Identification of the Threonine Phosphorylation Sites on the Polyomavirus Major Capsid Protein VP1: Relationship to the Activity of Middle T Antigen

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Phosphorylation of the polyomavirus major capsid protein VP1 was examined after in vivo ^{32}P labeling of virus-infected cells. Two phosphorylated peptide fragments of VP1 were identified by protease digestion, high-performance liquid chromatography purification, mass spectrometry, and N-terminal sequencing. The peptides from residues 58 to 78 and residues 153 to 173 were phosphorylated on threonine. Site-directed mutations were introduced at these threonine sites, and mutant viruses were reconstructed. A threonine-to-glycine change at residue 63 (mutant G63) and a threonine-to-alanine change at residue 156 (mutant A156) resulted in viruses defective in phosphorylation of the respective peptides after in vivo labeling. Growth of the mutant G63 virus was similar to that of the wild-type virus, but the mutant A156 was inefficient in assembly of 240S viral particles. Polyomavirus nontransforming host range (*hr-t*) mutants are defective in VP1 threonine phosphorylation when grown in nonpermissive cells (R. L. Garcea, K. Ballmer-Hofer, and T. L. Benjamin, J. Virol. 54:311–316, 1985). Proteolytic mapping of VP1 peptides after in vivo labeling from *hr-t* mutant virus infections demonstrated that both residues T-63 and T-156 were affected. These results suggest that the block in virion assembly in *hr-t* mutant viruses is associated with a defect in phosphorylation of threonine 156.

Polyomavirus has a nonenveloped capsid consisting of 72 pentamers of the major capsid protein VP1 arranged in a $T=7d$ icosahedral lattice (29). The virion also contains the two minor capsid proteins VP2 and VP3, which may serve to bridge the viral minichromosome with the VP1 capsid (18). In virus-infected cells, VP1 is posttranslationally modified by phosphorylation on both threonine and serine residues (2, 15). These modifications occur rapidly and probably precede virion assembly (10, 15). In addition to its role as a structural protein, VP1 is also involved in cell surface receptor recognition; the hemagglutination properties are determined by VP1 alone (6, 31), and a major determinant of “high tumor” (inducing a high incidence of tumors) versus “low tumor” (inducing a low incidence of tumors) virus strains has been localized to a point mutation in VP1 (12, 13).

The nontransforming host range (*hr-t*) mutant polyomaviruses (4) are defective in cell transformation in vitro and tumor induction in vivo and have a defect in virion assembly when infecting nonpermissive cells that is associated with underphosphorylation of VP1 on threonine (15, 16). The assembly and VP1 phosphorylation defects are related to the properties of the middle T antigen (17). However, if there is a direct association between VP1 phosphorylation and virus assembly, the mechanism may be complex. The unmodified recombinant VP1 protein purified after expression in *Escherichia coli* is capable of in vitro self-assembly into capsid-like aggregates (30). VP1 expressed in insect cells by using a recombinant baculovirus vector also assembles into capsids, which are exclusively localized within the nucleus (11, 27). Thus, VP1 intrinsically contains the information necessary for capsid

assembly in the correct subcellular compartment independent of VP2 and VP3, the viral genome, or threonine phosphorylation. However, the unmodified recombinant VP1 protein is unable to encapsidate viral DNA in vitro, and the particles produced in insect cells do not contain nucleic acid (27, 28).

The crystal structure at a 3.8-Å (1 Å = 0.1 nm) resolution of the related primate polyomavirus simian virus 40 has been determined (24), and, as inferred by primary sequence comparison, the structure of polyomavirus must be closely related. Electron density corresponding to phosphorylated residues has not yet been clearly identified in this structure, perhaps because only one or two VP1 proteins within a pentamer are similarly modified (5). In order to understand the function of VP1 phosphorylation in the polyomavirus life cycle, we have studied the in vivo sites of threonine phosphorylation by proteolytic mapping and reconstruction of mutant viruses. Two sites have been identified, both localized to loop domains on the virion exterior, and mutation of one site results in a phenotype similar to the assembly defect seen in *hr-t* mutant virus infection.

