Identification of a mutation in the SV40 capsid protein VP1 that influences plaque morphology, vacuolization, and receptor usage

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Abstract

A plaque variant of SV40 that was first isolated in the 1960s, designated SV40-LP(KT), was molecularly cloned and subjected to sequence analysis. The genome of SV40-LP(KT) was found to be nearly identical to the previously described isolate known as 777. However, SV40-LP(KT) contained a mutation in the VP1 coding region resulting in a change of histidine 136 to tyrosine. This VP1 mutation was identified as a genetic determinant influencing a number of phenotypes associated with SV40-LP(KT) such as plaque morphology, intracellular vacuole formation, and ganglioside receptor usage.

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Introduction

Polyomaviruses are small, non-enveloped viruses with double-stranded, circular DNA genomes of approximately 5000 base pairs that have been found in numerous species of birds and mammals (Imperiale and Major, 2007). The major structural protein VP1 of the polyomavirus SV40 assembles into 72 pentamers that form the icosahedral capsid (Liddington et al., 1991; Stehle et al., 1996). The VP1 pentamer forms a barrel-like structure around a conical hollow with its wider opening towards the interior of the virus (Stehle et al., 1996). This hollow accommodates one of the two minor capsid proteins, either VP2 or VP3. Thus, for the intact virus particle, VP2 and VP3 remain largely sequestered within the capsid, while the surface residues of VP1 provide the primary functional contacts between the virus particle and its environment. These general structural features are conserved among all polyomaviruses (Stehle et al., 1994).

A key interaction between the virus particle and its host occurs during virus entry into cells. The capsid must interact with specific receptors on the cell surface that allow subsequent endocytosis of the virus particle. Early studies identified the class I major histocompatibility complex (MHC) protein as a receptor for SV40 (Atwood and Norkin, 1989; Breau et al., 1992). However, class I MHC does not appear to be the sole determinant of infectivity and tropism as the expression of class I MHC does not strictly correlate with the ability of the virus to bind to the cell surface (Basak et al., 1992; Breau et al., 1992). Recently, the ganglioside GM1 has been shown to mediate the entry of SV40 into cells. A rat cell line deficient in ganglioside synthesis can display exogenously supplied gangliosides on the cell surface; supplying such a cell with GM1 greatly facilitates SV40 infection (Tsai et al., 2003). Other polyomaviruses also use specific gangliosides for cell entry. For example, the ganglioside GT1b is used by both BK virus and murine polyomavirus (Low et al., 2006; Tsai et al., 2003).

The features on the surface of SV40 that interact with cellular receptors are unknown. The crystal structures of murine polyomavirus complexed with small sialic acid-bearing carbohydrate ligands have been determined (Stehle and Harrison,
1996, 1997; Stehle et al., 1994), but similar data are not available for SV40 and related human polyomaviruses (JC virus and BK virus). The identification of surface amino acid residues on polyomavirus capsids that function in critical aspects of the virus life cycle, such as cell entry, can be exploited in a number of ways. For example, detailed capsid structure/function data should facilitate the development of serological assays with enhanced specificity that more effectively discriminate among polyomaviruses than existing assays.

In this communication, we report the whole-genome sequence analysis of an SV40 plaque variant that we have designated as SV40-LP(KT). This isolate is closely related to previously identified isolate 777. The structural basis for several novel phenotypes associated with SV40-LP(KT) resides in a point mutation that alters a single amino acid in the capsid protein VP1. It is of particular interest that a virus bearing this VP1 mutation appears to be deficient in its ability to interact with the ganglioside GM1.

