at the lowest concentration of serum, the mutant mT lines grew substantially better than normal F111 cells, though not quite as well as the clones expressing wild-type mT. At higher concentrations, the mutant lines remained intermediate between untransformed and wild-type-transformed cells, but the relative differences were less. When the cells were suspended in soft nutrient agar and examined for distribution of clone size after 6 days, it was evident that only the wild-type-transformed cells grew efficiently, giving rise to macroscopically visible colonies (approximately 20 cells or more). While neither of the mutant lines grew as well as the wild type, 315YF cells grew detectably better than 250YS cells, as determined by microscopic counts (Fig. 7). These results show that while both Shc and PI3K pathways are needed for efficient anchorage-independent growth, either pathway alone can promote substantial growth in low concentrations of serum. It should be noted that a mutant polyomavirus which fails to express mT but encodes normal small and large T antigens is ineffective in inducing anchorage-independent growth but can promote some growth in low serum (43). Since mutants 250YS and 315YS express normal small and large T antigens, these proteins most likely contribute to the levels of growth in low serum observed in the mT mutant lines (Fig. 6).

DISCUSSION

This study extends an earlier report on polyomavirus-induced elevation of ribosomal protein S6 phosphorylation (68) by demonstrating activation of pp70^{S6K} in a pathway mediated by mT and PI3K. Genetic and biochemical evidence supports the conclusion that PI3K binding to mT is required for activation of both the cytoplasmic (70-kDa) and nuclear (85-kDa) forms of S6 kinase. Polyomavirus mutant 315YF, which is

specifically defective in PI3K binding (69) and activation, fails to activate pp70^{S6K} despite activating pp60^{c-src} normally. Wortmannin at concentrations of <100 nM effectively blocks pp70^{S6K} activation in wild-type virus-infected cells in parallel with inhibiting mT-associated PI3K activity. mT thus acts similarly to a variety of stimuli in linking activated tyrosine kinases to pp70^{S6K} through PI3K (17, 19, 20, 24, 50, 57).

The results further indicate that binding of the Shc proteins, purportedly involved in a signalling network leading to Ras (47), is neither necessary nor sufficient for mT activation of pp70^{S6K}. This is indicated by the ability of mutant 250YS, which fails to bind Shc, to fully activate pp70^{S6K} and by the inability of mutant 315YF to stimulate pp70^{S6K} activity despite binding Shc normally. Although 250YS mT binds PI3K in vitro slightly less well than wild-type mT, 250YS activates PI3K in vivo to wild-type levels. Elevation in levels of S6 phosphorylation is also found in *v-ras*-transformed cells (9), but it is not

TABLE 1. Transforming abilities of polyomavirus mutants 250YS and 315YF

Virus	Transformation efficiency ^a (10 ⁻⁸ TFU/PFU)	
	Focus formation	Growth in agar ^b
Wild type	2,700	1,100
250YS	93	1
315YF	33	<1

^a F111 cells (4 × 10⁴) in 35-mm-diameter dishes were infected with 0.1 ml of virus stocks at a multiplicity of infection of 10² to 10⁴ PFU per cell. After 24 h, cells were divided equally between two 60-mm-diameter dishes to measure transforming units (TFU) as indicated by focus formation or growth in agar. Foci and agar clones were counted microscopically 16 days later.

^b Agar clones on the wild type frequently reached >64 cells. Agar clones on 315YF were usually <32 cells. Agar clones on 250YS were usually <16 cells.

FIG. 6. Effects of serum concentration on cell yields of F111-derived cell lines containing wild-type or mutant mT. Cell lines F111, PyF-250YS, PyF-315YF, and PyF-WT were seeded in DMEM containing 5% calf serum on 60-mm-diameter plates at a density of 10^4 cells per plate. After cells were allowed to attach overnight, the medium was replaced with DMEM containing 0.5, 2, or 10% calf serum and incubated for 6 days. Cells were trypsinized and counted in a hemacytometer. Data are averaged from results with two clones of each cell type except F111.

clear to what extent this requires activation of pp70^{S6K}. In human 293 cells, activation of pp70^{S6K} occurs by a pathway independent of p21^{ras} (49). Activation of pp70^{S6K} shows the same dependence on mT functions as does the elevation of glucose transport, which also requires interaction with PI3K and not with Shc (79). Although these results are consistent with the notion that Shc and PI3K binding to mT trigger separate parallel biochemical pathways, downstream interactions are expected to occur. Cross talk between Ras or Raf and PI3K has in fact been found in several systems (24, 61, 65).