MATERIALS AND METHODS

Cells and viruses. Infections with virus were performed with A31 mouse fibroblasts at approximately 50% confluence. Cell culture techniques have been previously described (17). The wild-type virus strain used was NG59RA. The *hr-t* mutant virus strains NG59 and NG18 (3, 8, 19) and the site-directed mutant viruses 1387T and 808A have also been described previously (9, 23).

In vivo [^{32}P]orthophosphate labeling and VP1 immunoprecipitation. Cells were grown in 100-mm-diameter plates and labeled with 2 mCi of [^{32}P]orthophosphate in 1 ml of phosphate-free Dulbecco's modified Eagle's medium with 2% dialyzed calf serum. Labeling of cells was carried out for 4 h

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between 24 and 28 h postinfection with wild-type virus or between 36 and 40 h postinfection with *hr-t* mutant virus. VP1 was immunoprecipitated with a rabbit anti-VP1 polyclonal antiserum (generated against recombinant VP1 protein, which recognizes all modified forms of VP1) and protein A-Sepharose CL-4B (Pharmacia) as described previously (27, 28).

Protease digestion. Immunoprecipitated VP1 was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE [7.5% polyacrylamide]) and electroblotted to nitrocellulose in CAPS buffer (10 mM 3-[cyclohexylamino]-1-propanesulfonic acid, 10% methanol [pH 11.0]) (1). Proteins were stained with 0.1% Ponceau S in 1% acetic acid for 5 min. Blots were destained in 1% acetic acid and washed with water. The VP1 band (approximately 3 to 5 μ g of protein by comparison with recombinant VP1 protein standards) was excised and incubated at 37°C for 30 min in 0.5% polyvinylpyrrolidone 40 dissolved in 100 mM acetic acid. For analyses of 32 P-labeled protein, typically one labeled gel band was combined with four unlabeled bands for digestion. Excess polyvinylpyrrolidone 40 was removed by washing with water five times, and the nitrocellulose paper was then cut into small pieces. Trypsin, endoproteinase Lys-C, and endoproteinase Asp-N were used to digest VP1 on the nitrocellulose. The following buffers were used for digestion: (i) trypsin–100 mM NaHCO₂ (pH 8.2), (ii) endoproteinase Lys-C–50 mM Tricine (pH 8.0)–10 mM EDTA, and (iii) endoproteinase Asp-N–10 mM Tris-HCl (pH 7.5). The ratio of enzyme to VP1 was approximately 1:20 (wt/wt). The reactions were performed in the solution of enzyme buffer-acetonitrile (95:5 [vol/vol]) at 37°C overnight. The nitrocellulose was then pelleted at 10,000 $\times$ g for 5 min, and the supernatant was lyophilized with a Speed-Vac concentrator.

Peptide analysis. The enzymatically cleaved peptides were dissolved in 0.1% trifluoroacetic acid and centrifuged for 5 min at 10,000 $\times$ g. The supernatant was then analyzed by reverse-phase high-performance liquid chromatography (HPLC) with C₄ and C₁₈ columns. The peptides were separated on a C₁₈ column with a linear gradient from 0 to 70% acetonitrile in 0.1% trifluoroacetic acid applied with a flow rate of 200 μ l/min over 60 min. The same flow rate with a gradient from 0 to 55% acetonitrile was used for a C₄ column over a 45-min period. The fractions were collected every 0.5 to 1 min. Phosphorylated peptides were detected by scintillation counting. The amino-terminal 8 to 10 amino acids of the 32 P-labeled peptide fragments were sequenced with an Applied Biosystems model 470A gas phase sequencer (21). The molecular weights of the peptide fragments were determined with a Lasermat mass spectrometer (Finnigan Mat Ltd., Hemel Hempstead, United Kingdom).

