Polyomavirus Middle T Antigen Induces Ribosomal Protein S6 Phosphorylation through pp60^{c-src}-Dependent and -Independent Pathways

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Phosphorylation of ribosomal protein S6 is elevated in polyomavirus-infected cells. This elevation results only in part from activation of S6 kinase activity. These effects appear to reflect independent activities of wild-type middle T antigen. Hr-t mutant NG59, encoding a defective middle T protein, and mutant Py808A, encoding no middle T protein, were unable to induce S6 kinase activity or elevate S6 phosphorylation. Two other site-directed mutants encoding altered middle T proteins did elevate S6 phosphorylation while only weakly stimulating S6 kinase activity. These results suggest at least two independent pathways leading to elevation of S6 phosphorylation. One pathway leads to induction of S6 kinase activity following activation of pp60^{c-src} by transformation-competent middle T antigen. Another pathway operates independently of S6 kinase induction and can be regulated by transformation-defective middle T mutants such as Py1387T. This mutant, encoding a truncated middle T protein that failed to associate with the plasma membrane and to activate pp60^{c-src}, caused increased levels of S6 phosphorylation without detectably increasing S6 kinase activity. The ability of mutants such as Py1387T to induce S6 phosphorylation correlated with their ability to increase phosphorylation of VP1, an event linked to maturation of infectious virions.

Transformation of cells by polyomavirus requires the interaction of the polyomavirus middle T antigen with the cellular proto-oncogene product pp60^{c-src} (15, 16). This interaction leads to the activation of the intrinsic tyrosine kinase activity of pp60^{c-src} and ultimately to transformation (10, 14). Although pp60^{c-src} activation is obligatory for transformation, mutant middle T's exist which interact with and activate pp60^{c-src} yet fail to transform infected cells. Full transformation in culture and tumor induction in mice may require the presence of an additional activity in middle T–pp60^{c-src} immune complexes which phosphorylates phosphatidylinositol (PI) (27, 51; D. Talmage, R. Freund, A. Young, C. Dawe, and T. Benjamin, manuscript in preparation).

Polyomavirus *hr-t* mutants encoding defective middle T and small T antigens grow poorly on NIH 3T3 cells, yielding bursts which are only 2 to 5% the size of those of wild-type virus (22). The low burst size results from inefficient or unstable assembly of viral components into infectious virions and is associated with underphosphorylation of the major viral capsid protein, VP1 (19, 20). Thus, the role of the *hr-t* gene in viral growth lies in inducing cellular "permissivity factors" which act, at least in part, to bring about stable phosphorylation of VP1. Activation of this phosphorylation pathway in NIH 3T3 cells does not require detectable interaction of middle T with pp60^{c-src} and therefore represents a middle T function which is separate from its known function during transformation (R. Garcea, D. Talmage, R. Freund, A. Harmatz, and T. Benjamin, manuscript in preparation).

The ribosomal protein S6 is highly phosphorylated in growing cells and rapidly becomes dephosphorylated when

growth is arrested by serum deprivation (48), contact inhibition, or heat shock (21). Phosphorylation of S6 is induced by a wide variety of mitogens and oncogene products, including insulin (31, 39, 46, 47, 50), insulinlike growth factors (23), epidermal growth factor (36, 37, 47), platelet-derived growth factor (PDGF) (34), fibroblast growth factor (38), tumor-promoting phorbol esters (5, 8, 50), and transforming gene products of Rous sarcoma virus (5, 8, 17), Abelson murine leukemia virus (30), simian virus 40, and polyomaviruses (4, 28). Increased phosphorylation of S6 is also seen in *Xenopus laevis* oocytes after progesterone or maturation-promoting factor stimulation of meiotic maturation (33).

In chicken embryo fibroblasts, S6 kinase activity is stimulated by calf serum, pp60^{v-src}, and phorbol esters, the last presumably acting through protein kinase C (8). pp60^{v-src} and serum can stimulate S6 kinase activity in cells depleted of protein kinase C, indicating that the pathways by which these two agents activate S6 kinase are distinct from the pathway by which phorbol esters are thought to activate S6 kinase (6).

The function of pp60^{c-src} during growth of normal fibroblasts is unknown. Activation by mutation or interaction with middle T can lead to cell transformation, but it is not known what role middle T activation of pp60^{c-src} might play in natural infections of cells by polyomavirus. The only reported activation of pp60^{c-src} by growth factors or hormones is the rapid, twofold increase of pp60^{c-src} kinase activity in quiescent Swiss 3T3 cells following PDGF stimulation (40). It remains to be determined whether pp60^{c-src} mediates the activation of S6 kinase in response to PDGF or any other external stimuli.

Phosphorylation of the ribosomal protein S6 is sensitive to both mitogenic and oncogenic stimuli. Since polyomavirus T antigens are both mitogenic and oncogenic, we looked at the state of S6 phosphorylation in polyomavirus-infected cells.

