Phosphorylation of the polyomavirus major capsid protein VP1 plays a role in virus assembly and may function in virus-cell recognition. Previous mapping of the in vivo phosphorylation sites on VP1 identified phosphorylation of threonine residues Thr-63 and Thr-156 (Li, M., and Garcea, R. L. (1994) J. Virol. 68, 320–327). Phosphoserine was detected in a tryptic phosphopeptide encompassing residues 58–78. Because of consensus casein kinase II (CK II) sites in this peptide, we examined the in vitro phosphorylation of the purified recombinant VP1 protein by CK II. CK II phosphorylated VP1 on serine, and the resulting tryptic phosphopeptide eluted in a 30–31 min high performance liquid chromatography fraction corresponding to residues 58–78. The VP1 tryptic phosphopeptide also co-migrated in two-dimensional peptide analysis with one of the tryptic phosphopeptides obtained from VP1 isolated after in vivo labeling of virus-infected cells. A site-directed mutant VP1 protein, Ser-66 to Ala, was phosphorylated poorly by CK II in vitro. As determined by electron microscopy, all of the mutant proteins were isolated in pentameric form similar to the wild-type protein, although the Ala-66 pentamers had a tendency to self-assemble in vitro into tubular as well as capsid-like structures. These findings identify Ser-66 as a site of VP1 phosphorylation in vitro, and suggest that VP1 may serve as a substrate for CK II in vivo.

The polyomavirus capsid is composed of 72 pentamers of the major coat protein VP1 (1). The minor capsid proteins VP2 and VP3 are present in approximately one-tenth the abundance of VP1 in the virion and play an unknown structural role (2). VP1 is synthesized late in the viral lytic cycle and transported to the nucleus of the infected cells where encapsidation of the viral minichromosome occurs. Studies have suggested that post-translational modifications of VP1 play an important role in virus assembly and cell infection (3–6). VP1 is phosphorylated in serine and threonine residues (7, 8). Recently, we mapped the phosphorylation sites of VP1 isolated from virus-infected mouse cells (9). Threonine phosphorylation of VP1 was identified on residues Thr-63 and Thr-156 in the BC and DE loops, respectively, which are exposed on the exterior viral surface (10). A defect in virus assembly was associated with a mutation at threonine 156 (9). Serine sites, although present in the same tryptic phosphopeptides as the threonine sites, could not be assigned because viruses reconstructed with mutations at these serine residues were nonviable.

Polyoma host-range nontransforming mutant viruses have genetic alterations in both middle and small tumor (T)-antigens, and are defective in cell transformation in vitro and tumor induction in vivo (11). The host-range nontransforming mutant viruses are blocked in virus assembly when grown on nonpermissive cells (3). The assembly defect of host-range nontransforming mutant viruses is associated with underphosphorylation of VP1 on threonine (8). In vivo phosphate labeling of VP1 during host-range nontransforming mutant virus infections showed that phosphorylation of VP1 on both residues Thr-63 and Thr-156 was defective (9). This regulation could be controlled by activation of a cellular kinase, e.g. pp60 src, or inactivation of a specific phosphatase, e.g. phosphatase 2A, both activities known to be associated with middle T-antigen (12–14). VP1 serine phosphorylation appears constitutive, however, at least in the presence of an intact large T-antigen (8).

Casein kinase II (CK II) is a ubiquitous cyclic nucleotide independent serine-threonine kinase present in the cell nucleus and cytoplasm (15, 16). CK II is activated rapidly when cells are treated with certain growth factors such as serum, epidermal growth factor, and insulin-like growth factor (17–19). These findings raise the possibility that CK II plays an important role in cellular activities related to cell growth and proliferation (20, 21). This enzyme has a number of substrates including MYC and p53 (22, 23). In addition, CK II activity is stimulated by many of the agents that activate c-fos transcription (18, 24, 25). These substrates suggest that CK II may link signal transduction pathways and nuclear proteins that control cell proliferation (26, 27). CK II has also been found to phosphorylate structural and nonstructural viral proteins such as SV40 large T-antigen and varicella-zoster virus glycoprotein (28, 29). The data presented in this report show that the polyomavirus major capsid protein VP1 is also phosphorylated by CK II.

