Mutations in the Putative Calcium-Binding Domain of Polyomavirus VP1 Affect Capsid Assembly†

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Calcium ions appear to play a major role in maintaining the structural integrity of the polyomavirus and are likely involved in the processes of viral uncoating and assembly. Previous studies demonstrated that a VP1 fragment extending from Pro-232 to Asp-364 has calcium-binding capabilities. This fragment contains an amino acid stretch from Asp-266 to Glu-277 which is quite similar in sequence to the amino acids that make up the calcium-binding EF hand structures found in many proteins. To assess the contribution of this domain to polyomavirus structural integrity, the effects of mutations in this region were examined by transfecting mutated viral DNA into susceptible cells. Immunofluorescence studies indicated that although viral protein synthesis occurred normally, infective viral progeny were not produced in cells transfected with polyomavirus genomes encoding either a VP1 molecule lacking amino acids Thr-262 through Gly-276 or a VP1 molecule containing a mutation of Asp-266 to Ala. VP1 molecules containing the deletion mutation were unable to bind ^{45}Ca in an in vitro assay. Upon expression in *Escherichia coli* and purification by immunoaffinity chromatography, wild-type VP1 was isolated as pentameric, capsomere-like structures which could be induced to form capsid-like structures upon addition of CaCl_2 , consistent with previous studies. However, although VP1 containing the point mutation was isolated as pentamers which were indistinguishable from wild-type VP1 pentamers, addition of CaCl_2 did not result in their assembly into capsid-like structures. Immunogold labeling and electron microscopy studies of transfected mammalian cells provided in vivo evidence that a mutation in this region affects the process of viral assembly.

The murine polyomavirus is composed of three structural proteins, designated VP1, VP2, and VP3, which are assembled onto a DNA-protein complex (43). A single virus capsid contains 72 pentameric capsomeres, each of which is composed of five molecules of the major capsid protein VP1. The presence of the divalent cation calcium in murine polyomavirus virions was indicated by X-ray fluorometry studies (9). A structural role for calcium was demonstrated in studies by Brady et al. (9, 10) which showed that chelation of calcium by ethylene glycol-bis- N,N,N',N' -tetraacetic acid (EGTA), in conjunction with disulfide bond disruption by the reducing agent dithiothreitol, results in the breakdown of the virion into its capsomere subunits and a DNA-protein complex. Interestingly, addition of exogenous CaCl_2 to this dissociated mixture permitted reassembly into intact virions which partially regained both infectivity and hemagglutination activity (8, 47). Calcium binding appears to contribute to the stability of a number of plant viruses as well (1, 14–16, 22, 34, 37), possibly by helping to ensure that these viruses release their RNAs only in the host-cell cytoplasm. A characteristic swelling of these viruses which occurs upon exposure to calcium chelators under basic conditions is believed to represent an initial step in their uncoating.

When polyomavirus proteins were separated chromatographically and the isolated VP1 protein was analyzed by electron microscopy in the absence of calcium, structures

which resembled capsomeres in both size and shape were observed (7). Polyomavirus VP1, which was expressed in *Escherichia coli* from pALVP1tac (24) and purified in the presence of EDTA and β -mercaptoethanol, was also isolated as pentameric structures that resembled viral capsomeres (38, 39). Removal of the chelator and reducing agent together with addition of CaCl_2 resulted in the assembly of these capsomeres into structures which resembled viral capsids. Besides further implicating a role for calcium in maintaining the structural integrity of the virus, these studies also suggested that the posttranslational modifications of VP1 which occur in eukaryotic cells (2, 3, 5, 18, 19, 26, 28, 29, 35) may not be necessary for proper capsid formation. These studies also indicated that the minor capsid proteins were not necessary for in vitro assembly.

A 16-kDa fragment of the polyomavirus major capsid protein VP1, which is generated by formic acid cleavage and extends from Pro-232 to Asp-364, was shown to have calcium-binding capabilities (27). This fragment contains a sequence of 12 amino acids (Asp-266 through Glu-277) that is quite similar to the 12-amino-acid loops which make up part of the EF hand structures found in many calcium-binding proteins, including parvalbumin, calmodulin, thrombospondin, and troponin C (13, 23, 42). The purpose of the present studies was to determine the effects that alterations within this domain would have on polyomavirus structure and functions. The work focused on a single site-directed point mutation within this domain, as well as a deletion mutant which eliminated the putative calcium-binding domain. Both mutations resulted in the inability to form infective viral progeny in cells transfected with polyomavirus DNA encoding the respective mutations. In addition, when expressed in *E. coli*, VP1 molecules lacking the 12-amino-acid motif were unable to bind calcium in an in vitro assay. Finally, when the

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VP1 gene harboring the point mutation was expressed in *E. coli* and purified, it was shown to be defective in the ability to assemble into capsid-like structures in the presence of calcium.