Phosphoamino acid analysis. VP1 protein was isolated by immunoprecipitation and SDS-PAGE as described above. Proteins were electroblotted to Immobilon-P membrane with CAPS transfer buffer (22). VP1 was stained with 0.1% Coomassie blue R-250 in 50% methanol for 5 min and destained with 50% methanol–10% acetic acid several times. The membrane was rinsed with water, cut into small pieces, and completely immersed in 5.7 N HCl. After purging with nitrogen, VP1 was hydrolyzed at 110°C for 2 h. The membrane was pelleted at 10,000 $\times$ g for 1 min. The supernatant was then lyophilized in a Speed-Vac concentrator. The phosphorylated amino acids were detected by one-dimensional thin-layer electrophoresis. Peptides were first dissolved in 5 μ l of buffer (7.5% acetic acid, 2.5% formic acid, 0.1% Orange G, 1 μ g of phosphoserine and phosphothreonine per μ l) and spotted onto Cel 300 thin-layer chromatography (TLC) plates (Alltech). Electrophoresis was performed in a running buffer (7.5%

acetic acid, 2.5% formic acid [pH 1.9]) for 60 min at 1,200 V. Phosphoamino acids were stained by being sprayed with 0.1% ninhydrin in acetone and were developed at 80°C. The plate was exposed to Kodak film at –70°C. The ratios of phosphoamino acids were compared by densitometry of the autoradiogram with a Hewlett-Packard Scan Jet Plus scanner and Macintosh Image 1.12c software.

Peptide mapping. VP1 protein was electroblotted to nitrocellulose and digested with trypsin as described above. The peptide fragments were dissolved in electrophoresis buffer (7.5% acetic acid, 2.5% formic acid) and spotted onto a Cel 300 TLC plate. The first-dimension electrophoresis was performed as described above for phosphoamino acid analysis. The second dimension was TLC in 38% *n*-butanol–25% pyridine–7.5% acetic acid and was orthogonal to the first dimension. The chromatography was stopped when the tracking dye had migrated 2 cm from the top. Phosphorylated peptides were detected by autoradiography.

Site-directed mutagenesis and construction of mutant viruses. Mutations were introduced into the VP1 coding region of polyomavirus with the Bio-Rad Muta-Gene M13 in vitro mutagenesis kit. The large *EcoRI*–*Bam*HI fragment of polyomavirus containing the VP1 sequence was cloned into M13mp19. A threonine-to-glycine mutation was introduced at residues 63 and 68 of VP1 with the 25-mers GGGCAGCCAC CCGCCCTGAAAGCC and CCTGAAAGCCTAGCAGAG GGAGGGC. A threonine-to-alanine mutation was introduced at residue 156 of VP1 with a 26-mer, GGAATTTCCGGTC CAGTGGAAGGCAG. The mutated VP1 sequence was verified by dideoxynucleotide chain termination sequencing with the Sequenase System (U.S. Biochemical Corp.). Mutant polyomavirus genomes were reconstructed by ligating the mutated large *EcoRI*–*Bam*HI fragment with the small *EcoRI*–*Bam*HI fragment derived from viral DNA. The reconstructed polyomavirus DNA was transfected into A31 cells by using the DEAE-dextran method (26). Virus lysates were obtained by subculturing the transfected cells every 3 to 4 days until an extensive cytopathic effect developed. Viral DNA was isolated by the method of Hirt (20) and purified with a GeneClean II Kit (Bio 101). Viruses were sequenced around the mutagenized site after isolation to verify the change.

Preparation of virion intermediates. A31 cells were infected with wild-type or mutant viruses in 100-mm-diameter plates with 50% confluence. At 24 h postinfection, cells were labeled with 30 μ Ci of [3 H]thymidine until 40 h postinfection. The polyomavirus virion intermediates were prepared as previously described (16, 17).

RESULTS

Identification of phosphothreonine peptide fragments. The sites of phosphorylation on VP1 were determined by proteolytic digestion and N-terminal sequencing of the cleaved peptide products. Recombinant VP1 protein, purified after expression in *E. coli*, was used to optimize enzymatic digestions and reverse-phase HPLC elution conditions. Recovery of cleaved products after *in situ* protease digestion with nitrocellulose (80%) was superior to that with Immobilon-P membranes (40%). The cleavage peptide products obtained after *in situ* digestion with VP1 on the nitrocellulose blot were first analyzed by reverse-phase HPLC with a C₁₈ column (Fig. 1A). A major phosphopeptide peak was found in the 30.5- to 31.0-min fraction and a minor peak was found in the 36.5- to 37.0-min fraction (Fig. 1C). N-terminal sequence analysis of these peptides demonstrated that the peptide obtained from the 36.5- to 37.0-min fraction began at residue 153 and the