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By using mutant viruses, we have shown that neither large T alone nor small T and large T antigens together are sufficient to induce high levels of S6 phosphorylation. Middle T antigen is required for this response. It accomplishes this in part by activating S6 kinases which are chromatographically indistinguishable from those activated in NIH 3T3 cells in response to calf serum and in part through a mechanism which is currently not understood but does not require measurable increases in S6 kinase activity. The former action requires a wild-type middle T capable of activating both pp60^{c-src} and PI kinase; the latter does not.

MATERIALS AND METHODS

Materials. ³⁵S translabel and H₃³²PO₄ were purchased from ICN. [γ -³²P]ATP was made by the method of Johnson and Walseth (26). Protein A-Sepharose CL4B was purchased from Pharmacia.

Cells and viruses. NIH 3T3 mouse fibroblasts were grown in Dulbecco modified Eagle medium supplemented with 10% calf serum. Polyomavirus stocks were prepared on baby mouse kidney primary cultures (52). The generation and characterization of the wild-type virus NG59RA (18) and the mutants NG59 (3), Py808A (29), Py1387T (11), Py1178T (12), and Py1178T/dl1014 (42) have been described previously.

Subconfluent monolayers were infected at multiplicities of 5 to 10 PFU/cell. Following adsorption of virus, fresh medium containing 1% calf serum was added. For labeling of ribosomes, the fluid was changed in monolayers 22 h postinfection to minimal essential medium lacking phosphate plus 1% calf serum. After 1 h, H₃³²PO₄ (0.25 mCi/60-mm dish) was added, and the cells were labeled for an additional 4 h. For T antigen analysis, infected cells were labeled with ³⁵S translabel (0.1 mCi/ml) in Hanks buffered saline from 22 to 24 h postinfection.

Protein kinase assays in cell-free lysates. Cell-free lysates were prepared and S6 kinase activity was measured as described previously with *Xenopus laevis* 40S ribosomal subunits as the substrate (5). Cell-free extracts were applied to a Mono S column (FPLC system, Pharmacia) in buffer C (20 mM Tris hydrochloride [pH 7.5], 5 mM EGTA [ethylene glycol-bis(β -aminoethyl) ether]-N,N,N',N'-tetraacetic acid], 1 mM EDTA, 10 mM MgCl₂, 10 mM 2-mercaptoethanol, 10% glycerol). Protein was eluted with a 100 to 350 mM NaCl gradient in buffer C, and fractions were assayed for S6 kinase activity. Two peaks of activity (fractions 14 to 18 and 22 to 26) were pooled, dialyzed against buffer C, and applied to a Mono Q column (FPLC system, Pharmacia). Protein was eluted with a 100 to 350 mM NaCl gradient in buffer C, and fractions were assayed for S6 kinase activity.

Ribosomal protein analysis. ³²P-labeled ribosomal proteins were prepared as described (17) and analyzed by electrophoresis on either sodium dodecyl sulfate (SDS)-12.5% polyacrylamide gels or Tris-borate (pH 8.5)-6 M urea-8% polyacrylamide gels as described previously (8, 47). Dried gels were exposed to X-ray film with Du Pont Lightning-Plus intensifying screens. S6 was excised from dried gels and dissolved in H₂O₂-NH₄OH (99:1) at 65°C, and the extent of labeling was quantitated by liquid scintillation spectroscopy.

Polyomavirus tumor antigen analysis. ³⁵S-labeled T antigens were extracted from monolayers and immunoprecipitated with anti-T ascites (44). Immunoprecipitates were separated on SDS-12.5% acrylamide gels and visualized by fluorography following impregnation with Autofluor (National Diagnostics).

TABLE 1. Stimulation of S6 kinase activity by serum and polyomavirus

Stimulant	Expt	Fold stimulation ^a
Calf serum (10%) ^b	1	3
	2	11.4
	3	3.6
	4	4.5
Wild-type virus ^c	1	3
	2	16.7
	3	7.2
	4	5.2

^a Fold stimulation was calculated as the ratio of ³²P transferred from [γ -³²P]ATP to S6 in serum-stimulated or NG59RA-infected lysates to that in lysates from mock-infected NIH 3T3 cells maintained in 1% calf serum.

^b Serum stimulation was for 2 to 4 h prior to preparation of cell-free lysates.

^c Lysates were made from infected cells 24 h postinfection.

RESULTS

Polyomavirus infection stimulates S6 kinase activity. NIH 3T3 cells infected with wild-type polyomavirus, stimulated with 10% calf serum or maintained in 1% calf serum, were assayed for protein kinase activity with *X. laevis* 40S ribosomal subunits as the substrate. Extracts from wild-type virus-infected cells or serum-stimulated cells were on average six- to sevenfold more active in phosphorylation of S6 than were extracts from uninfected cells maintained in low serum (Table 1). The level of activation in each case ranged from 3-fold to greater than 10-fold in four separate experiments.