MATERIALS AND METHODS

Mutagenesis. A 1.9-kb *HindIII*-*PvuII* fragment containing 1 kb of the VP1 gene as well as about 900 bp of 3' flanking DNA was excised from the polyomavirus genome and cloned into M13mp19 to generate M13mp19-VP1. Prior to ligation, the terminal *PvuII* site was converted to a *Bam*HI site to allow cloning into the *HindIII*-*Bam*HI-digested vector. A point mutation in the Asp-266 codon was created by using the Muta-gene in vitro mutagenesis kit (Bio-Rad) as described by the manufacturer and the phosphorylated mixed oligonucleotide primer 5'-CCCAACTCCATTTTCA (T/G)CTAG-3' (corresponding to polyomavirus nucleotides 3265 to 3285 [41]; the underlined G represents the mutation). Sequencing of single-stranded phage DNA by the dideoxy-chain termination method was used to determine whether the wild-type T nucleotide or the mutant G nucleotide (underlined) was present at the variable position. The oligonucleotide primer 5'-GCCCTGCACTTGGGGGAGCAT-3' (corresponding to polyomavirus nucleotides 3061 to 3081) (41), α -³⁵S-dATP (New England Nuclear) and the Sequenase kit (United States Biochemicals) were used in this determination. A phage clone whose DNA contained the mutation was termed M13mp19-VP1A (for alanine), and one which contained the wild-type T nucleotide at the position of interest was designated M13mp19-VP1D (for aspartic acid) and served as a control for the mutagenesis reactions.

To generate a deletion mutation in the region of the VP1 gene encoding the putative calcium-binding domain, the 504-bp *HincII* from wild-type polyomavirus DNA was first cloned into pUC19 to generate pCH101. This construct was subsequently treated with *ApaI* (which cleaves pCH101 at a unique site within the region encoding the putative calcium-binding domain corresponding to nucleotide 3264 in the polyomavirus genome [41]). *Bal* 31 slow exonuclease (International Biotechnologies, Inc.) was used to generate the deletion, and the shortened DNA fragment was recircularized with T4 DNA ligase. The recircularized DNA was used to transform HB101 cells, and transformants were screened by dideoxynucleotide sequencing to determine the extent of the deletions. Plasmid DNA from one colony contained a 42-bp deletion which eliminated the coding information for amino acids Thr-262 through Gly-276. An arginine codon resulted at the site of ligation, but the reading frame was preserved. This plasmid was designated pCH101Δ42.

Plasmid pPY501, which contains the entire 5.3-kb polyomavirus genome cloned into the *EcoRI* site of pGEM-7Zf(+) (Promega), was used to introduce the respective mutations into intact polyomavirus genomes. pPY501 and M13mp19-VP1A were digested with *HincII*, and the 504-bp *HincII* fragment (nucleotides 2962 to 3466) from M13mp19-VP1 was used to replace the corresponding wild-type *HincII* fragment in pPY501 to generate pPY501A. Similarly, the small *HincII* fragment (now 462 bp) from pCH101Δ42 was used to replace the corresponding wild-type *HincII* fragment in pPY501 to generate pPY501Δ42. The 504-bp *HincII* fragment derived from M13mp19-VP1D was used in an identical exchange to generate a wild-type recombinant DNA designated pPY501D. In each case, digestion with *ApaI* was employed to verify that the *HincII* fragment had been cloned in the correct

orientation, and DNA was sequenced to ensure the presence of the mutation.

To create vectors which would allow expression of mutated VP1 genes in *E. coli*, the bacterial VP1 expression vector pALVP1tac (provided by R. Garcea) (24) was digested with *HindIII* and *PvuII* to generate fragments of approximately 2.3 kb (vector DNA and the 5' end of the VP1 gene) and 1.9 kb (polyomavirus nucleotides 3918 to 2032). The gel-purified 1.9-kb fragment, which included the 504-bp *HincII* fragment, was cloned into pSP64polyA (Promega), a vector lacking *HincII* sites. The resulting construct was digested with *HincII*, and the *HincII* fragment from pPY501A or pPY501Δ42 was used to replace the corresponding wild-type *HincII* fragment. The respective 1.9-kb *HindIII*-*PvuII* fragments were then excised and religated to the gel-purified 2.3-kb fragment (saved from the original digestion) to create pALVP1Atac (expressing VP1 containing the point mutation) or pALVP1Δ42tac (expressing VP1 containing the deletion mutation). Each construct was transformed into *E. coli* RB791.

Cells and transfections. Primary cultures of mouse kidney cells (MKCs) were prepared as described previously (12, 40) and grown on coverslips in 35-mm dishes. In preparation for subsequent transfection studies, full-length viral DNA was released from pPY501D, pPY501A, or pPY501Δ42 by *EcoRI* digestion, and the gel-purified polyomavirus DNA fragments (approximately 5.3 kb) were self-ligated at a high dilution (1 μg/100 μl). Transfections were performed when cells were approximately 80% confluent with 10 μg of Lipofectin (Bethesda Research Laboratories) as recommended by the manufacturer and 500 ng of circularized polyomavirus DNA per dish. The DNA-Lipofectin mixture was allowed to remain on the cells for 16 h and was then replaced with Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum. The time of addition of this medium was considered the starting time point in all transfection studies. At the indicated times following the change of medium, cells were fixed and the presence of large T antigen or the virus structural proteins in the cell nuclei was determined by indirect immunofluorescence (31) with a monoclonal antibody against large T antigen (provided by J. Bolen) or rabbit antipolyomavirus immunoglobulin G as the respective primary antibodies.