MATERIALS AND METHODS

Cell Culture and Virus Infection—A31 (Balb/c3T3) mouse fibroblasts were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Cell culture was performed as previously described (30). The wild-type polyomavirus strain used was NG59RA. Virus infections were performed at multiplicities of 5–10 plaque forming units/cell in A31 mouse fibroblasts at approximately 50% confluence. Following adsorption of polyomavirus, fresh Dulbecco's modified Eagle's medium supplemented with 2% calf serum was added.

In Vivo [32 P]Orthophosphate Labeling—Cells were grown in 100-mm
plates and infected with polyomavirus. At 25-28 h postinfection, cells were washed with phosphate-free Dulbecco’s modified Eagle’s medium and labeled with 2 mCi of $^{32}$P orthophosphate (specific activity 900 mCi/mmol) in 1 ml of phosphate-free Dulbecco’s modified Eagle’s medium with 2% dialyzed calf serum for 4–6 h (9).

**VP1 Immunoprecipitation—Polyomavirus-infected monolayers were lysed in radiolabel immunoprecipitation assay buffer (RIPA: 150 mM NaCl, 50 mM Tris-HCl, pH 7.2, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS). Lysates were cleared by centrifugation at 12,000 $\times$ g for 20 min. The soluble lysate was incubated with a rabbit anti-VP1 polyclonal antiserum (I58) at 4°C for 1 h (31, 32). The immune complexes were adsorbed to protein A-Sepharose CL-4B beads (Pharmacia) by a further incubation for 1 h at 4°C. The Sepharose beads were washed three times with phosphate-buffered saline and twice with water. HPLC Phosphopeptide Mapping—Immunoprecipitated VP1 was removed from the protein A-Sepharose beads with SDS sample buffer (2% SDS, 5% 2-mercaptoethanol, 0.625 mM Tris-HCl, pH 6.8, 10% glycerol) by heating for 5 min at 100°C. VP1 was resolved by 7.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted to nitrocellulose membranes (37). VP1 was stained with 0.1% Ponceau S in 1% acetic acid for 5 min and destained with 0.1% Ninhydrin in acetone, and developed at 100°C. The plate was exposed to Kodak film at 4°C. The Sepharose beads were washed three times with water 5 times, and the nitrocellulose was then removed by centrifugation at 10,000 $\times$ g for 5 min and the supernatant was transferred to a fresh tube and lyophilized under vacuum. The trypsin peptide pellets 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 HPLC with a C18 column. Peptides were separated with a linear gradient from 0 to 70% acetonitrile in 0.1% trifluoroacetic acid applied with a flow rate of 200 $\mu$L/min over 60 min. The fractions were collected every 1 min. Phosphorylated peptides were detected by scintillation counting.

**Phosphoamino Acid Analysis**—The $^{32}$P-labeled VP1 isolated by immunoprecipitation from in vivo $^{32}$P-labeled polyomavirus-infected cells or recombinant protein phosphorylated in vitro was used for phosphoamino acid analysis. After SDS-PAGE and electroblotting to Immobilon-P membrane, the VP1 band was hydrolyzed with HCI (34). The hydrolysate was then dissolved in 5 ml of two-dimensional buffer (7.5% acetic acid, 2.5% formic acid, 0.2% Glacial acetic acid) and digested with trypsin in enzyme buffer (100 mM NaHCO3, pH 8.2), acetoni-trile, and 95:5 (v/v), at 37°C overnight. The ratio of trypsin to VP1 was approximately 1:20 (w/w). The tryptic digest was removed by centrifugation at 10,000 $\times$ g for 5 min and the supernatant was transferred to a fresh tube and lyophilized under vacuum. The tryptic peptide pellets 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 HPLC with a C18 column. Peptides were separated with a linear gradient from 0 to 70% acetonitrile in 0.1% trifluoroacetic acid applied with a flow rate of 200 $\mu$L/min over 60 min. The fractions were collected every 1 min. Phosphorylated peptides were detected by scintillation counting.