The mechanism by which mT binding of PI3K leads to phosphorylation and activation of pp70^{S6K} remains unknown, as does the identification of the possible PI3K products involved. Phosphorylation of pp70^{S6K} at multiple sites both outside and within an autoinhibitory pseudosubstrate domain activates the enzyme, although the relevant kinases have not been defined (18, 76, 77). The protein serine kinase activity of PI3K is blocked by wortmannin (41), raising the possibility that PI3K phosphorylates pp70^{S6K} directly. However, a recent study showing that a fragment of p70^{S6K} is not a substrate for PI3K in vitro rules against this possibility (76). Other possible downstream targets of PI3K in pp70^{S6K} regulation are members of the calcium-insensitive protein kinase C family (51, 72) and the recently identified serine/threonine protein kinase B/Akt (10, 31).

At the cellular level, the mT-PI3K and mT-Shc pathways act independently of one another to effect some parameters of transformation but must act synergistically to elicit others. In established rat embryo fibroblasts, each pathway by itself induces a characteristic type of morphological transformation which appears weak or incomplete compared with that induced by wild-type mT. Each of the pathways also leads to decreased serum dependence of growth, and their effects may be additive in reaching the level of serum independence achieved by wild-type mT. With respect to induction of anchorage-independent growth, interactions of mT with both Shc and PI3K are required, and the effects of the two pathways are clearly synergistic rather than additive. This result indicates a requirement for downstream integration of signals from both pathways for induction of anchorage-independent growth.

Activation of pp70^{S6K} through the mT-PI3K pathway most likely plays a role in the emergence of at least some transformation-related changes, given the known role of S6 kinase in normal mitogenesis (42, 60). The mutant 315YF, defective in the mT-PI3K pathway, stimulates growth of cells in low serum and, to a limited extent, in soft agar. While these effects of the mutant are most likely mediated primarily through the mT-Shc pathway, phosphorylation of S6 may still play a role since this mutant elevates S6 phosphorylation through some kinase-independent mechanism (68).

The partially transforming virus mutants 315YF and 250YS are both able to induce tumors upon inoculation of newborn mice, but with reduced efficiency, altered time course, and some changes in the spectrum and histological features of the tumors compared with wild-type virus (32, 34). Further studies

FIG. 7. Growth in soft agar of F111-derived cell lines containing wild-type or mutant mT. Cell lines were scored for agar growth as described in Materials and Methods. Data are averaged from results with two clones of each cell type except F111.

of the molecular and cell biological aspects of mutant virus infections in culture should enable a better understanding of the processes that accompany neoplastic transformation of a variety of different cell types in mice.