FIG. 1. HPLC analysis of phosphorylated peptide fragments of the polyomavirus major capsid protein VP1. Wild-type polyomavirus-infected A31 cells were labeled with [^{32}P]orthophosphate, and tryptic cleavage peptides were fractionated with reverse-phase HPLC. Phosphorylated peptide fragments were detected by scintillation counting. (A) Tryptic peptides separated by C_{18} column chromatography. (B) Peptides from the 30.0- to 32.0-min fractions of the C_{18} column rechromatographed on a C_4 column. (C) Phosphorylated peptide profile of peptides shown in panel A. (D) Phosphorylated peptide profile of peptides shown in panel B. mAU, milli-absorbancy units.

dominant sequence from the 30.5- to 31.0-min fraction began at residue 58 (Table 1 [see Fig. 3]). In order to verify the identification of the peptide in the 30.5- to 31.0-min fraction, four fractions (30.0 to 32.0 min) from the C_{18} column were pooled and refractionated with a C_4 column (Fig. 1B). The results of this analysis revealed that the 23.0- to 23.5-min fraction from this column contained a phosphopeptide (Fig. 1D) whose sequence was identical to the sequence from the 30.5- to 31.0-min fraction of the C_{18} column (Table 1).

Previous phosphoamino acid analysis of polyomavirus VP1 found that phosphorylation of VP1 occurs on both serine and threonine residues (2, 15). When phosphoamino acid analysis of the phosphopeptides obtained by HPLC was performed (Fig. 2B, lane 1), the major phosphopeptide in the 30.5- to

31.0-min fraction of the C_{18} column contained predominately phosphothreonine, with some phosphoserine (Fig. 2B, lane 2). The phosphopeptides in the 23.0- to 23.5-min fraction of the C_4 column (Fig. 2B, lane 4) and the minor phosphopeptide in the 36.5- to 37.0-min fraction from the C_{18} column contained only phosphothreonine (Fig. 2B, lane 3).

VP1 phosphopeptides were also analyzed by two-dimensional TLC. A major (spot 2) and two minor (spots 1 and 3) phosphopeptides were detected (Fig. 2A). To compare the two-dimensional TLC profiles of ^{32}P -labeled VP1 with that from the HPLC analysis, the HPLC fractions were subjected to two-dimensional analysis. Two-dimensional mapping of the HPLC peptide fractions showed that the phosphopeptide eluting in the 36.5- to 37.0-min fraction migrated to a position

TABLE 1. Identification of phosphorylated peptides by N-terminal sequencing and mass spectrometry

Enzyme	HPLC		N-terminal sequence ^a	Mass (calculated mass [Da]) ^b	Sequence of cleavage peptide (positions) ^c
	Reverse-phase column	Eluted fraction (min)			
Trypsin	C_{18}	30.5–31.0	MGQ	2,367 (2,341.5)	58–78
	C_{18}	36.5–37.0	GIS	3,218 (3,215.5)	153–183
	C_4	23.0–23.5	MGQ	2,361 (2,341.5)	58–78
Asp-N	C_{18}	39.0–39.5	DLV	5,116 (5,071.6)	39–85
	C_{18}	33.0–33.5	DTV	2,815 (2,803.1)	147–173
Lys-C	C_{18}	35.5–36.5	GIS	3,435 (3,442.8)	153–185

^a First three amino acids determined by N-terminal sequencing.

^b Mass determined by mass spectrometry. Values in parentheses are the calculated masses of the cleavage peptides.

^c Positions determined by mass spectrometry.

FIG. 2. Two-dimensional tryptic peptide mapping and phosphoamino acid analysis of VP1 in vivo labeled with [32 P]orthophosphate. (A) Two-dimensional phosphopeptide map of VP1 after trypsin digestion. Three major phosphorylated peptide fragments are indicated by arrowheads; o, origin. (B) Phosphoamino acid analysis of phosphopeptide from HPLC (Fig. 1). Lanes: 1, total VP1; 2, 30.0- to 32.0-min fractions from C_{18} column; 3, 36.0- to 37.0-min fractions from C_{18} column; 4, 23.0- to 24.0-min fractions from C_4 column. S, serine; T, threonine.

similar to that of spot 1, and phosphopeptides from the 30.5- to 31.0-min fraction migrated to the positions of spots 2 and 3 (data not shown). Phosphoamino acid analysis of these phosphopeptides showed that spots 1 and 2 contained phosphothreonine, while spot 3 contained phosphoserine (data not shown).