**In Vitro Phosphorylation of VP1 by Casein Kinase II**—VP1 obtained after the phosphocellulose purification step was dialyzed into kinase buffer (10 mM MgCl2, 150 mM KCI, 50 mM Tris-HCl, pH 7.5). The phosphorylation of VP1 was carried out in kinase buffer containing 30 $\mu$g of VP1, 10 $\mu$L of $^{32}$P-labeled ATP (specific activity 3000 mCi/mmol) and 200 microunits of CK II (Upstate Biotechnology) or 200 microunits of CK II (Boehringer-Mannheim) for 1 h at 30°C. Enzymes from both vendors gave equivalent results. CK II phosphorylation of VP1 immuno precipitates from virus-infected cells was performed by washing the immunoprecipitate twice with phosphate-buffered saline and once with kinase buffer. 10 $\mu$L of $^{32}$P-labeled ATP was then added with or without CK II. The peptides GQPPTPESLTEGGQYYGWSRGINC and DVHGFNYERTQGIGSRNCG and DVGHSFKNPTDTVNTKGISTPVEGC, corresponding to residues 59–81 and 138–160 of VP1, were used as substrates in phosphorylation in vitro. The phosphorylated peptides were purified using the Glu-63 recombinant VP1 protein, was incubated with CK II and $^{32}$P-labeled ATP, and the products analyzed by SDS-PAGE and autoradiography. Panel A, Coomassie Blue stained; and panel B, autoradiogram. Full-length VP1 (lanes 1 and 2) and $\Delta$NCOVP1 (lanes 3 and 4) were incubated with lanes 2 and 4 or without lanes 1 and 3. CK II. Panel C, phosphoamino acid analysis of the $^{32}$P-labeled VP1 (full-length, lane 1; $\Delta$NCOVP1, lane 2).

**RESULTS**

In Vitro Phosphorylation of VP1 by Casein Kinase II—In vivo phosphorylation site motif (S/T)XX(D/E) were identified by inspection. The presence of these putative sites prompted us to investigate whether CK II could phosphorylate VP1 in vitro. Recombinant VP1 proteins, purified after expression in E. coli, were used as substrates in vitro kinase reactions with CK II. These proteins are purified as pentamers which resemble the capsomeric subunits of the virion (39). Because the full-length protein has the potential for self-assembly into capsid-like structures in vitro (39), a carboxyl-terminal 63 amino acid deleted form of VP1 ($\Delta$NCOVP1; 38) which is unable to assemble into capsids but is otherwise structurally intact, was also tested as a substrate. As shown in Fig. 1, recombinant VP1 was phosphorylated by CK II. The minor Coomassie Blue-stained band in lanes 1 and 2 (Fig. 1A) migrating slightly above the position of the $\Delta$NCOVP1 band in

**FIG. 1. In vitro phosphorylation of VP1 by CK II.** Purified recombinant VP1 protein was incubated with CK II and [γ-32P]ATP, and the products analyzed by SDS-PAGE and autoradiography. Panel A, Coomassie Blue stained; and panel B, autoradiogram. Full-length VP1 (lanes 1 and 2) and $\Delta$NCOVP1 (lanes 3 and 4) were incubated with lanes 2 and 4 or without lanes 1 and 3. CK II. Panel C, phosphoamino acid analysis of the $^{32}$P-labeled VP1 (full-length, lane 1; $\Delta$NCOVP1, lane 2). Expresses site-directed mutant and carboxyl-terminal deleted forms of VP1 in E. coli were prepared using ptacVP1. The site-directed mutant VP1 was constructed by inserting the HindIII-Xbal fragment of the reconstructed mutant polyomavirus into HindIII-Xbal-digested ptacVP1. Carboxyl-terminal deletions of VP1 were constructed as described (38) with slight modifications. The plasmid ptacVP1 containing the introduced mutation was digested with Ncol, and the large DNA fragment was isolated using Genedean II Kit (BIO 101). This fragment was then digested with S1 nuclelease. The blunt-ended DNA was ligated into T4 DNA ligase. The truncated VP1 protein was purified as described (37).