Purification of polyomavirus VP1 from *E. coli*. Wild-type or mutated polyomavirus VP1 was expressed in *E. coli* RB791 from pALVP1tac (24) and pALVP1Atac, respectively, and purified from bacterial cell lysates. VP1 expression was induced beginning at early logarithmic phase by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a 1 mM final concentration. The total growth time was approximately 6 h, after which time the cells were pelleted and frozen at -20°C until used. The initial steps of VP1 purification, including the preparation and sonication of bacterial cell lysates, were performed essentially as described by Leavitt et al. (24) with a buffer composed of 50 mM Tris (pH 7.2), 2 mM EDTA, and 250 mM NaCl. The subsequent chromatography step dictated the omission of β-mercaptoethanol from this buffer. Following centrifugation of the sonicated lysate (10,000 × g for 20 min), VP1 was purified from the supernatant by immunoaffinity chromatography. In the case of VP1 expressed from pALVP1Δ42tac, the bacterial cell pellet was sonicated in distilled water and centrifuged under conditions identical to those used in the purification of VP1 from pALVP1tac and pALVP1Atac. After centrifugation, the pellet was reserved for separation by

sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described below.

To prepare the immunoaffinity column for purification of VP1 expressed from pALVP1tac and pALVP1Atac, purified rabbit antipolyomavirus immunoglobulin G was coupled to Affi-Gel HZ hydrazide gel (Bio-Rad) as described by Chang et al. (11). The bacterial cell lysate was allowed to mix with the column matrix by rocking overnight at room temperature. The column was then treated with 20 volumes of wash buffer (50 mM Tris [pH 7.2], 2 mM EDTA, 500 mM NaCl). Bound VP1 was subsequently collected after application of elution buffer (100 mM glycine [pH 2.5], 150 mM NaCl) to the column and was immediately neutralized by collecting 0.8-ml fractions in 0.2 ml of 1 M Tris (pH 8.0)–150 mM NaCl. The amounts of protein were determined by measuring the A_{280} of the fractions, and the peak fractions were pooled. The purity of an aliquot of the recombinant VP1 was evaluated by SDS-PAGE and Western blotting with anti-polyomavirus serum.

In vitro calcium-binding assay. To serve as a control in the $^{45}\text{CaCl}_2$ -binding studies, polyomavirus virions were isolated from infected primary MKCs and purified as described previously (9). VP1 expressed in *E. coli* RB791 from pALVP1tac, pALVP1Atac, and pALVP1 Δ 42tac was obtained as described above. Polyomavirus virions and recombinant VP1 proteins (2.5 μg each) were resolved by SDS-PAGE (12.5% acrylamide). In the case of the recombinant VP1 deletion mutant, 10 μg of the *E. coli* lysate pellet (generated as described above) was electrophoresed because of the purification difficulties resulting from VP1 insolubility. The proteins were transferred to a NitroBind filter (Micron Separations, Inc., Westboro, Mass.), and the filter was incubated in 10 mM EGTA (pH 6.8) overnight with gentle shaking. The filter was briefly rinsed three times with Ca^{2+} -binding buffer (0.06 M KCl, 5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 10 mM imidazole-HCl [pH 6.8]) and then incubated in Ca^{2+} -binding buffer for 1 h with shaking. Blots were probed for 15 min in 20 ml of Ca^{2+} -binding buffer containing 200 μCi of $^{45}\text{CaCl}_2$ (ICN, Irvine, Calif.; specific activity, 14.5 mCi/mg of Ca), washed once with 20 ml of 0.47 mM CaCl_2 , allowed to air dry, and autoradiographed. All distilled water used to make the solutions described above was further processed through a chelating resin column (Sigma, St. Louis, Mo.) to remove residual cations.

The filters that had been probed with $^{45}\text{CaCl}_2$ were then subjected to Western blotting to identify the VP1 proteins. The filters were first incubated in 10 mM EGTA overnight at room temperature with gentle shaking. They were then washed three times with water to remove the $^{45}\text{Ca}^{2+}$ and autoradiographed to ensure that no radioactive signal remained. A 5% solution of nonfat dry milk in Tris-buffered saline (TBS; 50 mM Tris [pH 7.4], 150 mM NaCl) was used for blocking. The filters were then incubated overnight with rabbit antipolyomavirus sera (1 to 300 dilution), washed five times with TBS, incubated for 2 h with ^{125}I -labeled *Staphylococcus aureus* protein A (5×10^5 cpm/ml), washed five times with TBS, air dried, and autoradiographed.

Electron microscopy of recombinant VP1 proteins. Affinity-purified VP1 derived from pALVP1tac and pALVP1Atac was dialyzed against 10 mM Tris (pH 7.2)–1 mM EDTA–150 mM NaCl–15 mM β -mercaptoethanol–5% glycerol for 3 days at room temperature as described previously (39). To induce self-assembly of purified VP1 into capsid-like structures, the preparations were subsequently dialyzed for 3 days against the dialysis buffer described above supplemented with 0.5 mM CaCl_2 but lacking EDTA and β -mercaptoethanol. For

electron microscopy analysis of purified recombinant VP1 protein under alternate buffer conditions, the sample was placed on a pioloform-coated grid, and the protein was allowed to adsorb to the grid for 5 min. After a distilled-water rinse, the sample was stained with a 1% aqueous uranyl acetate solution for 5 min and examined with a Philips 201 electron microscope operating at 60 kV.