**Results**

**Sequencing of SV40-LP(KT)**

A large plaque variant of SV40 that we designate as SV40-LP (KT) was obtained from K. Takemoto, National Institutes of Health (Takemoto et al., 1966); Takemoto originally referred to this isolate as SV40-L. We have amended the designation to reflect its source. The genome of SV40-LP(KT) was molecularly cloned and subjected to sequence analysis. The sequence comparisons between SV40-LP(KT) (GenBank accession number EF579803) and two previously reported isolates of strain 777 are summarized in Table 1. SV40-LP(KT) differed from the sequence designated 777 (GenBank accession number AF332562) in only two ways: (1) SV40-LP(KT) contained a complex, non-archetypal arrangement in the regulatory region of the genome (an imperfect duplication with approximately two incomplete copies of the 72 base pair enhancer), whereas 777 has an archetypal regulatory region (containing a single copy of the enhancer); and (2) SV40-LP(KT) contained a single nucleotide alteration in the late region of the genome resulting in a coding change for the structural protein VP1 (histidine 136 altered to a tyrosine). In comparison with the 5244 base pair genome sequence termed 777* (GenBank accession number AF332562), an isolate recovered from a rodent cell transformed by 777 (Forsman et al., 2004), the 5244 base pair sequence of SV40-LP(KT) differed by only 3 nucleotides, all found in the late region of the genome (Table 1); one of the three differences reflected the previously mentioned VP1 H136 → Y change. Notably, the regulatory region of SV40-LP(KT) was identical to that of 777*.
Fig. 2. Vacuolization phenotype in CV-1 cells. Confluent monolayers of cells grown in T75 flasks were inoculated with $10^5$ pfu of the indicated virus. Phase-contrast micrographs were taken at 200× magnification on post-infection day 6 through day 9.
Fig. 3. Transmission electron micrographs of infected CV-1 cells. Confluent cells grown in 24-well culture plates were infected with 50,000 pfu/well of the indicated virus. At day 6 post-infection, the samples were fixed and prepared for electron microscopy as described in Materials and methods. (A) A cell infected by 776 at 1600× magnification. (B) A higher magnification (20,000×) of the same cell shown in panel A. (C) A cell infected by 776 at a later stage of infection at 1600× magnification. (D) A higher magnification (20,000×) of the same cell shown in panel C. (E) A cell infected by 776 VP1 H136→Y at 1600× magnification. (F) A higher magnification (20,000×) of the same cell shown in panel E. (G) A cell infected by 776 VP1 H136→Y at a later stage of infection at 1600× magnification. (H) A higher magnification (20,000×) of the same cell shown in panel G.
VP1 His136→Tyr mutation influences plaque morphology

The original assignment of SV40-LP(KT) as a large-plaque variant is based on its behavior in primary African green monkey kidney cells. However, in the CV-1 cell line (derived from African green monkey kidney cells), SV40-LP(KT) produces small plaques (Takemoto et al., 1966, 1968). We sought to determine whether or not the H136→Y VP1 mutation occurring in SV40-LP(KT) could explain this unusual plaque phenotype by introducing this mutation into the 776 strain background. As shown in Fig. 1, both SV40-LP(KT) and the 776 VP1 H136→Y mutant produced ~1 mm diameter plaques in CV-1 cells, whereas the parental 776 virus produced 3–5 mm plaques. In contrast, the plaques produced by the 776 VP1 H136→Y mutant in primary African green monkey kidney cells were similar to SV40-LP(KT) plaques (5–10 mm plaques) and were larger than those produced by 776 (3–5 mm plaques). These results demonstrate that the cell type-dependent plaque behavior of SV40-LP(KT) is conferred by the H136→Y change alone and is independent of other genetic differences between SV40-LP(KT) and 776. Despite having different plaque phenotypes in CV-1 cells, the titers of 776 and 776 VP1 H136→Y produced in CV-1 cells were comparable and typically greater than 10^7 pfu/mL (data not shown). This suggests that the VP1 mutation has minimal effect on replication efficiency in these cells.

VP1 His136→Tyr mutation confers a minimal vacuolization phenotype

During the course of studying SV40-LP(KT), we noticed that this isolate exhibited a hitherto unreported cytopathic phenotype. SV40 typically induces a distinctive cytopathology that involves the extensive formation of intracellular vacuoles (Sweet and Hilleman, 1960). In the experiment shown in Fig. 2,
confluent monolayers of CV-1 cells grown in T75 flasks were inoculated with $10^5$ pfu of the indicated virus (SV40-LP(KT), 776, or 776 VP1 H136→Y). SV40 776 induced detectable cytopathic effect (CPE) in CV-1 cells starting at day 4 post-infection. By day 6, most of the cells were extensively vacuolated. In contrast, SV40-LP(KT) produced a relatively nondescript CPE with very few vacuolated cells, and when vacuoles occurred, they tended to be smaller and less noticeable than those produced by 776. The 776 VP1 H136→Y mutant behaved in a manner similar to SV40-LP(KT) (Fig. 2). Thus, the VP1 H136→Y mutation appears to be sufficient to confer what we have termed a “minimal vacuolization” phenotype. The data suggest that a determinant of the vacuolization phenotype of SV40 maps to VP1. For all viruses, CPE was nearly complete by day 9 post-infection. Similar results were obtained with experiments using primary African green monkey kidney cells (data not shown). Thus, this phenomenon appears to be controlled by the virus rather than the host cell.