In vitro phosphorylation site motif (S/T)XX(D/E) were identified by inspection. The presence of these putative sites prompted us to investigate whether CK II could phosphorylate VP1 in vitro. Recombinant VP1 proteins, purified after expression in E. coli, were used as substrates in vitro kinase reactions with CK II. These proteins are purified as pentamers which resemble the capsomeric subunits of the virion (39). Because the full-length protein has the potential for self-assembly into capsid-like structures in vitro (39), a carboxyl-terminal 63 amino acid deleted form of VP1 ($\Delta$NCOVP1; 38) which is unable to assemble into capsids but is otherwise structurally intact, was also tested as a substrate. As shown in Fig. 1, recombinant VP1 was phosphorylated by CK II. The minor Coomassie Blue-stained band in lanes 1 and 2 (Fig. 1A) migrating slightly above the position of the $\Delta$NCOVP1 band in
lanes 3 and 4, represents a proteolytic cleavage product of VP1, which has deleted the first 27 amino acids of the protein (31). Both the partially proteolyzed species and ΔNCOVP1 were substrates for CK II (Fig. 1B, lanes 2 and 4). Phosphoamino acid analysis of the kinased proteins revealed that CK II phosphorylated both VP1 and ΔNCOVP1 on serine only (Fig. 1C).

Immunoprecipitates of VP1 from virus-infected cells were also tested for associated kinase activity by direct incubation with [γ-32P]ATP (data not shown). No significant VP1 "auto-phosphorylation" was detected and the addition of CK II to the immunoprecipitates did not lead to increased VP1 phosphorylation. Mitogen-associated protein (p44), cdc2 (p34), and cdk2 kinases were also tested for their ability to phosphorylate VP1 in vitro. Mitogen-associated protein kinase did not phosphorylate VP1. cdc2 and cdk2 kinases did not phosphorylate VP1 in vitro, but the tryptic phosphopeptides resulting from these reactions did not correspond with those seen in vivo (see below) and these enzymes were therefore not further characterized.

Analysis of Phosphopeptides by HPLC and Two-dimensional Peptide Mapping—To characterize the site(s) phosphorylated by CK II, we analyzed VP1 tryptic phosphopeptides by HPLC. Fig. 2 shows that two phosphopeptide peaks (30–31 min and 36–37 min fractions) were detected in VP1 isolated after in vivo 32P labeling of polyomavirus-infected cells. In contrast, only one major phosphopeptide peak (30–31 min fraction) was detected for the recombinant VP1 protein phosphorylated by CK II in vitro, with a variable shoulder peak (28–29 min fraction).

Phosphoamino acid analysis of VP1 isolated from polyomavirus-infected cells showed that the phosphopeptide in the 36–37 min fraction is phosphorylated only on threonine residues (9) and the phosphopeptide in the 30–31 min fraction is phosphorylated on both threonine and serine residues (Fig. 2B, lane 1). The phosphopeptide in the 30–31 min fraction of in vitro CK II-phosphorylated recombinant VP1 contained only phosphoserine (Fig. 2B, lane 2). These results suggest that phosphorylation of VP1 by CK II was primarily on serine sites between residues 58 and 78. In order to verify that the in vivo and in vitro phosphopeptides were identical, they were further analyzed by two-dimensional phosphopeptide mapping (Fig. 3). The two-dimensional mapping showed that VP1 isolated from polyomavirus-infected mouse cells has three phosphopeptide spots (Fig. 3A) (9). Phosphopeptide spots 1 and 2 contained phosphothreonine, and spot 3 phosphoserine (9) (data not shown). Recombinant VP1 protein phosphorylated by CK II showed a major phosphopeptide spot containing phosphoserine (Fig. 3B; data not shown). When 32P-labeled VP1 from polyomavirus-infected cells was mixed with the recombinant VP1 phosphorylated by CK II, spot 3 from the in vivo labeled VP1 co-migrated with the phosphopeptide generated by CK II (Fig. 3C). HPLC analysis showed that both spots 2 and 3 eluted in the 30–31 min fraction (data not shown). This result may be due to two different peptides eluting coincidentally from the 30–31 min fraction, or the same peptide which has been differentially modified. The two-dimensional chromatography analysis supports the latter, suggesting that the peptide from residues 58 to 78 is phosphorylated on either threonine or serine residues, but not both, and each of these modifications yields a distinctive species in...
the two-dimensional chromatogram (spot 2 or 3).