Fixation and embedding of transfected MKCs. Forty hours after transfection of MKCs (as described above), the medium was removed and the cells were rinsed with phosphate-buffered saline (PBS; 10 mM sodium phosphate [pH 7.2], 150 mM NaCl). The cells were released from the dish by a brief treatment with Viokase to better preserve cell integrity. The cells were then treated for 5 min with fixative (2% [wt/vol] paraformaldehyde, 0.5% [wt/vol] glutaraldehyde) made in Earle's salts solution, transferred to an Eppendorf tube, and pelleted. The supernatants were removed, and fresh fixative was added to the cells, which were incubated for 30 min at room temperature. Free aldehydes were quenched by rinsing the cell pellet three times (30 min total) with serum-free Dulbecco's modified Eagle's medium. Following three distilled-water rinses, the cells were dehydrated in 50% ethanol and stained with 2% [wt/vol] uranyl acetate in 70% ethanol (17). After a 70% ethanol rinse, the cells were infiltrated (4, 33) by treatment with a 1:1 mixture of LR White resin (Polysciences, Inc.) and 70% ethanol solution (1:1) for 60 min. This was followed by three treatments with 100% LR White over a 24-h time period. Curing was performed at 55°C for 2 days in fresh LR White.

Immunogold labeling of thin sections and electron microscopy. After embedding and hardening, sectioning was performed with a diamond knife by using a Reichart OMV-2 ultramicrotome, and silver sections were collected on pioloform-coated nickel grids. All subsequent immunogold-labeling procedures were performed at room temperature by floating the grids on droplets of the indicated solutions. Sections were first treated for 30 min at room temperature with a saturated solution of sodium metaperiodate to expose the antigenic sites. Following a series of washes with distilled water, the sections were blocked with 3% bovine serum albumin (BSA) made in EMG buffer (0.01 M phosphate buffer [pH 7.2], 0.5 M NaCl, 0.05% Tween 20) and were treated for 90 min with rabbit antipolyomavirus serum. After being washed with EMG buffer, the grids were treated for 90 min with a 1:100 dilution of goat anti-rabbit immunoglobulin G conjugated to colloidal gold (average particle diameter, 5 nm) (Amersham). Antibody incubations were carried out in the presence of 0.5% BSA in EMG buffer. Samples were postfixed with 2% glutaraldehyde in PBS for 10 min and poststained with 3% uranyl acetate for 15 min and lead citrate (36) for 10 min. The sections were viewed with a Philips 201 electron microscope operating at 60 kV.

RESULTS

Generation of polyomavirus genomes encoding site-directed and deletion mutations. A previous study (27), together with similarities in amino acid sequence to the calcium-binding EF hand structure, suggested that amino acids Asp-266 to Glu-277 of polyomavirus VP1 may have a role in calcium binding by this virus. Oligonucleotide-directed mutagenesis of Asp-266 was performed to determine the effects that substitution of this residue with a neutral amino acid (alanine) would have on the virus structure and function(s). In addition, a mutation which resulted in the deletion of VP1 residues Thr-262 to Gly-276 and the creation of an arginine

FIG. 1. Immunofluorescence staining of MKCs transfected with polyomavirus genomes derived from pPY501D (A and B), pPY501A (C and D), and pPY501Δ42 (E and F). Cells were fixed and stained by immunofluorescence for the presence of large T antigen (A, C, and E) or for the presence of the late structural proteins (B, D, and F).

codon at the deletion junction was generated. The strategy for introducing DNA fragments containing the mutations into intact polyomavirus genomes is described in Materials and Methods. The plasmid pPY501A contains a copy of the viral genome which encodes the mutation of Asp-266 to Ala in VP1, while pPY501Δ42 contains a copy of the viral genome that encodes the deletion mutation in VP1.

Transfection of wild-type and mutated polyomavirus genomes into primary MKCs. Primary MKCs grown on coverslips were transfected with polyomavirus DNA derived from pPY501D (wild type), pPY501A (point mutant), or pPY501Δ42 (deletion mutant). Immunofluorescence staining for the presence of large T antigen as well as for the late structural proteins was performed on cells which were fixed 44 h after the addition of serum-containing Dulbecco's modified Eagle's medium. The results, shown in Fig. 1, indicated that both large T antigen (Fig. 1A, C, and E) and the late viral proteins (Fig. 1B, D, and F) were synthesized in successfully transfected cells and properly transported to the nucleus, the site of viral assembly. This was true in cells transfected with polyomavirus DNA encoding the mutation of Asp-266 to Ala in VP1 (Fig. 1C and D) and viral DNA encoding a VP1 molecule lacking amino acids Thr-262 to Gly-276 (Fig. 1E and F) as well as in cells transfected with wild-type polyomavirus DNA (Fig. 1A and B).