**VP1 His136→Tyr mutation diminishes the interaction between virus particles and intracellular membranes**

The possibility that a structural protein of SV40 might be involved in the formation of cytoplasmic vacuoles prompted us to visualize the relationship between virus particles and intracellular membranes through transmission electron microscopy. CV-1 cells were infected with 776 or 776 VP1 H136→Y and fixed for analysis at a late stage of CPE. Most of the cells infected by 776 were vacuolated; a typical cell is shown in Fig. 3A. Examination of the same cell at a higher magnification (Fig. 3B) revealed that the nucleus was filled with virus particles. However, relatively few particles were located in the cytoplasm, and their interaction with vacuolar membranes was not obvious. Occasionally, a cell at an even later stage of CPE was encountered (Fig. 3C). Even though the overall architecture of such a cell appeared intact, cell permeabilization had occurred to some extent as evidenced by a noticeable loss of intracellular electron density (compare Figs. 3A and C). Virus particles were abundant throughout the cytoplasm of such a cell and consistently appeared to array in close apposition to intracellular membrane surfaces (Fig. 3D). The tendency of SV40 to align on membranous structures was reported in earlier electron micrographic studies (Granboulan et al., 1963; Oshiro et al., 1967). This non-random distribution suggests that the SV40 particle has an intrinsic affinity for membranes (or some constituent of membranes). As expected, a cell infected by 776 VP1 H136→Y contained few vacuoles (Fig. 3E). Examination of the same cell at a higher magnification revealed a nucleus filled with virus particles; cytoplasmic particles were rather sparse (Fig. 3F). Again, cytoplasmic particles were observed in abundance only in cells that were somewhat permeabilized (Fig. 3G). However, the mutant particles did not appear to associate with intracellular membranes to the same extent as 776 particles (compare Figs. 3D and H).

**VP1 His136→Tyr mutation confers resistance to inhibition by soluble ganglioside GM1**

A critically important interaction between SV40 particles and cellular membranes occurs at the cell surface during virus entry. Recent studies have identified the ganglioside GM1 as a receptor for SV40 (Tsai et al., 2003). We performed plaque assays in CV-1 cells in the absence or presence of soluble gangliosides. When GM1 was present in the inoculum at a concentration of 5 μM, 776 plaque formation was inhibited by 4.3-fold. In contrast, plaque formation by 776 VP1 H136→Y was not significantly affected under the same conditions (Figs. 4A and B). The ganglioside GT1b, which can serve as a receptor for both mouse polyomavirus as well as BKV (Low et al., 2006; Tsai et al., 2003), had no significant effect on the plaque-forming efficiency of 776 or 776 VP1 H136→Y; thus, differential inhibition appears to be specific to GM1.

**Discussion**

The novel VP1 H136→Y variation harbored by SV40-LP (KT) influences several phenotypes including plaque morphology, vacuolization, intracellular membrane attachment, and resistance to inhibition by soluble ganglioside GM1. Histidine 136 is situated in the surface DE loop of VP1 and guards the
entrance to the central cavity of the VP1 pentamer, which aligns with the main axis of symmetry (Fig. 5) (Liddington et al., 1991; Stehle et al., 1996). This cavity, or surface features in its vicinity, might contain one or more binding sites for cellular factors that function as mediators in the manifestation of the aforementioned phenotypes.

One such factor may be the ganglioside GM1. Our data suggest that the DE loop of VP1 may be directly interacting with GM1. A recent study demonstrated that delivery of ganglioside GM1 to C6 rat glioma cells facilitates virus cell entry and large T-antigen expression; this enhancement is competitively inhibited when infection occurs in the presence of soluble GM1 (Tsai et al., 2003). To our knowledge, our study is the first demonstration that GM1 might also play a role in the infection of a simian cell type normally permissive to infection by SV40.