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REFERENCES

- Auger, K. R., and L. C. Cantley. 1991. Novel polyphosphoinositides in cell growth and activation. *Cancer Cells* 3:263–270.
- Auger, K. R., C. L. Carpenter, S. E. Shoelson, H. Piwnicka-Worms, and L. C. Cantley. 1992. Polyoma virus middle T antigen-pp60^{src} complex associates with purified phosphatidylinositol 3-kinase in vitro. *J. Biol. Chem.* 267:5408–5415.
- Auger, K. R., L. A. Serunian, and L. C. Cantley. 1990. Separation of novel polyphosphoinositides, p. 159–166. *In* F. Irvine (ed.), *Methods in inositide research*. Raven Press, New York.
- Baxter, R. M., P. Cohen, A. Obermeier, A. Ullrich, C. P. Downes, and Y. N. Doza. 1995. Phosphotyrosine residues in the nerve-growth-factor receptor (Trk-A). Their role in the activation of inositolphospholipid metabolism and protein kinase cascades in pheochromocytoma (PC12) cells. *Eur. J. Biochem.* 234:84–91.
- Benjamin, T. L. 1970. Host range mutants of polyoma virus. *Proc. Natl. Acad. Sci. USA* 67:394–399.
- Berridge, M. J. 1993. Inositol triphosphate and calcium signalling. *Nature (London)* 361:315–325.
- Blenis, J. 1993. Signal transduction via the MAP kinases: proceed at your own RSK. *Proc. Natl. Acad. Sci. USA* 90:5889–5892.
- Blenis, J., J. Chung, E. Erikson, D. A. Alcorta, and R. L. Erikson. 1991. Distinct mechanisms for the activation of the RSK kinases/MAP2 kinase/pp90^{src} and pp70-S6 kinase signaling systems are indicated by inhibition of protein synthesis. *Cell Growth Differ.* 2:279–285.
- Blenis, J., and R. L. Erikson. 1984. Phosphorylation of the ribosomal protein S6 is elevated in cells transformed by a variety of tumor viruses. *J. Virol.* 50:966–969.
- Burging, B. M. T., and P. J. Coffer. 1995. Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature (London)* 376:599–602.
- Campbell, K. S., E. Ogris, B. Burke, W. Su, K. R. Auger, B. J. Druker, B. S. Schaffhausen, T. M. Roberts, and D. C. Pallas. 1994. Polyoma middle tumor antigen interacts with SHC protein via the NPTY (asn-pro-thr-tyr) motif in middle tumor antigen. *Proc. Natl. Acad. Sci. USA* 91:6344–6348.
- Cantley, L. C., K. R. Auger, C. Carpenter, B. Duckworth, A. Graziani, R. Kapeller, and S. Soltoff. 1991. Oncogenes and signal transduction. *Cell* 64:281–302.
- Carmichael, G., B. S. Schaffhausen, G. Mandel, T. J. Liang, and T. L. Benjamin. 1984. Transformation by polyoma virus is drastically reduced by substitution of phenylalanine for tyrosine at residue 315 of middle-sized tumor antigen. *Proc. Natl. Acad. Sci. USA* 81:679–683.
- Carmichael, G., and T. L. Benjamin. 1980. Identification of DNA sequence changes leading to loss of transforming ability in polyoma virus. *J. Biol. Chem.* 255:230–235.
- Carmichael, G., B. S. Schaffhausen, D. I. Dorsky, D. B. Oliver, and T. L. Benjamin. 1982. Carboxy terminus of polyoma middle-sized tumor antigen is required for attachment to membranes, associated protein kinase activities, and cell transformation. *Proc. Natl. Acad. Sci. USA* 79:3579–3583.
- Carpenter, C. L., K. R. Auger, B. C. Duckworth, W. M. Hou, B. Schaffhausen, and L. C. Cantley. 1993. A tightly associated serine/threonine protein kinase regulates phosphoinositide 3-kinase activity. *Mol. Cell. Biol.* 13:1657–1665.