A time course study was performed to examine the progression of infection in cells transfected with these DNAs. Cells on duplicate coverslips were fixed and stained for the presence of both large T antigen and the late viral

proteins at various times after transfection. Figure 2 shows the results obtained from cells transfected with DNA derived from pPY501D and pPY501A. Approximately 2% of the cells transfected with either DNA sample stained positive for the presence of viral proteins 2 days after transfection. Similar results were observed whether staining was for large T antigen (Fig. 2A) or for the late structural proteins (Fig. 2B). The percentage of cells transfected with wild-type viral DNA (pPY501D) which stained positive increased dramatically between 2 and 3 days after transfection and continued to increase throughout the course of the experiment. This increase was presumably due to infection by progeny virions which were produced in cells that took up the viral DNA. No such increase was seen in cells transfected with DNA derived from pPY501A, nor was the increase detected in cells transfected with DNA derived from pPY501Δ42 (data not shown). It is likely that the small percentage of these cells which stained positive throughout the experiment was simply the result of successful transfection events. Abundant cytopathic effects were also evident in cells transfected with the wild-type genomes, while no cytopathic effects were observed in cells transfected with DNA derived from pPY501A or pPY501Δ42 even 10 days after transfection. These results demonstrated that while expression and nuclear transport of the mutant proteins appear normal, infective progeny virions are not produced. Finally, unlike lysates from cells transfected with viral DNA from pPY501D, lysates from cells transfected with viral DNA derived from pPY501A or pPY501Δ42 were not infective (data not

FIG. 2. Time course study of transfected MKCs. Primary MKCs were transfected with DNA derived from pPY501D or pPY501A. Duplicate plates were fixed 1, 2, 3, 4, and 6 days after transfection, and the presence of large T antigen (A) or polyomavirus structural proteins (B) was assayed by indirect immunofluorescence. The percentage of cells exhibiting nuclear fluorescence at each time point was determined after 1,000 cells (500 cells on each of two coverslips) were counted. +, wild-type; Δ, point mutant.

shown). This observation confirms that infective viral progeny do not result from transfection with polyomavirus genomes encoding either the point mutation or the deletion mutation into normally susceptible cells. These results also revealed that large quantities of mutated VP1 protein could not be obtained from transfected cell cultures for further analysis.

Calcium-binding analysis of recombinant VP1 proteins. To obtain quantities of mutated polyomavirus VP1 suitable for additional analysis, we expressed the proteins in *E. coli* from versions of the VP1 expression vector, pALVP1tac (24), generated as described in Materials and Methods. pALVP1Atac expresses VP1 containing the Asp-266-to-Ala mutation, while pALVP1Δ42tac expresses VP1 containing the deletion mutation. Wild-type VP1 and VP1 containing the Asp-266-to-Ala mutation were purified from bacterial cell lysates by immunoaffinity chromatography for use in calcium-binding analysis. An aliquot from each preparation was also resolved by SDS-PAGE. Coomassie blue staining and Western blotting indicated that both proteins had been purified to near homogeneity (data not shown). Although Western blotting studies of the *E. coli* pellets generated after the initial sonication and centrifugation steps indicated that

FIG. 3. Analysis of the ^{45}Ca -binding abilities of purified viral proteins and recombinant wild-type and mutant VP1 molecules. Proteins were separated by SDS-PAGE (12.5% polyacrylamide), transferred to NitroBind, and analyzed by both a ^{45}Ca -binding assay and Western blotting. Lanes 1 and 2 contain 2.5 μg of purified polyomavirus virion proteins detected by Western blotting (lane 1) and analyzed for their ability to bind ^{45}Ca (lane 2); lanes 3 (Western blot) and 4 (^{45}Ca blot) contain 2.5 μg of purified recombinant wild-type VP1; lanes 5 (Western blot) and 6 (^{45}Ca blot) contain 2.5 μg of purified recombinant VP1 with the Asp-266-to-Ala mutation; lanes 7 (Western blot) and 8 (^{45}Ca blot) contain 10 μg of the pellet generated by centrifugation of the bacterial cell lysate from the *E. coli* strain expressing the deletion mutant. The arrow to the left of the figure identifies VP1.

VP1 was expressed from pALVP1Δ42tac, soluble VP1 was not detected in the supernatant component (data not shown). Perhaps the deletion resulted in a folding difficulty which rendered the protein insoluble under the buffer conditions used. Since we were unable to obtain the VP1 deletion mutant in a soluble form for application to the immunoaffinity column, the insoluble portion of the lysate from *E. coli* expressing this mutant protein was used in the calcium-binding analysis shown in Fig. 3.

After resolution by SDS-PAGE and blotting, proteins from purified polyomavirus virions as well as recombinant wild-type and mutated VP1 were examined for their ability to bind ^{45}Ca (Fig. 3, lanes 2, 4, 6, and 8). The isotope was then removed and the presence and location of VP1 on the filter were confirmed by Western blotting (Fig. 3, lanes 1, 3, 5, and 7). Analysis of the ^{45}Ca -binding abilities of proteins derived from purified virions (lane 2) reveals a major signal as well as several minor signals. The corresponding Western blot (lane 1) demonstrates that the principal ^{45}Ca -binding protein seen in lane 2 is VP1, consistent with previous studies (27). These studies had also shown that the low-molecular-weight ^{45}Ca -binding entities represent C-terminal VP1 fragments resulting from breakdown of the protein (27) and that the minor capsid proteins (detected by the polyclonal antisera in lane 1) do not bind ^{45}Ca .