Chromatographic analysis of serum-stimulated and virus-induced S6 kinase activities. Several enzymes have been shown to phosphorylate S6. Swiss 3T3 mouse fibroblasts contain serum-stimulated activities which fractionate into two peaks following chromatography on Mono S cation-exchange columns. The two peaks coelute from Mono Q anion-exchange columns and cochromatograph with the activity from chicken embryo fibroblasts which is responsive to both serum and pp60^{v-src} (C. K. Kuo and J. Blenis, manuscript in preparation). Extracts from uninfected, uninfected and serum-stimulated, and polyomavirus-infected NIH 3T3 cells were fractionated by chromatography on Mono S and Mono Q columns. Serum-stimulated S6 kinase activity from NIH 3T3 cells eluted as two peaks from the Mono S column (Fig. 1A). S6 kinase activity from polyomavirus-infected cells also eluted as two peaks at the same positions as the two peaks seen following serum stimulation (Fig. 1A). The ratio of activities between peak I and peak II differed between serum-stimulated extracts and virus-infected extracts; the significance of this difference is not known.

When the Mono S peaks were further fractionated on a Mono Q anion-exchange column, the virus- and serum-stimulated activities again coeluted (Fig. 1B and C). Interestingly, both peak I and peak II from the cation-exchange column coeluted as a single peak when rerun on the anion-exchange column, raising the possibility that the chromatographically resolved activities are related enzymes. These results are consistent with the notion that polyomavirus and calf serum are activating the same S6 kinase(s).

Polyomavirus activation of S6 kinase requires wild-type middle T. S6 phosphorylation is responsive to a broad spectrum of mitogenic and transforming agents. The early region of polyomavirus encodes three proteins, each of which has been described as having either mitogenic or

FIG. 1. Chromatographic comparison of S6 kinase in serum-stimulated and polyomavirus-infected cells. Lysates from serum-starved ($\circ$), serum-stimulated ($\blacksquare$), or NG59RA-infected ($\bullet$) cells were applied to a Mono S cation-exchange column in buffer C. Proteins were eluted with a linear, 100 to 350 mM NaCl gradient in buffer C, and fractions were assayed for S6 kinase. The peaks indicated in panel A, peak I (fractions 14 to 18, panel B) and peak II (fractions 22 to 26, panel C), from each Mono S profile were dialyzed against buffer C and applied to Mono Q anion-exchange columns, and protein was eluted with a linear, 100 to 350 mM NaCl gradient. Fractions were assayed for S6 kinase activity.

transforming abilities (13, 29, 35, 41, 43, 49, 53). To investigate the role of middle T antigen in the stimulation of S6 kinase activities, we infected NIH 3T3 cells with mutant polyomaviruses.

The virus mutants used in this study fall into two classes, both altered in the middle T gene but differing in retention of middle T functions (Fig. 2). The first class includes the host range-selected transformation-defective *hr-t* mutant NG59, encoding a defective middle T antigen (3), and the site-directed mutant Py808A, which encodes no middle T (29). The second class includes two additional site-directed mutants encoding altered middle T proteins, Py1387T, which

FIG. 2. Polyomavirus middle T mutants. The mutant middle T's used in this study are shown. The hatched region indicates the hydrophobic stretch necessary for middle T association with the membrane (11). The single-letter amino acid code is used. Stop indicates a translational termination codon. Tf, Ability to induce dense focus formation and growth in soft agar following infection of F111 rat fibroblasts; Growth, ability to grow in *hr-t*-nonpermissive cells, typically NIH 3T3 cells; P-K, ability of the middle T antigen to associate with and activate the tyrosine kinase activity of pp60^{c-src}.

encodes a cytoplasmic form of middle T with a truncated C terminus (11), and Py1178T, in which the middle T has a phenylalanine substituted for tyrosine at position 315 (12). Py1178T/dl1014 has an additional deletion of 3 amino acids, including tyrosine 322 (42). This double mutant is biologically indistinguishable from Py1178T (42) and has been used interchangeably with Py1178T. The mutation in NG59 also affects small T. The other three mutants encode normal small T proteins, although the level of expression of small T in Py808A-infected cells is reduced compared with the wild type (29). All mutants encode functional large T proteins. Of the four mutants, only Py1178T is capable of activating pp60^{c-src} (Talmage et al., in preparation). This mutant transforms cells only very weakly (12) and fails to induce tumors in mice (Talmage et al., in preparation). The other mutants are all transformation defective.

Virus titers were matched by PFU and T antigen expression. Levels of large T were nearly identical in the five viral infections (Fig. 3). Since large T is not functionally altered by any of the mutations, its expression served as an appropriate index of the extent of infection.