- Cheatham, B., C. J. Vlahos, L. Cheatham, L. Wang, J. Blenis, and C. R. Kahn. 1994. Phosphatidylinositol 3-kinase activation is required for insulin stimulation of pp70 S6 kinase, DNA synthesis, and glucose transporter translocation. *Mol. Cell. Biol.* 14:4902–4911.
- Cheatham, L., M. Monfar, M. M. Chou, and J. Blenis. 1995. Structural and functional analysis of pp70^{S6k}. *Proc. Natl. Acad. Sci. USA* 95:11696–11700.
- Chou, M. M., and J. Blenis. 1995. The 70 kD S6 kinase: regulation of a kinase with multiple roles in mitogenic signalling. *Curr. Opin. Cell Biol.* 7:806–814.
- Chung, J., T. C. Grammer, K. P. Lemon, A. Kazlauskas, and J. Blenis. 1994. PDGF- and insulin-dependent pp70^{S6k} activation mediated by phosphatidylinositol-3-OH kinase. *Nature (London)* 370:71–75.
- Chung, J., C. J. Kuo, G. R. Crabtree, and J. Blenis. 1992. Rapamycin-FKBP specifically blocks growth-dependent activation of and signaling by the 70 kD S6 protein kinases. *Cell* 69:1227–1236.
- Clarke, J. F., P. W. Young, K. Yonezawa, M. Kasuga, and G. D. Holman. 1994. Inhibition of the translocation of GLUT1 and GLUT4 in 3T3-L1 cells by the phosphatidylinositol 3-kinase inhibitor, wortmannin. *Biochem. J.* 300:631–635.
- Courtneidge, S. A., and A. E. Smith. 1983. Polyoma virus transforming protein associates with the product of the *c-src* cellular gene. *Nature (London)* 303:435–439.
- Cross, D. A. E., D. R. Alessi, J. R. Vandenheede, H. E. McDowell, H. S. Hundal, and P. Cohen. 1994. The inhibition of glycogen synthase kinase-3 by insulin or insulin-like growth factor 1 in the rat skeletal muscle cell line L6 is blocked by wortmannin, but not by rapamycin: evidence that wortmannin blocks activation of the mitogen-activated protein kinase pathway in L6 cells between Ras and Raf. *Biochem. J.* 303:21–26.
- Cross, M. J., A. Stewart, M. N. Hodgkin, D. J. Kerr, and J. O. Wakelam. 1995. Wortmannin and its structural analogue demethoxyviridin inhibit stimulated phospholipase A₂ activity in Swiss 3T3 cells. *J. Biol. Chem.* 270:25352–25355.
- Dahl, J., U. Thathamangalam, R. Freund, and T. L. Benjamin. 1992. Functional asymmetry of the regions juxtaposed to the membrane-binding sequence of polyomavirus middle T antigen. *Mol. Cell. Biol.* 12:5050–5058.
- Dilworth, S. M., C. E. Brewster, M. D. Jones, L. Lanfrancone, G. Pelicci, and P. G. Pelicci. 1994. Transformation by polyoma virus middle T-antigen involves the binding and tyrosine phosphorylation of Shc. *Nature (London)* 367:87–90.
- Druker, B. J., L. E. Ling, B. Cohen, T. M. Roberts, and B. S. Schaffhausen. 1990. A completely transformation-defective point mutant of polyomavirus middle T antigen which retains full associated phosphatidylinositol kinase activity. *J. Virol.* 64:4454–4461.
- Druker, B. J., L. Sibert, and T. M. Roberts. 1992. Polyomavirus middle T-antigen NPTY mutants. *J. Virol.* 66:5770–5776.
- Fluck, M. M., and T. L. Benjamin. 1979. Comparisons of two early gene functions essential for transformation in polyoma virus and SV-40. *Virology* 96:205–228.