Purified recombinant wild-type VP1 and recombinant VP1 containing the mutation of Asp-266 to Ala also bound ^{45}Ca in this assay (Fig. 3, lanes 4 and 6). Western blotting (Fig. 3, lanes 3 and 5) confirmed the identity of these proteins as VP1. Additional signals seen in lanes 3 through 6 represent recognition of proteolytic breakdown products of VP1, generated during purification. Lane 8 reveals that the recombinant VP1 deletion mutant did not bind ^{45}Ca in this assay,

FIG. 4. Electron microscopic analysis of purified recombinant wild-type and mutated polyomavirus VP1 protein under different buffer conditions. (A) VP1 expressed from pALVP1tac in buffer containing EDTA and β -mercaptoethanol but lacking calcium. (B) VP1 expressed from pALVP1tac after removal of EDTA and β -mercaptoethanol and the addition of CaCl_2 ; (C) VP1 expressed from pALVP1Atac under the conditions described for panel A; (D) VP1 expressed from pALVP1Atac under the conditions described for panel B. The bar shown in panel C is 100 nm and applies to panel A as well; the bar shown in panel D is 100 nm and applies to panel B as well.

while lane 7 identifies the location of VP1 on the filter. These data strongly suggest that amino acids Thr-262 to Gly-276 are required for calcium binding by VP1 *in vitro*. However, a mutation of Asp-266 to Ala does not prevent *in vitro* calcium binding.

Electron microscopy analysis of recombinant VP1 proteins. Salunke et al. (38, 39) demonstrated that when polyomavirus VP1 was expressed in *E. coli* RB791 from pALVP1tac and later purified under appropriate conditions, it was found in pentameric structures which were indistinguishable from viral capsomeres and could assemble into capsid-like structures upon addition of calcium. Figure 4 shows a series of electron micrographs of purified VP1 expressed from pALVP1tac and pALVP1Atac under different buffer conditions. Our inability to obtain purified VP1 from pALVP1 Δ 42tac precluded its use in these studies. Figure 4A shows purified wild-type VP1 in buffer containing EDTA and β -mercaptoethanol. The protein formed pentameric capsomere-like structures under these conditions, consistent with previous reports (38, 39). Likewise, removal of the chelator and reducing agent, coupled with the addition of CaCl_2 , resulted in assembly of the pentamers into capsid-like structures (Fig. 4B). VP1 expressed from pALVP1Atac and purified under conditions favoring pentamer formation was indistinguishable from wild-type VP1 (Fig. 4C). This similarity appears to indicate that the mutation does not cause a gross folding abnormality in the protein. However, addition of CaCl_2 did not result in assembly into capsid-like struc-

tures. Rather, aggregates of the pentamers with variable sizes resulted, as shown in Fig. 4D. Thus, assembly of the pentamers derived from the mutated VP1 protein into capsid-like structures, a process shown to be calcium dependent, was impeded.

Immunogold staining of virus proteins in transfected cells.

In an attempt to study the assembly properties of the VP1 point mutant in mammalian cells, wild-type polyomavirus DNA or DNA encoding the Asp-266-to-Ala point mutation of VP1 was first transfected into primary MKCs. Forty-eight hours after transfection, the cells were fixed and prepared for thin-section electron microscopy and immunogold labeling as described in Materials and Methods. A thin section of a normal cell which was subjected to immunogold labeling is presented in Fig. 5A, while Fig. 5B shows the insert portion of Fig. 5A at higher magnification. The complete lack of immunogold particles in these normal cells demonstrates that the gold particles do not bind to the sections nonspecifically.

An immunogold-labeled thin section of a typical cell transfected with wild-type polyomavirus DNA, presented in Fig. 5C, reveals a concentration of assembled virus particles in an electron-dense region within the nucleus. Figure 5D shows the insert portion of Fig. 5C at higher magnification. Most of the immunogold particles in this region are associated with intact virions. Closer examination of Fig. 5C reveals the presence of additional gold particles scattered throughout the nucleus. These particles do not appear to be

FIG. 5. Immunogold labeling and electron microscopy of thin sections of transfected MKCs. Specimens were prepared as described in Materials and Methods. (A) Normal cell; (B) insert region of cell shown in panel A, under higher magnification; (C) cell transfected with DNA derived from pPY501D; (D) insert region of cell shown in panel C under higher magnification; (E) cell transfected with DNA derived from pPY501A; (F) insert region 1 of cell shown in panel E, under higher magnification; (G) insert region 2 of cell shown in panel E, under higher magnification. The bar in panel E is 500 nm and applies to panels A and C as well; the bar in panel G is 100 nm and applies to panels B, D, and F as well. Nu, nucleus; Cy, cytoplasm.

associated with intact virions and are presumably bound to viral proteins which have not yet assembled into recognizable structures.