In order to define the carboxy termini of the digested peptide fragments, the phosphopeptide fractions from the HPLC were analyzed by mass spectrometry. The masses of the HPLC peptide fragments, determined by mass spectrometry, and the calculated values of enzymatically digested products are given in Table 1. For all peptides, the mass spectrometry data were consistent with complete digestion at the appropriate sites. The largest deviation (less than 1%) between the experimental and the theoretical mass among these data was 44.4 Da (39.0- to 39.5-min fraction of Asp-N digestion). This correspondence of the mass with the predicted sequence indicates that the peptides were approximately 99% pure. The mass of a phosphate group in addition to the peptide mass would not have been detected, given the likely presence of unmodified VP1 molecules in the fraction in combination with a 1% mass deviation.

Figure 3 shows a summary of phosphopeptides identified from different enzymatic digestions. Phosphopeptides determined by tryptic digestion were verified by Lys-C and Asp-N digestions. We conclude from overlapping fragments that the major and minor phosphothreonine residues are located in fragments from residues 58 to 78 and 153 to 173.

We could not identify the site(s) of serine phosphorylation. Serine phosphate was detected in the C_{18} 30.5- to 31.0-min fraction but not in the C_4 refractionated peaks. This behavior

FIG. 3. Summary of phosphorylated peptide fragments of VP1 cleaved by different enzymes. The in vivo- 32 P-labeled VP1 was digested with trypsin (■), endoproteinase Asp-N (▬), and Lys-C (▮), fractionated with reverse-phase HPLC, and analyzed by N-terminal sequencing.

could have resulted from the overall low yield of material transferred to and analyzed on the second column or separation of a phosphoserine peptide from phosphothreonine species on the C_4 column. In addition, when the C_{18} fraction was analyzed by two-dimensional chromatography, threonine and serine phosphopeptides migrated differently. Again, this pattern may have resulted from two peptides with different sequences that eluted coincidentally from the C_{18} column (although the purity of the C_{18} peptide was estimated at 99% by mass spectrometry) or the same peptide modified differentially. In order to test whether serine phosphorylation occurred in the major C_{18} 30.5- to 31.0-min peptide, the two serines between residues 58 to 78 were mutagenized to glycine and the mutant viruses were reconstructed. Neither mutant virus (G66 and G77) could be isolated (data not shown), and VP1 protein from infected cells could not be obtained to determine whether serine phosphorylation was abolished.

In vivo threonine phosphorylation of VP1 occurs on residues 63 and 156. Site-directed mutagenesis was performed to verify the phosphothreonine sites predicted from protease mapping. The phosphopeptide from residues 58 to 78 contains two threonine residues at positions 63 and 68, and the phosphopeptide from residues 153 to 173 contains only threonine 156. These three sites were mutated, and the three mutant viruses G63, G68, and A156 were constructed from wild-type poly-

omavirus by changing the threonine residues at positions 63 and 68 to glycine and changing the threonine residue at position 156 to alanine. HPLC analysis of *in vivo*- ^{32}P -labeled VP1 from these mutant viruses was performed. The mutant virus G63 showed a significant decrease of phosphopeptide in the HPLC 30.5- to 31.0-min fraction, and the mutant virus A156 showed a decrease in the 36.5- to 37.0-min fraction (Fig. 4A and B). The mutant virus G68 showed the same phosphopeptide profile as the wild type (Fig. 4C). Residual counts per minute in the 30.5- to 31.0-min fraction (Fig. 4A) could be attributed to serine phosphate in this fraction. These results confirmed that the threonine residues at positions 63 and 156 were the phosphate acceptors. The pattern of eluted peptides determined by optical absorbance (data not shown) did not change for these mutant viruses, indicating that the C_{18} column did not separate the modified from the unmodified species of these particular peptides.