- Franke, T. F., S.-I. Yang, T. O. Chan, K. Datta, A. Kazlauskas, D. K. Morrison, D. R. Kaplan, and P. N. Tsichlis. 1995. The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell* 81:727–736.
- Freund, R., and T. L. Benjamin. Unpublished results.
- Freund, R., R. T. Bronson, and T. L. Benjamin. 1992. Separation of immortalization from tumor induction with polyoma large T mutants that fail to bind the retinoblastoma gene product. *Oncogene* 7:1979–1987.
- Freund, R., C. J. Dawe, J. P. Carroll, and T. L. Benjamin. 1992. Changes in frequency, morphology, and behavior of tumors induced in mice by a polyoma virus mutant with a specifically altered oncogene. *Am. J. Pathol.* 141:1409–1425.
- Freund, R., A. Sotnikov, R. T. Bronson, and T. L. Benjamin. 1992. Polyoma virus middle T is essential for virus replication and persistence as well as for tumor induction in mice. *Virology* 191:716–723.
- Herman, P. K., and S. D. Emr. 1990. Characterization of VPS34, a gene required for vacuolar protein sorting and vacuole segregation in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 10:6742–6754.
- Jefferies, H. B. J., C. Reinhard, S. C. Kozma, and G. Thomas. 1994. Rapamycin selectively represses translation of the “polypyrimidine tract” mRNA family. *Proc. Natl. Acad. Sci. USA* 91:4441–4445.
- Jefferies, H. B. J., G. Thomas, and G. Thomas. 1994. Elongation factor-1 α mRNA is selectively translated following mitogenic stimulation. *J. Biol. Chem.* 269:4367–4372.
- Joly, M., A. Kazlauskas, F. S. Fay, and S. Corvera. 1994. Disruption of PDGF receptor trafficking by mutation of its PI-3 kinase binding sites. *Science* 263:684–687.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* 227:680–685.
- Lam, K., C. L. Carpenter, N. B. Ruderman, J. C. Friel, and K. L. Kelly. 1994. The phosphatidylinositol 3-kinase serine kinase phosphorylates IRS-1: stimulation by insulin and inhibition by wortmannin. *J. Biol. Chem.* 269:20648–20652.
- Lane, H. A., A. Fernandez, N. J. Lamb, and G. Thomas. 1993. p70^{S6k} function is essential for G1 progression. *Nature (London)* 363:170–172.
- Liang, T. J., G. G. Carmichael, and T. L. Benjamin. 1984. A polyoma mutant that encodes small T antigen but not middle T antigen demonstrates uncoupling of cell surface and cytoskeletal changes associated with cell transformation. *Mol. Cell. Biol.* 4:2774–2783.
- Ling, L. E., B. J. Druker, L. C. Cantley, and T. M. Roberts. 1992. Transformation-defective mutants of polyomavirus middle T antigen associate with phosphatidylinositol 3-kinase (PI 3-kinase) but are unable to maintain wild-type levels of PI 3-kinase products in intact cells. *J. Virol.* 66:1702–1708.
- Markland, W., B. A. Oostra, R. Harvey, A. F. Markham, W. H. Colledge, and A. E. Smith. 1986. Site-directed mutagenesis of polyomavirus middle-T antigen sequences encoding tyrosine 315 and tyrosine 250. *J. Virol.* 59:384–391.
- Matthews, J. T., and T. L. Benjamin. 1986. 12-O-Tetradecanoylphorbol-13-acetate stimulates phosphorylation of the 58,000-M_r form of polyomavirus middle T antigen in vivo: implications for a possible role of protein kinase C in middle T function. *J. Virol.* 58:239–246.