Figure 5E depicts a thin section of a representative cell transfected with polyomavirus DNA derived from pPY501A. Here, an electron-dense region is again observed, but it does not appear to be composed of assembled virus particles. Figure 5F (showing insert region 1 of Fig. 5E at higher magnification) and Fig. 5G (showing higher magnification of insert region 2) establish that immunogold particles are seen in both the electron-dense region (Fig. 5F) and in a non-electron-dense region (Fig. 5G) of the nucleus. As in Fig. 5C, closer examination of Fig. 5E reveals the presence of gold

particles throughout the nucleus. They are also observed in the cytoplasm of cells transfected with polyomavirus genomes derived from either pPY501D or pPY501A (data not shown). Unlike the situation seen in Fig. 5C and D, none of the immunogold particles are associated with structures resembling virions in any region of these cells (Fig. 5E, F, and G). In addition, their presence is not the result of nonspecific binding, since no gold particles are seen in untransfected cells (Fig. 5A and B). These immunogold particles must therefore have bound to viral proteins which were not assembled into identifiable structures. Coupled with the immunofluorescence studies of cells transfected with DNA from pPY501A which indicated the presence of

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		1	2	3	4	5	6	7	8	9	10	11	12
VP1	266	Asp	Glu	Asn	Gly	Val	Gly	Pro	Leu	Cys	Lys	Gly	Glu ₂₇₇
TnC													
II	66	Asp	Glu	Asp	Gly	Ser	Gly	Thr	Ile	Asp	Phe	Glu	Glu ₇₇
IV	142	Asp	Lys	Asn	Asn	Asp	Gly	Arg	Ile	Asp	Phe	Asp	Glu ₁₅₃
CaM													
II	56	Asp	Ala	Asp	Gly	Asn	Gly	Thr	Ile	Asp	Phe	Pro	Glu ₆₇
III	93	Asp	Lys	Asp	Gly	Asn	Gly	Tyr	Ile	Ser	Ala	Ala	Glu ₁₀₄

FIG. 6. Sequence comparison of polyomavirus VP1 amino acids Asp-266 to Glu-277 and representative 12-amino-acid calcium-binding loops from troponin C (TnC) (45) and calmodulin (CaM) (44). The Roman numerals indicate which calcium-binding loop of TnC or CaM is being shown, and the asterisks designate the amino acids which tend to be the most conserved.

virus proteins in the nucleus (Fig. 1C and D) and with electron microscopy studies of recombinant VP1 expressed from pALVP1Atac (Fig. 4C and D), the results shown in Fig. 5E, F, and G strongly suggest that this mutation results in a deficiency in virus assembly. Specifically, the calcium-dependent assembly of pentamers into capsid-like structures appears to be affected.

DISCUSSION

Studies employing purified polyomavirus virions (7–10, 47) have implicated calcium as having a role in maintaining the virion structure as well as in viral uncoating and assembly. In addition, calcium was shown to contribute to the assembly of recombinant polyomavirus VP1 expressed in *E. coli* into capsid-like structures (38, 39). Further evidence of the importance of calcium in capsid assembly was obtained when polyomavirus VP1 was expressed in *Spodoptera frugiperda* cells from a baculovirus construct (32). Here, VP1 was not assembled into recognizable structures in the cytoplasm, but it was found to form capsid-like structures in the nucleus. The authors proposed that differences in the concentrations of available calcium between the cytoplasm and the nucleus could account for this result, and they further demonstrated that addition of the calcium ionophore ionomycin to the cells resulted in cytoplasmic assembly of VP1 into capsid-like structures.

Ludlow and Consigli (27) identified a stretch of amino acids from Pro-232 to Asp-364 of polyomavirus VP1 which, itself, has calcium-binding capabilities. Within this polypeptide, a stretch of amino acids from Asp-266 to Glu-277 shows a high degree of sequence similarity to the calcium-binding portion of the EF hand motif found in many proteins (13, 42). A comparison between this domain of polyomavirus VP1 and representative EF hand loops from other calcium-binding proteins is shown in Fig. 6. These calcium-binding EF hand motifs usually contribute either six or seven oxygen ligands to the metal ion. Coordination can occur through side chain oxygens (particularly from acidic residues), main chain carbonyl oxygens, or oxygens from water molecules in the area. In these EF hand structures, amino acids at positions 1, 3, 5, 7, 9, and 12 tend to be involved in calcium coordination, with the conserved glutamic acid residue at position 12 supplying both oxygen atoms from its carboxyl group to the cation (13, 42). Although sequence conservation among EF hand motifs is not maintained at every residue, amino acids at certain positions are well conserved. A

comparison of sequences of 165 EF hands from various calcium-binding proteins revealed that amino acids at positions 1 (Asp [98%]), 3 (Asx [97%]), 6 (Gly [89%]), and 12 (Glu [86%]) are the most conserved (13). In addition, a glycine residue was found at position 4 in 51% of the sequences examined. Each of the amino acids is properly placed within the domain of polyomavirus VP1 extending from Asp-266 to Glu-277. Considerable variability exists at several other positions of the motif, including positions 2, 6, 9, 10, and 11, where no single amino acid was found to be present in more than one-third of the EF hands (13).

The data from our transfection experiments indicate that deletion of the putative calcium-binding domain or mutation of a residue within this domain does not affect viral gene expression or nuclear transport. However, these mutations do result in a defect in the production of infective viral progeny. Evidence that an assembly problem, and more specifically an inability to bind calcium, may be the cause of this defect comes from several sources. First, recombinant VP1 containing the deletion mutation was unable to bind ⁴⁵Ca in vitro. Second, VP1 molecules containing a single point mutation within the putative calcium-binding domain were unable to carry out the calcium-dependent in vitro self-assembly step from pentameric, capsomere-like structures to capsid-like structures. Finally, despite the fact that VP1 protein containing the point mutation could be detected in the nuclei of transfected cells, there was no evidence that these proteins were participating in viral assembly.