To confirm that VP1 residues 58–78 contained a serine that was a substrate for CK II, a peptide of VP1 corresponding to residues 59–81 was synthesized. This peptide was incubated with CK II in vitro, and phosphoamino acid analysis of the reaction product demonstrated phosphoserine (Fig. 4, lane 1). In addition, a peptide corresponding to residues 138–160 (the DE loop of VP1) was also phosphorylated by CK II (Fig. 4, lane 2). This peptide contains a potential consensus CK II site (residue Thr-156), a site phosphorylated in vivo. In vitro the intact protein is not phosphorylated in this region, but the synthetic peptide is phosphorylated on both serine and threonine. These results suggest that in the intact VP1 pentamer, potential CK II sites are conformationally distinct for kinase recognition.

VP1 is phosphorylated by CK II on Serine 66—Two serine residues, Ser-66 and Ser-77, are found between residues 58 and 78. Serine 66 is a CK II phosphorylation site based on the motif of (S/T)XX(D/E), whereas Ser-77 is not a consensus CK II site. Site-directed mutations were introduced into the polyomavirus genome, substituting alanine for serine at these residues. Neither of the mutant viruses (Ala-66 and Ala-77) could be isolated (data not shown), and therefore determination of the in vivo phosphorylation site could not be confirmed in this manner. Both the Ala-66 and Ala-77 mutations were also introduced into the VP1 expression vector, pTAcVP1. The mutant recombinant VP1 proteins were purified and analyzed for phosphorylation by CK II. The wild-type VP1 and two additional proteins Gly-63 (Thr-63 to Gly) and Ala-156 (Thr-156 to Ala) as well as Ala-77 were phosphorylated by CK II, both as full-length and carboxyl-terminal deleted proteins (Fig. 5B). The mutant protein Ala-66 was not significantly phosphorylated by CK II (Fig. 5B, lanes 2 and 7). This result demonstrates that VP1 is phosphorylated in vitro on Ser-66, and not Ser-77, by CK II.

Electron Microscopy of Mutant VP1 Proteins—The purified recombinant VP1 protein is isolated as pentamers resembling viral capsomers, which can self-assemble in vitro into capsid-like structures (39). In order to assess the structural integrity of the mutant VP1 proteins they were analyzed by electron microscopy for pentamer structure and in vitro capsid self-assembly. All mutant proteins (Ala-66, Ala-77, Gly-63, and Ala-156) appeared as pentamers resembling wild-type pentamers (data not shown). However, when subjected to conditions which promote in vitro capsid assembly, i.e. high ionic strength and calcium (39, 40), the Ala-66 mutant protein had a tendency to form tubular structures as well as capsid-like aggregates (Fig. 6). Thus, although the Ala-66 mutation did not affect pentamer formation, changes in the Ser-66 residue may influence the formation of higher order aggregates of VP1.

**DISCUSSION**

These data demonstrate that the polyomavirus major capsid protein VP1 is an in vitro substrate for phosphorylation by CK II. VP1, purified after expression in E. coli, was phosphorylated in vitro by CK II, and phosphoamino acid analysis demonstrated only phosphoserine residues. The major phosphorylation site modified by casein kinase II was located in the tryptic peptide encompassing residues 58 to 78. A site-directed mutant VP1 protein, with a serine-to-alanine change at residue 66, was defective in CK II phosphorylation. Therefore, Ser-66 is the phosphorylation site modified by CK II in vitro, and the data suggest that the in vivo phosphorypeptide representing residues 58–78 is also phosphorylated on Ser-66.