47. McCormick, F. 1993. How receptors turn Ras on. *Nature (London)* **363**:15–16.
48. McGlade, J., A. Cheng, G. Pelicci, P. G. Pelicci, and T. Pawson. 1992. Shc proteins are phosphorylated and regulated by the v-Src and v-Fps protein-tyrosine kinases. *Proc. Natl. Acad. Sci. USA* **89**:8869–8873.
49. Ming, X.-F., B. M. T. Burgering, S. Wennström, L. Claesson-Welsh, C.-H. Heldin, J. L. Bos, S. C. Kozma, and G. Thomas. 1994. Activation of p70/p85 S6 kinase by a pathway independent of p21^{ras}. *Nature (London)* **371**:426–429.
50. Monfar, M., K. P. Lemon, T. C. Grammer, L. Cheatham, J. Chung, C. J. Vlahos, and J. Blenis. 1995. Activation of pp70/85 S6 kinases in interleukin-2-responsive lymphoid cells is mediated by phosphatidylinositol 3-kinase and inhibited by cyclic AMP. *Mol. Cell. Biol.* **15**:326–337.
51. Nakanishi, H., K. A. Brewer, and J. H. Exton. 1993. Activation of the zeta isozyme of protein kinase C by phosphatidylinositol 3,4,5-triphosphate. *J. Biol. Chem.* **268**:13–16.
52. Okada, T., Y. Kawano, T. Sakakibara, O. Hazeki, and M. Ui. 1994. Essential role of phosphatidylinositol 3-kinase in insulin-induced glucose transport and antilipolysis in rat adipocytes—studies with a selective inhibitor wortmannin. *J. Biol. Chem.* **269**:3568–3573.
53. Pallas, D. C., H. Fu, L. C. Haehnel, W. Weller, R. J. Collier, and T. M. Roberts. 1994. Association of polyomavirus middle tumor antigen with 14-3-3 proteins. *Science* **265**:535–537.
54. Pallas, D. C., C. Schley, M. Mahoney, E. Harlow, B. S. Schaffhausen, and T. M. Roberts. 1986. Polyomavirus small t antigen: overproduction in bacteria, purification, and utilization for monoclonal and polyclonal antibody production. *J. Virol.* **60**:1075–1084.
55. Pallas, D. C., L. K. Shahrík, B. L. Martin, S. Jaspers, T. B. Miller, D. L. Brautigam, and T. M. Roberts. 1990. Polyoma small and middle T antigens and SV40 small t antigen form stable complexes with protein phosphatase 2A. *Cell* **60**:167–176.
56. Pelicci, G., L. Lanfrancone, F. Grignani, J. McGlade, F. Cavallo, G. Fornì, I. Nicoletti, T. Pawson, and P. G. Pelicci. 1992. A novel transforming protein (SHC) with an SH2 domain is implicated in mitogenic signal transduction. *Cell* **70**:93–104.
57. Petritsch, C., R. Woscholski, H. M. L. Edelmann, P. J. Parker, and L. M. Ballou. 1995. Selective inhibition of p70 S6 kinase activation by phosphatidylinositol 3-kinase inhibitors. *Eur. J. Biochem.* **230**:431–438.
58. Powis, G., R. Bonjouklian, M. M. Berggren, A. Gallegos, R. Abraham, C. Ashendel, L. Zalkow, W. F. Matter, J. Dodge, G. Grindey, and C. J. Vlahos. 1994. Wortmannin, a potent and selective inhibitor of phosphatidylinositol 3-kinase. *Cancer Res.* **54**:2419–2423.
59. Raptis, L., H. Lamfrom, and T. L. Benjamin. 1985. Regulation of cellular phenotype and expression of polyomavirus middle T antigen in rat fibroblasts. *Mol. Cell. Biol.* **5**:2476–2486.
60. Reinhard, C., A. Fernandez, N. J. C. Lamb, and G. Thomas. 1994. Nuclear localization of p85^{S6K}: functional requirement for entry into S phase. *EMBO J.* **13**:1557–1565.
61. Rodriguez-Viciana, P., P. H. Warne, R. Dhand, B. Vanhaesebroeck, I. Gout, M. J. Fry, M. D. Waterfield, and J. Downward. 1994. Phosphatidylinositol-3-OH kinase as a direct target of Ras. *Nature (London)* **370**:527–532.
62. Rozakis-Adcock, M., J. McGlade, G. Mbamalu, G. Pelicci, R. Daly, W. Li, A. Batzer, S. Thomas, J. Brugge, P. G. Pelicci, J. Schlessinger, and T. Pawson. 1992. Association of the Shc and Grb2/Sem5 SH2-containing proteins is implicated in activation of the Ras pathway by tyrosine kinases. *Nature (London)* **360**:689–692.