Even though it appears to be defective in the calcium-dependent assembly steps both in vitro and in vivo, VP1 containing the Asp-266-to-Ala mutation was able to bind ⁴⁵Ca in vitro. Calcium binding by native VP1 in intact polyomavirus virions is probably of a cooperative nature, with ligands for calcium binding being contributed by domains from more than one VP1 molecule. Mutation of even a single amino acid within this domain may have altered the three-dimensional relationship among the molecules in such a way that cooperative calcium binding was no longer possible. However, unlike the situation with the deletion mutant, a single amino acid substitution may not have been dramatic enough to alter the calcium-binding properties of VP1 on nitrocellulose, where such spatial relationships are less germane.

Although the high-resolution crystallographic structure of the murine polyomavirus has not yet been reported, the structure of the related simian virus 40 (SV40) has been determined to a resolution of 3.8 Å (0.38 nm) (25). This study has aided in the understanding of how the SV40 capsomere subunits interact to form a virus capsid. The C-terminal arms of the individual VP1 molecules extend away from the pentamer of origin and into a subunit of an adjacent pentamer. Deletion of the corresponding C-terminal region from polyomavirus VP1 resulted in an assembly defect in vitro (20). In SV40, the arms are locked into place by a clamp derived from sequences near the N terminus of a VP1 molecule in the invaded pentamer. A possible site of divalent cation binding to SV40 VP1 was identified in these studies. Because of solubility considerations, the trivalent cation gadolinium (Gd³⁺) was used in place of divalent calcium ions to produce difference maps between crystals treated or not treated with EGTA. It was postulated that Gd³⁺ would bind to the Ca²⁺-binding site of SV40, as was reported to occur in similar studies of tomato bushy stunt virus (22). The difference maps produced in the SV40 studies suggested that Gd³⁺ forms a bridge which links the C-terminal arm from one pentamer with an internal loop provided by a VP1

molecule in a neighboring pentamer. The investigators noted that two glutamate residues (157 and 160) from the loop of one VP1 molecule and an aspartate residue (345) from the C-terminal arm of the other VP1 molecule were in the correct location to coordinate the Gd^{3+} . Whether the results obtained with trivalent gadolinium ions can be extrapolated to divalent calcium ions in SV40 is not known for certain. In addition, the lack of a high-resolution crystallographic structure of the murine polyomavirus makes it unclear whether an identical mechanism of calcium binding is utilized by this virus. This is especially important in light of the fact that the amino acid sequences of SV40 VP1 and polyomavirus VP1 have only 50% identity (43). Therefore, significant structural variations are quite likely. In terms of spatial relationship, it is interesting that a sequence of amino acids found in SV40 VP1, which is nearly identical to Asp-266 through Glu-277 of polyomavirus VP1, is in close proximity to the SV40 calcium-binding site proposed by Liddington et al. (25).

On the basis of the available structural and biochemical data, we propose that the amino acid stretch from Asp-266 to Glu-277 of polyomavirus VP1 is involved in calcium binding. This binding would involve an intermolecular interaction which would include the EF hand-like region of one VP1 molecule and either the corresponding region or a separate region of another VP1 molecule. The sensitivity of intact virus particles to treatment with chelating agents, as previously demonstrated in virion dissociation experiments by Brady et al. (9, 10), suggests that calcium is bound to exposed regions of the capsid.

The early events of polyomavirus infection have been examined by electron microscopy (30), binding studies (6), isolation of virion-containing monopinocytotic vesicles (21), and nuclear uncoating studies (46). These studies demonstrated that uncoating of infecting virions begins upon nuclear entry, as evidenced by the isolation of uncoating intermediates in the nuclei of infected cells (46). We have postulated that the action of a calcium-binding protein(s) in this cellular compartment, together with a microenvironment which provides the necessary reducing conditions, may contribute to viral uncoating. The available information suggests that calcium is also involved in capsid assembly, a late event in the virus life cycle (8, 32, 38, 39, 48). The findings presented in the present study demonstrate that mutations in the putative calcium-binding domain affect capsid assembly both *in vitro* and *in vivo*. It remains possible that the mutations created in this study affected some aspect of capsid structure and/or assembly other than calcium binding; however, it must be stressed that the deletion mutant was unable to bind ^{45}Ca in an *in vitro* assay. In addition, the step of capsid assembly previously shown to be calcium dependent was specifically hindered in VP1 molecules containing this point mutation. This mutation may interfere with assembly either by eliminating a calcium coordination site or by leading to a local conformational change which renders the viral proteins unable to bind calcium.

In summary, although it is fairly well established that polyomavirus VP1 binds calcium as a means of maintaining its structural integrity, the exact nature of the binding is not yet understood. The X-ray crystallographic determination of the polyomavirus structure at high resolution will be beneficial in clarifying the exact nature of calcium binding by its major capsid protein VP1.

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