Although clarification will require the identification of other SV40 VP1 mutants, we speculate that the common denominator that underlies the varied phenotypic observations examined in our study may, in fact, be the interaction between the virus particle and ganglioside GM1. Murine polyomavirus VP1 mutants provide precedence for this idea. A small plaque (SP) strain of murine polyomavirus has a substantially higher affinity for branched disialyl receptor structures than a large plaque (LP) strain (Cahan et al., 1983). This difference in receptor interaction maps to a single amino acid change in VP1 at amino acid residue 92 in the BC loop (glutamate for LP and glycine for SP) (Freund et al., 1991). By analogy, the SV40 VP1 H136→Y alteration, which apparently reduces interaction with GM1, also clearly influences plaque morphology. Even our observation that the VP1 H136→Y mutant virus particle attaches to a lesser extent to intracellular membranes might be indicative of decreased binding to gangliosides (and related sugar moieties) contained in those membranes. However, we typically observe that 776 virus particles decorate the cytoplasmic surface of internal membranes. Carbohydrates in the form of glycolipids and glycoproteins are normally found in the lumenal surface of biosynthetic compartments. A possible explanation for this topological inconsistency can be found in a recent study demonstrating that SV40 structural proteins have intrinsic membrane-permeabilizing activity (Daniels et al., 2006); membrane permeabilization might allow the virus particles to access and bind sugar moieties exposed on the lumenal face of intracellular membranes. The permeabilized nature of the cells that exhibit virus-to-membrane attachment is consistent with this possibility.

We have found what appears to be a novel vacuolization phenotype associated with the VP1 H136→Y mutation. Vacuolization has been considered a hallmark of the cytopathology induced by SV40; in fact, early reports refer to SV40 as the “vacuolating agent” (Sweet and Hilleman, 1960). However, the mechanism that produces cytoplasmic vacuoles is not understood. Our data suggest that VP1 might be a determinant of the vacuolization phenotype. However, transmission electron micrographs demonstrate that vacuoles occur at a stage of infection when relatively few virus particles are localized in the cytosol. It is possible that VP1 monomers or pentamers (which are not readily visible by electron microscopy), rather than assembled particles, might be the direct mediators involved in the morphogenesis of cytoplasmic vacuoles. Alternatively, VP1 might produce vacuoles via an indirect mechanism (i.e., initiation of a signaling event leading to vacuole formation).

**Summary**

We report the complete genomic sequence of SV40-LP(KT) and describe the phenotypic properties of a viable SV40 mutant harboring a single amino acid change in VP1 that is found to occur in SV40-LP(KT). The mechanism of cell entry by SV40 and related polyomaviruses is an actively investigated subject (Pelkmans et al., 2001). The VP1 mutant virus that we have characterized might be a useful tool to study the dependence of SV40 endocytosis on gangliosides. Furthermore, a mutation that alters receptor usage would also be expected to influence tropism; thus, it might be possible to exploit this mutation in the development of SV40-based gene therapy vectors (Strayer et al., 2005).

**Materials and methods**

**Cells and virus stocks**

SV40 large plaque (SV40-LP(KT)) was obtained from K. Takemoto, National Institutes of Health (Takemoto et al., 1966); the stock bottle was dated 1965. CV-1 cells were obtained from the American Type Culture Collection (Manassas, Virginia). Primary African green monkey kidney cells were obtained from Diagnostic Hybrids (Athens, Ohio). All cells were grown in DMEM supplemented with 10% fetal bovine serum (HyClone; Logan, Utah) and 4 mM glutamine. A working stock of SV40-LP(KT) was generated by inoculating 0.5 mL of Takemoto’s original stock into a confluent T75 flask of primary African green monkey kidney cells.

**Cloning of SV40-LP(KT)**

Confluent flasks of primary African green monkey kidney cells were infected with 0.5 mL of Takemoto’s original stock of SV40-LP(KT). Cells were harvested when CPE was ~1 + SV40 genomic DNA was isolated according to the method of Hirt (Hirt, 1967; Peden et al., 1980). Hirt DNA was digested with BamHI and cloned into BamHI-digested pBluescript; the resulting construct was referred to as pSV-LP.