NIH 3T3 cells infected with these viruses were maintained in 1% calf serum for 24 h. Extracts were prepared and assayed for their ability to phosphorylate *X. laevis* 40S ribosomal subunits. Extracts from mock-infected cells maintained in 1% serum or stimulated with 10% serum were also assayed. Serum-stimulated and wild-type-infected cells contained the highest levels of active S6 kinase (Fig. 4). Extracts from cells infected by NG59 or Py808A contained little or no active S6 kinase above the level seen in extracts from uninfected cells maintained in 1% serum. Infection by Py1387T or Py1178T/dl1014 gave either no or slight stimulation of S6 kinase activity, never exceeding roughly 30% of the level seen with 10% serum or wild-type virus.

Thus, full activation of S6 kinase by polyomavirus required the expression of wild-type middle T. Neither large T alone (NG59) nor large T and small T together (Py808A, Py1387T, and Py1178T/dl1014) were sufficient to induce this activity. The strongest evidence that a wild-type middle T antigen is essential for induction of S6 kinase activity was

FIG. 3. T-antigen profiles in infected NIH 3T3 cells. NIH 3T3 cells were labeled from 22 to 24 h postinfection with no virus (lane A), NG59 (lane B), Py808A (lane C), Py1387T (lane D), Py1178T (lane E), or NG59RA (lane F). T antigens were immunoprecipitated with anti-T ascites and resolved on an SDS-12.5% polyacrylamide gel. Large T (LT), middle T (mT), and small T (sT) were visualized by fluorography, and their positions are indicated. The middle T antigen of Py1387T migrated more rapidly than the wild type, consistent with its smaller size.

the inability of Py1178T/dl1014 to appreciably activate S6 kinase, since the middle T antigen encoded by this virus is capable of interacting with and activating the tyrosine kinase activity of pp60^{c-src} (Talmage et al., in preparation). The minimal stimulation of S6 kinase activity in cells infected by Py1178T/dl1014 or Py1387T most likely results from their mutant middle T's, although it might also reflect elevated expression of small T relative to Py808A (Fig. 4) (29).

FIG. 4. S6 kinase activity in polyomavirus-infected cells. S6 kinase activity in cell-free lysates of NIH 3T3 cells was measured by using 40S ribosomal subunits prepared from *X. laevis* oocytes. Lysates were prepared and assayed from mock-infected cells maintained in 1% serum (lane A), mock-infected cells stimulated for 4 h with 10% calf serum (lane B), or cells infected with NG59RA (lane C), NG59 (lane D), Py808A (lane E), Py1387T (lane F), or Py1178T/dl1014 (lane G). Phosphorylated S6 (indicated) was visualized by autoradiography.

FIG. 5. Stimulation of S6 phosphorylation in polyomavirus-infected cells. Acid-soluble ribosomal proteins were isolated from ³²P-labeled NIH 3T3 cells and resolved on a 12.5% polyacrylamide gel. Cells were labeled in 1% calf serum (lane A), 10% calf serum (lane B), or 1% calf serum from 22 to 26 h postinfection with NG59RA (lane C), NG59 (lane D), Py808A (lane E), Py1387T (lane F), or Py1178T (lane G). Phosphoproteins were visualized by autoradiography. The positions of phosphorylated S6 are indicated.

Polyomavirus middle T mutants elevate S6 phosphorylation in vivo without activating the serum-induced S6 kinase. Stimulation of S6 kinase activity in cells should be paralleled by an increase in S6 phosphorylation in vivo. This is clearly the case in cells stimulated with serum, 12-*O*-tetradecanoylphorbol-13-acetate, or epidermal growth factor or in cells transformed by pp60^{v-src} (4, 47, 48). We examined the phosphorylation state of S6 in cells infected by wild-type polyomavirus and the mutants described above. Infected cells were maintained in 1% calf serum for 22 h and then labeled with ³²PO₄ for 4 h. For comparison, uninfected cells were also labeled for 4 h in the presence of either 1 or 10% calf serum. ³²P-labeled, acid-soluble ribosomal proteins were resolved by SDS-polyacrylamide gel electrophoresis. Following infection with wild type, Py1178T, or Py1387T, S6 phosphorylation was equal to or greater than that seen in serum-stimulated, uninfected cells (Fig. 5). Infection by NG59 or Py808A, however, failed to induce this elevated level of S6 phosphorylation, although incorporation of ³²P into S6 was elevated slightly over levels in uninfected cells grown and labeled in low serum.

The increased S6 phosphorylation seen in cells infected with Py1178T or Py1387T resulted from multiple phosphorylations. This was seen by the decreased mobility on SDS-polyacrylamide gels of phosphorylated S6 from cells infected with these viruses (as well as from serum-stimulated cells or NG59RA-infected cells) compared with S6 from unstimulated cells or cells infected with NG59 or Py808A (Fig. 5). Electrophoresis in Tris-borate-urea gels indicated that up to 4 phosphates were associated with S6 in cells stimulated with serum or infected by Py1178T or wild type, but not with S6 from unstimulated cells or cells infected with NG59 or Py808A (Fig. 6).