The phosphoamino acid analysis of total ^{32}P -labeled VP1 and the HPLC fractions of mutants G63 and A156 are shown in Fig. 5. Mutant A156 is completely deficient in phosphothreonine in the HPLC 36.5- to 37.0-min fraction (Fig. 5, lane 6). A significant decrease in the amount of phosphothreonine also was found in the 30.5- to 31.0-min fraction for mutant G63 (Fig. 5, lane 2). These results indicate that threonine 63 serves as a major phosphorylation site and threonine 156 serves as a minor site.

To examine the effect of the type of host cell infected on VP1 phosphorylation, baby mouse kidney cells were infected with mutants G63 and A156 and were *in vivo* labeled with [^{32}P]orthophosphate. HPLC analysis of tryptic peptides from ^{32}P -labeled VP1 from these infections showed the defect of threonine phosphorylation at residue 63 for G63 and at residue 156 for A156 (data not shown). These studies demonstrate that threonine phosphorylation sites are the same for both A31 fibroblasts and baby mouse kidney cells.

The mutant viruses NG18, NG59, and 808A are defective in phosphorylation on both threonine residues 63 and 156. We analyzed four mutant viruses affected in middle and/or small T antigens for defects in phosphorylation at T-63 and T-156. The *hr-t* mutant viruses NG18 and NG59 have genetic alterations in both the middle and small T proteins (8, 19). The site-directed mutant virus 808A encodes small T but no middle T (23). The site-directed mutant virus 1387T expresses a carboxy-terminal truncated middle T (9). Previous analysis of total VP1 phosphate from these mutant viruses (17) demonstrated that NG18 and NG59 have a 12-fold decrease, 808A has a 7-fold decrease, and 1387T has a 2-fold decrease in threonine content from wild-type infection levels.

The VP1 phosphorylation of these mutants was examined by HPLC analysis. Because unlabeled and ^{32}P -labeled protein bands were combined for this analysis, specific activities (i.e., counts per minute per peptide absorbance unit) could not be compared between infections. Therefore, because the amount of serine phosphorylation was previously found to be unaffected compared with that of the wild type for these mutant viruses (17), the ratio of phosphothreonine to phosphoserine in each HPLC fraction was compared. For 1387T, the HPLC phosphopeptide profile was similar to that of the wild type (data not shown), and 1387T was therefore considered to be an additional wild-type control. Figure 6 shows the results from phosphoamino acid analysis of HPLC phosphopeptide fractions obtained from *hr-t*, 808A, and 1387T mutant virus infections. The phosphopeptide spots were subjected to densitometry for comparison. In the 30.0- to 32.0-min fractions (residue T-63), the ratios of phosphothreonine to phosphoserine were 0.6 (NG18), 1.1 (NG59), 2.2 (808A), 3.5 (1387T),

FIG. 4. HPLC analysis of phosphorylated peptides of wild-type and site-directed mutant viruses affecting threonine residues 63, 68, and 156 of VP1. A threonine-to-glycine change at residues 63 (mutant G63) and 68 (mutant G68) and a threonine-to-alanine change at residue 156 (mutant A156) of VP1 were introduced into polyomavirus. The VP1 proteins of the reconstructed mutant polyomaviruses were labeled *in vivo* with [^{32}P]orthophosphate, digested with trypsin, and analyzed by HPLC. (A) Wild type (○) and mutant G63 (●). (B) Wild type (○) and mutant A156 (●). (C) Wild type (○) and mutant G68 (●).

FIG. 5. Phosphoamino acid analysis of HPLC peptide fragments from the ^{32}P -labeled VP1 of site-directed threonine mutant viruses. The VP1 of mutant G63 (lanes 1 to 3) and A156 (lanes 4 to 6) was labeled in vivo with [^{32}P]orthophosphate, digested with trypsin, and fractionated by HPLC. The total VP1 (lanes 1 and 4) and the peptides from the 30.0- to 32.0-min fractions (lanes 2 and 5) and the 36.0- to 37.0-min fractions (lanes 3 and 6) were analyzed by thin-layer electrophoresis. S, serine; T, threonine.

and 3.8 for wild-type virus (not shown). To compare threonine phosphorylation of VP1 on residue T-156, the ratio of phosphothreonine in the 36.0- to 37.0-min fraction to phosphoserine in the 30.0- to 32.0-min fractions was determined. The *hr-t* mutant viruses NG18 and NG59 gave a ratio of 0.30 to 0.32, 808A gave a ratio of approximately 0.39, and 1387T and the wild type gave a ratio of approximately 1.20. These data indicate that phosphorylation of both T-63 and T-156 was affected in the *hr-t* and 808A mutant virus infections.