63. Schaffhausen, B., and T. L. Benjamin. 1981. Comparison of phosphorylation of two polyomavirus middle T antigens in vivo and in vitro. *J. Virol.* **40**:184–196.
64. Silver, J., B. Schaffhausen, and T. Benjamin. 1978. Tumor antigens induced by nontransforming mutants of polyoma virus. *Cell* **15**:485–496.
65. Sjölander, A., K. Yamamoto, B. E. Huber, and E. G. Lapetina. 1991. Association of p21^{ras} with phosphatidylinositol 3-kinase. *Proc. Natl. Acad. Sci. USA* **88**:7908–7912.
66. Sturgill, T. W., L. B. Ray, E. Erikson, and J. L. Maller. 1988. Insulin-stimulated MAP-2 kinase phosphorylates and activates ribosomal protein S6 kinase II. *Nature (London)* **334**:715–718.
67. Su, W., W. Liu, B. S. Schaffhausen, and T. M. Roberts. 1995. Association of Polyomavirus middle tumor antigen with phospholipase C- γ 1. *J. Biol. Chem.* **270**:12331–12334.
68. Talmage, D. A., J. Blenis, and T. L. Benjamin. 1988. Polyomavirus middle T antigen induces ribosomal protein S6 phosphorylation through pp60^{c-src}-dependent and -independent pathways. *Mol. Cell. Biol.* **8**:2309–2315.
69. Talmage, D. A., R. Freund, A. T. Young, J. Dahl, C. J. Dawe, and T. L. Benjamin. 1989. Phosphorylation of middle T by pp60^{c-src}: a switch for binding of phosphatidylinositol 3-kinase and optimal tumorigenesis. *Cell* **59**:55–65.
70. Terada, N., R. A. Franklin, J. J. Lucas, J. Blenis, and E. W. Gelfand. 1993. Failure of rapamycin to block proliferation once resting cells have entered the cell cycle despite inactivation of p70 S6 kinase. *J. Biol. Chem.* **268**:12062–12068.
71. Thelen, M., M. P. Wymann, and H. Langen. 1994. Wortmannin binds specifically to 1-phosphatidylinositol 3-kinase while inhibiting guanine nucleotide-binding protein-coupled receptor signaling in neutrophil leukocytes. *Proc. Natl. Acad. Sci. USA* **91**:4960–4964.
72. Toker, A., M. Meyer, K. K. Reddy, J. R. Falck, R. Aneja, S. Aneja, A. Parra, D. J. Burns, L. M. Ballas, and L. C. Cantley. 1994. Activation of protein kinase C family members by the novel polyphosphoinositides Ptd-3,4-P-2 and Ptd-3,4,5-P-3. *J. Biol. Chem.* **269**:32358–32367.
73. Turler, H., and P. Beard. 1985. Simian virus 40 and polyoma virus: growth, titration, transformation and purification of viral components, p. 169–192. *In* B. W. J. Mahy (ed.), *Virology: a practical approach*. IRL Press, Oxford.
74. Ulug, E. T., P. T. Hawkins, M. R. Hanley, and S. A. Courtneidge. 1990. Phosphatidylinositol metabolism in cells transformed by polyomavirus middle T antigen. *J. Virol.* **64**:3895–3904.
75. Walter, G., R. Ruediger, C. Slaughter, and M. Mumby. 1990. Association of protein phosphatase 2A with polyoma virus medium tumor antigen. *Proc. Natl. Acad. Sci. USA* **87**:2521–2525.
76. Weng, Q.-P., K. Andrabi, A. Klippel, M. T. Kozlowski, L. T. Williams, and J. Avruch. 1995. Phosphatidylinositol 3-kinase signals activation of p70 S6 kinase *in situ* through site-specific p70 phosphorylation. *Proc. Natl. Acad. Sci. USA* **92**:5744–5748.
77. Weng, Q.-P., K. Andrabi, M. T. Kozlowski, J. R. Grove, and J. Avruch. 1995. Multiple independent inputs are required for activation of the p70 S6 kinase. *Mol. Cell. Biol.* **15**:2333–2340.
78. Whitman, M., C. P. Downes, M. Keeler, T. Keller, and L. Cantley. 1988. Type I phosphatidylinositol kinase makes a novel inositol phospholipid, phosphatidylinositol-3-phosphate. *Nature (London)* **332**:644–646.
79. Young, A. T., J. Dahl, S. F. Hausdorff, P. H. Bauer, M. J. Birnbaum, and T. L. Benjamin. 1995. Elevation of glucose transport by polyoma virus occurs by increased translocation of GLUT1 mediated by PI 3-kinase binding to middle T. *Proc. Natl. Acad. Sci. USA* **92**:11613–11617.