**Sequencing of SV40**

Sequencing of cloned SV40 was performed using Big Dye Terminator version 3.1 and an ABI Prizm 3100 machine. The sequencing primers are described elsewhere (Peden, et al. in press). For sequencing the SV40-LP(KT) genome, primers were spaced ~400 nucleotides apart on both strands; sequence coverage was minimally three-fold throughout the genome. The genomic sequence for SV40-LP(KT) was submitted to GenBank and assigned the accession number EF579803.
Site-directed mutagenesis of SV40

pUC19 was digested with BamHI and HindIII in the multiple cloning site, and the vector fragment was blunt-ended and recircularized; the resulting vector, termed pHM1, was disrupted for all restriction sites between (and including) BamHI and HindIII. The SV40 776 genome from pSV-B3 (Peden et al., 1980) was excised by restriction digest with BamHI, re-ligated to circles, and digested and cloned into pHM1 using the restriction enzyme Asp718 (an isoschizomer of KpnI); the resulting construct was named pSV776.1. Site-directed mutagenesis of pSV776.1 to introduce the VP1 His136→Tyr mutation was performed using the QuikChange XL Kit (Stratagene; La Jolla, CA); the resulting mutant construct, pSV776.VP1.1, was sequenced in the relevant region to verify the presence of the mutation.

Generation of infectious SV40 by transfection of cloned SV40 DNA

Two micrograms of cloned SV40 DNA (pSV-LP, pSV776.1, or pSV776.VP1.1) was digested with the cloning enzyme (either Asp718 or BamHI) and re-ligated to circles. The ligation reaction was transfected into CV-1 cells using the PolyFect reagent (Qiagen; Valencia, CA) according to the manufacturer’s protocol. The monolayer was lysed within 10 days. The lysate was frozen and thawed three times, subjected to low speed centrifugation (~200 RCF for 5 min) to remove debris, and stored frozen at -20 °C until use.

Transmission electron microscopy

CV-1 cells were plated in 24-well tissue culture plates. Confluent cells were inoculated with 50,000 pfu of virus per well. At 6 days post-infection, samples were fixed overnight at 4 °C with 2.5% glutaraldehyde in isotonic Millonig’s phosphate buffer pH 7.4. Following washes with Millonig’s phosphate buffer, the samples were post-fixed with 1% osmium tetroxide in Millonig’s phosphate buffer for 1 h and stained with 2% uranyl acetate for 1 h. The samples were then dehydrated in a graded series of ethanol solutions. After embedding in Epon 812, the samples were sectioned to 600–800 Å thickness with a Reichert Ultracut E ultramicrotome. Thin sections were mounted on copper grids and stained with 0.4% lead citrate for 1.5 min. The grids were examined using a Zeiss EM10 CA transmission electron microscope.

Plaque assay

Plaque assays were performed using CV-1 cells or primary African green monkey kidney cells. Confluent cells grown in 60-mm dishes were infected with dilutions of virus made in culture medium (1 mL inoculum/dish). Following incubation at 37 °C for 1 h, the inoculum was removed, and an overlay (4 mL) of modified Eagle’s medium with 5% fetal bovine serum, 4 mM glutamine, and 0.75% Difco Noble agar was added. Fresh overlay (3 mL) was added on day 4 and day 8. An overlay (2 mL) containing neutral red at a final concentration of 50μg/mL was added on day 13. Plaques were assessed on day 14. In order to make plaques more visible, the overlay was routinely stripped, and the cells were fixed with methanol and stained with crystal violet (0.04%). In experiments involving neutralization with gangliosides, stock solutions of gangliosides (GM1 and GT1b) obtained from Sigma (St. Louis, Missouri) were prepared in ethanol at a concentration of 1 mM. Equal volumes of virus dilution and 10 μM ganglioside solution (prepared by diluting into culture medium) were mixed and incubated at 37 °C for 15 min; thus, the final concentration of ganglioside was 5 μM. One milliliter of the mixture was added to each 60-mm dish of CV-1 cells. From this point, plaque assays were carried through as described above.

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