Recently the VP1 threonine phosphorylation sites were mapped (9). Two major phosphopeptides, in residues 58–78 and residues 153–173, were identified from in vivo 32P labeling polyomavirus-infected cells. Viruses with site-directed mutations confirmed that VP1 was phosphorylated on Thr-63 and Thr-156. However, the serine phosphorylation site(s) was unidentified because mutant viruses with substitutions at possible serine residues (Ser-66 and Ser-77) were non-viable. We conclude from this previous result that Ser-66 is essential for virus growth. HPLC analysis from in vivo 32P labeling polyomavirus-infected cells showed that the peptides eluted in the 30–31 min fraction were phosphorylated on both threonine and serine residues. Two-dimensional peptide mapping showed that the peptide fragments eluted from this fraction migrated in different locations (Fig. 2). However, the fragments in the 30–31 min fraction contained only one peptide sequence from residue 58 to 78 determined by NH2-terminal sequencing and mass spectrometry (9). Therefore, we conclude that phosphorylation of Thr-63 and Ser-66 occur in different VP1 monomers. VP1 isolated from polyomavirus-infected cells contains at least four isoelectric subspecies identified by two-dimensional protein gel analysis (3, 4). Several of these subspecies may represent distinct monophosphorylated molecules rather than multiply modified forms.

In two other well characterized examples of the consequences of CK II phosphorylation, skeletal muscle glycogen synthase and acetyl-CoA carboxylase (41, 42), the initial phosphorylation does not itself alter the activity of the substrate but is necessary for subsequent regulatory phosphorylation or dephosphorylation events. Serine phosphorylation of VP1 appears constitutive relative to the activity of middle T-antigen which appears to regulate VP1 threonine phosphorylation (8, 30). Because only a fraction, estimated by two-dimensional protein gel electrophoresis as 50% or less, of the VP1 monomers are modified it is possible that serine modification by a cellular CK II enzyme may affect the subsequent choice of VP1 molecules for threonine modification. This choice may dictate that a particular VP1 within a pentameric capsomere is phosphorylated either on serine or threonine but not both.
The in vitro assembly properties of the Ala-66 mutant VP1 protein demonstrated that this protein was structurally intact, although this mutant protein had a tendency to self-assemble into not only capsid-like but also tubular structures. Because inter-capsomeric bonds are formed using the carboxyl termini of VP1 molecules between pentamers (38, 43), this result suggests that changes in Ser-66, located on the surface of the VP1 pentamer (43), may influence interactions between VP1 carboxyl termini. This structural interaction may be relevant to wild-type VP1 molecules phosphorylated at Ser-66, in that such a modification may facilitate formation of alternative carboxyl-terminal bonding interactions in the final capsid (43). In addition, a perturbation of proper inter-capsomeric bonding may explain why a virus with the Ala-66 mutation could not be isolated (9). However, additional studies of the Ala-66 mutant protein under a variety of assembly conditions (40) will be necessary before the significance of these structural interactions can be further assessed.

For the related SV40 virus, the large T-antigen-p53 complex isolated from virus-infected cells is associated (in immunoprecipitates) with a serine-specific kinase activity (44, 45). This activity autophosphorylates T-antigen on many of the same sites seen for in vitro phosphorylation (29). The two-dimensional phosphopeptide map of SV40 large T-antigen was nearly reproduced in vitro by the combination of CK I and CK II, and some of the associated kinase activity may be related to terminal bonding interactions in the final capsid (43). In addition, a perturbation of proper inter-capsomeric bonding may explain why a virus with the Ala-66 mutation could not be isolated (9). However, additional studies of the Ala-66 mutant protein under a variety of assembly conditions (40) will be necessary before the significance of these structural interactions can be further assessed.

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Fig. 6. In vitro assembly of mutant VP1 proteins. The Ala-77 (A) and Ala-66 (B) mutant VP1 proteins were incubated under assembly conditions and examined by electron microscopy (see "Materials and Methods"). The Ala-77 protein formed typical capsid-like aggregates similar to the wild-type protein, whereas the Ala-66 protein formed both tubular and capsid-like structures. Bar equals 100 nm.
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