Virion assembly defect of mutant A156. A decrease in VP1 threonine phosphorylation is associated with a defect in virion assembly for *hr-t* mutant viruses (15, 17). The relationship between threonine phosphorylation and virus assembly for mutants G63 and A156 was further examined by sedimentation analysis of subvirion complexes labeled in vivo with [^3H]thymidine. Figure 7 shows the sucrose gradient profile of the complexes from A31 cells infected with wild-type and mutant viruses. This sedimentation analysis in 1 M NaCl (17) separates stable virions (240S) from viral DNA (22S), the latter derived from unstable virions (200 to 240S) and replicating (95S) and

FIG. 7. Comparison of viral intermediates from wild-type and site-directed mutant viruses. Cells were infected with polyomavirus and labeled with [^3H]thymidine. Viral intermediates were separated by sucrose gradient sedimentation analysis. (A) Wild type ($\square$) and mutant G63 ($\blacksquare$). (B) Wild type ($\square$) and mutant A156 ($\blacksquare$). The 240S peak (fractions 8 to 12) represents stable virions, and the 22S peak (fractions 21 to 24) represents all other viral DNA species.

FIG. 6. Phosphoamino acid analysis of HPLC peptide fragments from VP1 isolated from four mutant viruses affected in middle and/or small T antigens. The ^{32}P -labeled VP1 and peptide fragments of NG59 (A, lanes 1 to 3) and NG18 (A, lanes 4 to 6) and site-directed mutants 808A (B, lanes 1 to 3) and 1387T (B, lanes 4 to 6) were analyzed by thin-layer electrophoresis. Lanes: 1 and 4, total VP1; 2 and 5, 30.0- to 32.0-min fractions from the C_{18} column; 3 and 6, 36.0- to 37.0-min fractions from the C_{18} column. S, serine; T, threonine.

mature (70S) minichromosomes (14, 16). Mutant G63 had the same profile as wild-type virus, indicating no virion assembly defect (Fig. 7A). Mutant A156 was defective in the production of stable 240S virions, but the total amount of labeled viral DNA was equivalent to that seen for the wild-type virus. This result suggests that phosphorylation at threonine 156 affects virus assembly. To examine the effect on virus assembly of altering both residues T-63 and T-156, both sites were mutated by substitution of T-63 with glycine and T-156 with alanine or glycine. DNA sequence analysis of these mutant viruses recovered from A31 cell lysates showed that the mutation at residue T-156 reverted to the wild type, but the second change at residue T-63 was stable (data not shown), suggesting that the threonine phosphorylation of VP1 protein at residue T-156 is advantageous for virus growth.

DISCUSSION

In order to understand further the role of VP1 phosphorylation in the polyomavirus life cycle, we have determined the sites of VP1 phosphorylation after in vivo ^{32}P labeling of virus-infected cells. Two phosphopeptides (encompassing residues 58 to 78 and 153 to 173) were identified by N-terminal protein sequencing and mass spectrometry. The first peptide contains two threonine sites (T-63 and T-68), and the second peptide contains one threonine site (T-156). Site-directed mutant viruses were constructed by replacing threonine with glycine or alanine at these positions, and in vivo labeling of VP1 after infection with these mutant viruses confirmed that T-63 and T-156 were phosphate acceptors. Previous study of VP1 phosphorylation by two-dimensional TLC and deduction of peptide identity from predicted chemical cleavage sites identified a major site of phosphorylation between residues 122 and 228 and suggested that the site was between residues 143 and 159 (2). This site could correspond to T-156.