DISCUSSION

The major effects on S6 phosphorylation following polyomavirus infection are mediated by middle T antigen. Our

FIG. 6. S6 is multiply phosphorylated. 32 P-labeled, acid-soluble ribosomal proteins were separated on a Tris-borate gel and visualized by autoradiography. The hatch marks indicate the various phosphorylated forms of S6 from cells labeled in 1% calf serum (lane A) or 10% calf serum (lane B) or infected by NG59 (lane C), Py808A (lane D), Py1178T (lane E), or NG59RA (lane F). Electrophoresis was towards the cathode (+ to -) at 175 V for 20 h. All lanes are from the same gel. Exposure time was 18 h at -70°C with an intensifying screen.

results point to two distinct pathways by which middle T antigen elevates the level of S6 phosphorylation: one requiring the activation of $\text{pp60}^{\text{c-src}}$ and leading to stimulation of S6 kinase activity, the other requiring activation of neither of these kinases. S6 kinase activity is stimulated about sixfold by wild-type middle T antigen. The S6 kinase activity elution profiles from both cation- and anion-exchange columns were similar between serum-stimulated cells and polyomavirus-infected cells. The only difference seen was in the relative amounts of activity in each of the two peaks resolved by cation-exchange chromatography. The significance of this difference is not clear but suggests that serum and middle T might differentially activate two S6 kinases. In chicken embryo fibroblasts, the S6 kinase which is activated by calf serum is chromatographically similar to the enzyme(s) activated by the oncogene product $\text{pp60}^{\text{v-src}}$, phorbol esters (6), or calcium (Kuo and Blenis, in preparation). Thus, activation of $\text{pp60}^{\text{c-src}}$, either by mutation as in $\text{pp60}^{\text{v-src}}$ or by protein-protein interaction as in $\text{pp60}^{\text{c-src}}$ -middle T complexes, results in functional alterations of $\text{pp60}^{\text{c-src}}$ leading to S6 kinase induction in chicken and mouse cells.

The mechanisms by which middle T activates S6 kinases are not known. It is likely that this occurs as the result of second messengers generated at the plasma membrane. Immune complexes containing $\text{pp60}^{\text{v-src}}$ or $\text{pp60}^{\text{c-src}}$ and middle T also contain a PI kinase activity, while immune complexes containing normal $\text{pp60}^{\text{c-src}}$ do not (45, 51). This led to the suggestion that a primary function of oncogenic activation of $\text{pp60}^{\text{c-src}}$ is the activation of this PI kinase. The outcome of this proposed activation is predicted to be an increase in PI turnover, generating diacylglycerol and inositol trisphosphate. Addition to cells of phorbol esters (5) that mimic diacylglycerol by activating protein kinase C or of Ca^{2+} (Kuo and Blenis, in preparation) leads to activation of the chicken S6 kinase(s). It is reasonable to propose that middle T, through its interaction with $\text{pp60}^{\text{c-src}}$, activates S6 kinase by generating one or both of these second messengers.

This suggestion is strengthened by results with the mutant middle T encoded by Py1178T. Polyomavirus middle T antigens lacking tyrosine 315, a major site of phosphorylation by $\text{pp60}^{\text{c-src}}$ in immune complexes, have impaired transforming potential (12, 42). The defect is not the result of the mutant middle T's inability to activate $\text{pp60}^{\text{c-src}}$ as a tyrosine kinase, since these complexes phosphorylate enolase essentially as well as those containing wild-type middle

T (Talmage et al., in preparation). Since the mutants lacking tyrosine 315 do not stimulate S6 kinase activity to the level seen following serum stimulation or following infection with wild-type polyomavirus and are indeed no more effective than Py1387T middle T, which fails to activate $\text{pp60}^{\text{c-src}}$ in any detectable way, activation of $\text{pp60}^{\text{c-src}}$'s intrinsic tyrosine kinase alone is not sufficient for S6 kinase activation. We have shown that although Py1178T or Py1178T/dl1014 middle T activates $\text{pp60}^{\text{c-src}}$, immune complexes containing these proteins do not contain appreciable levels of PI kinase (Talmage et al., in preparation). Based on these observations, it is reasonable to conclude that it is the generation of the PI metabolites diacylglycerol and inositol trisphosphate that leads to S6 kinase activation.

Results with Py1387T, Py1178T, and NG59RA point to a second pathway by which middle T induces S6 phosphorylation. Increases in S6 phosphorylation were found that did not correlate with elevated levels of S6 kinase activity. The increased levels of S6 phosphorylation induced by these viruses appear to be selective and are not simply a reflection of differences in total cellular protein phosphorylation, since the total acid-precipitable incorporation varied little from sample to sample and the level of phosphorylation of other cellular proteins, in particular histones H1, H2a, and H3, remained the same regardless of the infecting virus or serum treatment.

Although the cellular components of this second pathway remain obscure, the possibility that middle T can act to stabilize the phosphates associated with S6 is supported by the results of preliminary experiments involving *in vivo* labeling of ribosomes with $^{32}\text{PO}_4$. Andres et al. (1) recently reported that phosphorylated S6 is a substrate for both phosphatases 1 and 2B. In light of earlier data from *in vitro* trials showing inhibition of phosphatase 1 following tyrosine phosphorylation by $\text{pp60}^{\text{v-src}}$ (25), it is intriguing to speculate that the second pathway involves regulation of protein phosphatase activity. Mutant (Py1387T and Py1178T) middle T's, as well as wild-type middle T, might act to inhibit a protein phosphatase normally active against S6. The slight and variable elevation of S6 kinase activity in Py1387T-infected cells may also result from such an inhibition; it has been suggested by others that regulation of S6 kinase activity involves phosphorylation and dephosphorylation of the enzyme (2, 24, 32, 37).

The viral mutants NG59 and Py808A, which encode normal large T antigen and, in the case of Py808A, an apparently normal small T antigen, induced minor yet reproducible increases in S6 phosphorylation. This induction was distinct from that seen with the other viruses in that it occurred on only one or two sites and was not stable. This low-level induction could result either from virus capsid binding to cellular receptors or from the expression of large T antigen, both of which have been shown to be mitogenic (43, 53). With respect to the latter possibility, Bockus and Schaffhausen (9) have also suggested that large T induces nuclear phosphorylations which are also responsive to the growth state of infected cells.

It is somewhat surprising that Py808A was no more effective than NG59 at inducing S6 phosphorylation, since the small T encoded by Py808A has been shown to contribute to stimulation of cell growth in low serum (29) and to increase the amount of viral DNA synthesized (viral DNA replication is dependent on host enzymes and proteins expressed primarily in S phase), as well as to induce the continued expression of *c-myc* and *c-fos* mRNAs (53). Small T alone, when expressed in NIH 3T3 cells, also has positive

effects on cell growth (13, 35). This suggests that the mitogenic potential of small T acts through a pathway independent of S6 phosphorylation; it may in fact be acting directly in the nucleus of infected cells (35).

The role of middle T and its interactions with cellular components in normal productive infections remains poorly understood. It is interesting, however, that the ability of various middle T mutants to increase S6 phosphorylation via this proposed second pathway parallels the ability of the same mutants to induce cellular permissivity factors for growth (22). The growth defect of *hr-t* mutants on NIH 3T3 cells has been associated with a reduction in phosphorylation of the major capsid protein, VP1 (20). The middle T's from both Py1178T and Py1387T induce permissivity factors in NIH 3T3 cells and consequently wild-type levels of phosphorylated VP1 (Garcea et al., in preparation). Since these mutants have little effect on the activity of S6 kinase, this enzyme does not appear to be the VP1 kinase. This is consistent with the inability of partially purified S6 kinase to phosphorylate VP1 in vitro (7). Thus, the stabilization of VP1 phosphorylation may occur as a reflection of the same mechanism that leads to increased S6 phosphorylation via the pp60^{c-src}-independent pathway. An important implication of these results that needs further study is that regulation of permissivity factors by middle T may occur via a cellular phosphoprotein phosphatase.