Two-dimensional gel analysis of VP1 isolated from infected cells has identified at least four isoelectric focusing subspecies of VP1, with approximately 50% migrating as unmodified protein. The analysis of VP1 phosphopeptides is therefore complicated by the presence of the same peptide being differentially modified. From our analysis, we conclude that the C_{18} reverse-phase HPLC column did not separate subspecies of the same peptide, while the two-dimensional TLC analysis was capable of distinguishing phosphoserine from phosphothreonine forms of the same peptide. We could not identify the site(s) of serine phosphorylation, although the peptide from residues 58 to 78 may contain phosphoserine. These sites may be essential for virus growth, as evidenced by their persistence during *hr-t* mutant virus infections and our inability to obtain viable virus after mutation of serines in this region.

Polyomavirus *hr-t* mutant viruses have a defect in viral encapsidation associated with underphosphorylation of VP1 on threonine (15, 16). We analyzed two of these mutant viruses and found that the decrease in threonine phosphorylation corresponded to both T-63 and T-156 sites. The site-directed mutant virus 808A, defective only in middle T antigen, demonstrated similar decreases in both residues, while the mutant virus 1387T, which grows as well as the wild type, maintained a wild-type VP1 phosphorylation profile. Thus, the growth defect of these viruses is associated with decreased phosphorylation at both sites, and middle T antigen is responsible for these threonine phosphorylations. This effect could be mediated by activation of a cellular threonine kinase, e.g., $\text{pp60}^{\text{c-src}}$, or inactivation of a specific phosphatase, e.g., phosphatase 2A, both activities known to be associated with middle T antigen. When the mutant viruses G63 and A156 were tested for defects in virion maturation, A156 demonstrated a phenotype similar to that seen for the *hr-t* mutant viruses, i.e., an accumulation of viral minichromosomes with poor 240S virion production. In contrast to the *hr-t* assembly defect, however, viral DNA accumulation was the same for both A156 and the wild type. *hr-t* mutant viruses produce 10 to 30% the amount of DNA produced by the wild type, and this difference is probably due to the presence of intact middle and small T antigens, whose activities include stimulation of viral DNA synthesis, in the A156 mutant virus.

The 3.8-Å structure of simian virus 40 shows that the BC, DE, and HI peptide loops of each VP1 subunit in a pentameric capsomere interdigitate in forming the exterior surface of the virion (24). Comparison of the polyomavirus VP1 sequence with that of simian virus 40 predicts that T-156 is located in the DE loop and that T-63 is located in the BC loop. The BC loop

of polyomavirus has been implicated directly in cell surface recognition and hemagglutination. A mutant polyomavirus with a G-to-E substitution at position 92 within this loop has an altered plaque morphology and pH dependence for hemagglutination and a distinctive in vivo pattern of tumor induction (12, 13). These data suggest that T-63 (and perhaps T-156) is functionally involved in virus-cell recognition. The defect in assembly with mutations at T-156, as well as the inability to mutate putative serine sites, suggests that the virus may control for the correct residues necessary for receptor recognition by linking modification at this site with assembly.

Virions and empty capsids appear to have a different complement of VP1 isoelectric focusing subspecies, although site-specific differences in phosphorylation of these subspecies have not been detected (2). Because virions and capsids (or recombinant VP1 protein) from the same virus strain hemagglutinate erythrocytes equivalently, phosphorylation probably does not play a role in sialoglycoprotein recognition (6, 31). However, capsids (containing VP2 and VP3) are unable to inhibit infection by virions (7), suggesting that specific VP1 phosphorylation sites may play a role in cell recognition. This hypothesis is supported by the location of the identified phosphorylation sites in the virion structure and previous data suggesting that virions with distinct isoelectric focusing phosphorylation patterns generated by infection of different mouse cell types have different efficiencies of in vitro infectivity (25). Studies with the T-156 and T-63 mutant viruses concerning the properties of in vitro and in vivo infectivity are now in progress to determine the relationship of these functions to VP1 phosphorylation.

ACKNOWLEDGMENTS

We thank Jim Lee and Paul Morrison of the Dana-Farber peptide facility for assistance with the HPLC analysis, peptide sequencing, and mass spectrometry and Lynne Montross for excellent technical assistance.

This work was supported by Public Health Service grant CA37667 to R. Garcea from the National Cancer Institute, Department of Health and Human Services.

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