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LITERATURE CITED

- Andres, J. L., J. W. Johansen, and J. L. Maller. 1987. Identification of protein phosphatases 1 and 2B as ribosomal protein S6 phosphatases in vitro and in vivo. *J. Biol. Chem.* **262**:14389-14393.
- Ballou, L. M., P. Jenö, and G. Thomas. 1988. Protein phosphatase 2A inactivates the mitogen-stimulated S6 kinase from Swiss mouse 3T3 cells. *J. Biol. Chem.* **263**:1188-1194.
- Benjamin, T. L. 1970. Host range mutants of polyoma virus. *Proc. Natl. Acad. Sci. USA* **67**:394-399.
- 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.
- Blenis, J., and R. L. Erikson. 1985. Regulation of a ribosomal protein S6 kinase by the Rous sarcoma virus transforming protein, serum growth factors, or phorbol ester. *Proc. Natl. Acad. Sci. USA* **82**:7621-7625.
- Blenis, J., and R. L. Erikson. 1986. Stimulation of ribosomal protein S6 kinase activity by pp60^{v-src} or by serum: dissociation from phorbol ester-stimulated activity. *Proc. Natl. Acad. Sci. USA* **83**:1733-1737.
- Blenis, J., C. J. Kuo, and R. L. Erikson. 1987. Identification of a ribosomal protein S6 kinase regulated by transformation and growth-promoting stimuli. *J. Biol. Chem.* **262**:14373-14376.
- Blenis, J., J. G. Spivak, and R. L. Erikson. 1984. Phorbol ester, serum and Rous sarcoma virus transforming gene product induce similar phosphorylations of ribosomal protein S6. *Proc. Natl. Acad. Sci. USA* **81**:6408-6412.
- Bockus, B., and B. Schaffhausen. 1987. Phosphorylation of polyomavirus large T antigen: effects of viral mutations and cell growth state. *J. Virol.* **61**:1147-1154.
- Bolen, J. B., C. J. Thiele, M. A. Israel, W. Yonemoto, L. A. Lipsich, and J. S. Brugge. 1984. Enhancement of cellular *src* gene product associated tyrosine kinase activity following polyoma virus infection and transformation. *Cell* **38**:767-777.
- Carmichael, G., B. S. Schaffhausen, D. Dorskey, D. Oliver, and T. L. Benjamin. 1982. Carboxy terminus of polyoma virus 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.
- 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.
- Cheriton, V., B. Morgan, B. M. Spiegelman, and T. M. Roberts. 1986. Recombinant retroviruses that transduce individual polyoma tumor antigens: effects on growth and differentiation. *Proc. Natl. Acad. Sci. USA* **83**:4307-4311.
- Courtneidge, S. A. 1985. Activation of the pp60^{c-src} kinase by middle T antigen binding or by dephosphorylation. *EMBO J.* **4**:1471-1477.
- 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.
- Courtneidge, S. A., and A. E. Smith. 1984. The complex of polyoma virus middle T antigen and pp60^{c-src}. *EMBO J.* **3**:585-591.
- Decker, S. 1981. Phosphorylation of ribosomal protein S6 in avian sarcoma virus-transformed chicken embryo fibroblasts. *Proc. Natl. Acad. Sci. USA* **78**:4112-4115.
- Feunteun, J., L. Sompayrac, M. Fluck, and T. L. Benjamin. 1976. Localization of gene functions in polyoma virus DNA. *Proc. Natl. Acad. Sci. USA* **73**:4169-4173.
- Garcea, R. L., and T. L. Benjamin. 1983. Host range transforming gene of polyoma virus plays a role in virus assembly. *Proc. Natl. Acad. Sci. USA* **80**:3613-3617.
- Garcea, R. L., K. Ballmer-Hofer, and T. L. Benjamin. 1985. Virion assembly defect of polyomavirus *hr-t* mutants: underphosphorylation of major capsid protein VP1 before viral DNA encapsulation. *J. Virol.* **54**:311-316.
- Glover, C. V. C. 1982. Heat shock induces rapid dephosphorylation of a ribosomal protein in *Drosophila*. *Proc. Natl. Acad. Sci. USA* **79**:1781-1785.
- Goldman, E., and T. L. Benjamin. 1975. Analysis of host range of nontransforming polyoma virus mutants. *Virology* **66**:372-384.
- Hasselbacher, G. K., R. E. Humbel, and G. Thomas. 1979. Insulin like growth factor, insulin or serum increase phosphorylation of ribosomal protein S6 during transition of stationary chick embryo fibroblasts into early G1 phase of the cell cycle. *FEBS Lett.* **100**:185-190.
- Jenö, P., L. M. Ballou, I. Novak-Hofer, and G. Thomas. 1988. Identification and characterization of a mitogen-activated S6 kinase. *Proc. Natl. Acad. Sci. USA* **85**:406-410.
- Johansen, J. W., and T. S. Ingebritsen. 1986. Phosphorylation and inactivation of protein phosphatase 1 by pp60^{v-src}. *Proc. Natl. Acad. Sci. USA* **83**:207-211.
- Johnson, R. A., and T. F. Walseth. 1979. The enzymatic preparation of [α -³²P]ATP, [α -³²P]GTP, [³²P]cAMP, and [³²P]cGMP and their use in the assay of adenylate cyclase and guanylate cyclase and cyclic nucleotide phosphodiesterases. *Adv. Cyclic Nucleotide Res.* **10**:135-167.
- Kaplan, D. R., M. Whitman, B. Schaffhausen, L. Raptis, R. L. Garcea, D. Pallas, T. M. Roberts, and L. Cantley. 1986. Phosphatidylinositol metabolism and polyoma-mediated transformation. *Proc. Natl. Acad. Sci. USA* **83**:3624-3628.
- Kennedy, I. M., and D. P. Leader. 1981. Increased phosphorylation of ribosomal protein S6 in hamster fibroblasts transformed by polyoma virus and simian virus 40. *Biochem. J.* **198**:235-237.
- Liang, T. J., G. C. Carmichael, and T. L. Benjamin. 1984. A polyomavirus 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.
- Maller, J. L., J. G. Foulkes, E. Erikson, and D. Baltimore. 1985. Phosphorylation of ribosomal protein S6 on serine after microinjection of the Abelson murine leukemia virus tyrosine-specific

- protein kinase into *Xenopus* oocytes. *Proc. Natl. Acad. Sci. USA* **82**:272–276.
31. **Martin-Perez, J., M. Siegmann, and G. Thomas.** 1984. EGF, PGF₂ and insulin induce the phosphorylation of identical S6 peptides in Swiss mouse 3T3 cells: effect of cAMP on early sites of phosphorylation. *Cell* **36**:287–294.
32. **Matsuda, Y., and G. Guroff.** 1987. Purification and mechanism of activation of a nerve growth factor-sensitive S6 kinase from PC12 cells. *J. Biol. Chem.* **262**:2832–2844.
33. **Nielsen, P. J., G. Thomas, and J. L. Maller.** 1982. Increased phosphorylation of ribosomal protein S6 during meiotic maturation of *Xenopus* oocytes. *Proc. Natl. Acad. Sci. USA* **79**:2937–2941.
34. **Nishimura, J., and T. F. Deuel.** 1983. Platelet derived growth factor stimulates the phosphorylation of ribosomal protein S6. *FEBS Lett.* **156**:130–134.
35. **Noda, T., M. Satake, T. Robins, and Y. Ito.** 1986. Isolation and characterization of NIH 3T3 cells expressing polyomavirus small t antigen. *J. Virol.* **60**:105–113.
36. **Novak-Hofer, I., and G. Thomas.** 1984. An activated S6 kinase in extracts from serum and epidermal growth factor stimulated Swiss 3T3 cells. *J. Biol. Chem.* **259**:5995–6000.
37. **Novak-Hofer, I., and G. Thomas.** 1985. Epidermal growth factor mediated activation of an S6 kinase in Swiss mouse 3T3 cells. *J. Biol. Chem.* **260**:10314–10319.
38. **Pelech, S. L., B. B. Olwin, and E. G. Krebs.** 1986. Fibroblast growth factor treatment of Swiss 3T3 cells activates a subunit S6 kinase that phosphorylates a synthetic peptide substrate. *Proc. Natl. Acad. Sci. USA* **83**:5968–5972.
39. **Perisic, O., and J. A. Traugh.** 1983. Protease-activated kinase II as the potential mediator of insulin-stimulated phosphorylation of ribosomal protein S6. *J. Biol. Chem.* **258**:9589–9592.
40. **Ralston, R., and J. M. Bishop.** 1985. The product of the protooncogene *c-src* is modified during the cellular response to platelet-derived growth factor. *Proc. Natl. Acad. Sci. USA* **82**:7845–7849.
41. **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**:2476–2485.
42. **Schaffhausen, B. S., T. J. Liang, G. G. Carmichael, and T. L. Benjamin.** 1985. Residual transforming activity of Py1178T, a mutant lacking the principal in vitro tyrosine phosphorylation site, is not affected by removal of the secondary tyrosine phosphorylation site at residue 322. *Virology* **143**:671–675.
43. **Schlegel, R., and T. L. Benjamin.** 1978. Cellular alterations dependent upon the polyoma virus hr-t function: separation of mitogenic from transforming capacities. *Cell* **14**:587–599.
44. **Silver, J., B. S. Schaffhausen, and T. L. Benjamin.** 1978. Tumor antigens induced by nontransforming mutants of polyoma virus. *Cell* **15**:485–496.
45. **Sugimoto, Y., M. Whitman, L. C. Cantley, and R. L. Erikson.** 1984. Evidence that the Rous sarcoma virus transforming gene product phosphorylates phosphatidylinositol and diacylglycerol. *Proc. Natl. Acad. Sci. USA* **81**:2117–2121.
46. **Tabarini, D., J. Heinrich, and O. M. Rosen.** 1985. Activation of S6 kinase activity in 3T3-L1 cells by insulin and phorbol ester. *Proc. Natl. Acad. Sci. USA* **82**:4369–4373.
47. **Thomas, G., J. Martin-Perez, M. Siegmman, and A. M. Otto.** 1982. The effect of serum, EGF, PGF₂ and insulin on S6 phosphorylation and the initiation of protein and DNA synthesis. *Cell* **30**:235–242.
48. **Thomas, G., M. Siegmman, A. Kubler, J. Gordon, and L. Jimenez de Asua.** 1980. Regulation of 40s ribosomal protein S6 phosphorylation in Swiss 3T3 cells. *Cell* **19**:1015–1023.
49. **Treisman, R., U. Novak, J. Favalaro, and R. Kamen.** 1981. Transformation of rat cells by an altered polyoma virus genome expressing only the middle T protein. *Nature (London)* **292**:595–600.
50. **Trevillyan, J. M., O. Peresic, J. A. Traugh, and C. V. Byus.** 1985. Insulin- and phorbol ester-stimulated phosphorylation of ribosomal protein S6. *J. Biol. Chem.* **260**:3041–3044.
51. **Whitman, M., D. R. Kaplan, B. Schaffhausen, L. Cantley, and T. M. Roberts.** 1985. Association of phosphatidylinositol kinase activity with polyoma middle T competent for transformation. *Nature (London)* **315**:239–242.
52. **Winocour, E.** 1963. Purification of polyomavirus. *Virology* **19**:158–168.
53. **Zullo, J., C. D. Stiles, and R. L. Garcea.** 1987. Regulation of *c-myc* and *c-fos* mRNA levels by polyomavirus: distinct roles for the capsid protein VP1 and the viral early proteins. *Proc. Natl. Acad. Sci. USA* **84**:1210–1214.