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Mutations in the GM1 Binding Site of Simian Virus 40 VP1 Alter Receptor Usage and Cell Tropism

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Polyomaviruses are nonenveloped viruses with capsids composed primarily of 72 pentamers of the viral VP1 protein, which forms the outer shell of the capsid and binds to cell surface oligosaccharide receptors. Highly conserved VP1 proteins from closely related polyomaviruses recognize different oligosaccharides. To determine whether amino acid changes restricted to the oligosaccharide binding site are sufficient to determine receptor specificity and how changes in receptor usage affect tropism, we studied the primate polyomavirus simian virus 40 (SV40), which uses the ganglioside GM1 as a receptor that mediates cell binding and entry. Here, we used two sequential genetic screens to isolate and characterize viable SV40 mutants with mutations in the VP1 GM1 binding site. Two of these mutants were completely resistant to GM1 neutralization, were no longer stimulated by incorporation of GM1 into cell membranes, and were unable to bind to GM1 on the cell surface. In addition, these mutant viruses displayed an infection defect in monkey cells with high levels of cell surface GM1. Interestingly, one mutant infected cells with low cell surface GM1 more efficiently than wild-type virus, apparently by utilizing a different ganglioside receptor. Our results indicate that a small number of mutations in the GM1 binding site are sufficient to alter ganglioside usage and change tropism, and they suggest that VP1 divergence is driven primarily by a requirement to accommodate specific receptors. In addition, our results suggest that GM1 binding is required for vacuole formation in permissive monkey CV-1 cells. Further study of these mutants will provide new insight into polyomavirus entry, pathogenesis, and evolution.
and stimulates infection by SV40 and SV40-derived viral vectors (28, 49). In contrast, treating SV40 preparations with GM1 neutralizes infection (49). JCV can bind to LSTc, a linear pentasaccharide present on glycolipids and glycoproteins, and uses it as an attachment receptor for infection (34). The use of LSTc rather than a ganglioside may explain the difference in the route of entry employed by JCV and its restricted cell tropism. In addition to their role at the cell surface, gangliosides mediate sorting of intracellular polyomavirus particles from the endocytic trafficking vesicles to the ER (39).

The crystal structure of the SV40 VP1 pentamer bound to GM1 pentasaccharide revealed that GM1 binds to a shallow V-shaped groove constructed from the BC, DE, and HI loops of three VP1 monomers. In this groove, VP1 directly interacts with the terminal NeuNaC and terminal galactose on the two branches of GM1. VP1 also makes minor contacts with the N-acetylgalactosamine of GM1. Because there is a rigid spacer in the SV40 VP1 groove, polysaccharides that bind to VP1 must be aligned in a Y shape to fit into the groove (36). This requirement is believed to narrow the specificity of SV40 to GM1, which has a dominant Y shape in solution (40).

As SV40, BKV, and JCV share over 70% nucleotide sequence similarity with each other, it is believed that these viruses evolved from a common ancestor (21). The VP1 sequences of SV40, BKV, and JCV are largely conserved. The structures of SV40 and JCV in complex with their respective receptors, GM1 and LSTc, show that both bind sialic acid in the same orientation and with similar contacts. A small number of additional residues outside the sialic acid binding pocket also contact the oligosaccharide and enable each virus to specifically engage distinct oligosaccharide conformations and linkages (34, 36). These residues are located in the BC, DE, and HI loops, the most divergent regions of VP1, and define the context in which sialic acid must be placed in order for it to interact with SV40 or JCV VP1. BKV also binds sialylated oligosaccharides, including the gangliosides GD1b and GT1b, but it is not known how BKV achieves specificity (26). In addition to the amino acids in the receptor-binding regions of VP1, there are numerous other differences scattered throughout VP1 proteins from different polyomaviruses. It is not known if amino acids in the SV40 binding site are sufficient to specify physiologically relevant differences in receptor usage and cell tropism or if divergent amino acids elsewhere in the VP1 structure influence the conformation of the oligosaccharide binding site and indirectly contribute to determining the specific receptors used for infection and the specific cell types that are infected.

Amino acid substitutions in VP1 can alter receptor specificity and have a profound effect on pathogenicity. A highly pathogenic strain of mPyV possesses amino acid substitutions in VP1 that decrease its affinity for its receptors, allowing the virus to spread rapidly in the host (4, 14). JCV variants with mutations in the receptor-binding site of VP1 are frequently found in the blood and cerebrospinal fluid of patients with progressive multifocal leukoencephalopathy (PML), the disease caused by this virus. These variants display a decreased ability to bind to sialic acid and altered binding to gangliosides and cells, suggesting that these mutations have changed the specificity of JCV VP1 for its cell surface receptor (18, 34, 46).

Here, we used SV40 as a model to study how differences in VP1 might determine the receptors used for polyomavirus infection and cell tropism. We hypothesized that it would be possible to change the receptor used by SV40 by introducing mutations in the GM1 binding site on VP1 and that changing the interaction with its receptor would alter the tropism of the virus. To address this hypothesis, we conducted two sequential screens to select for viable SV40 mutants with altered receptor usage, and we isolated two mutant viruses that no longer appear to use GM1 for infection. One of these viruses, which contains mutations at residues in the GM1 binding site of VP1, also displayed altered cell tropism and infected cells with low cell surface GM1 more efficiently than wild-type (WT) virus, suggesting it uses a receptor other than GM1. Strikingly, these mutations also inhibited the ability of SV40 to induce vacuolization in permissive monkey cells, a hallmark of SV40 infection, but did not impair virus production.

**Materials and Methods**

**Cells.** HeLa S3, CV-1, 293T, LLC-MK2, and Vero cells were purchased from the American Type Culture Collection (ATCC) (Manassas, VA). Normal diploid human foreskin fibroblasts (HFFs) were obtained from the Yale Skin Diseases Research Center (YSDRC). Human embryonic kidney (HEK) 293 cells were provided by Tae Hoon Kim (Yale University), and 293TT cells were provided by Chris Buck (National Cancer Institute). All the cells listed above were grown in Dulbecco’s minimal essential medium (DMEM) with 10% fetal bovine serum (FBS), standard antibiotics, 10 mM l-glutamine, and 10 mM HEPES, pH 7.2 (with standard supplements, DMEM10). Hybridomas expressing polyclonal antibody (Pab) 108 mouse anti-large T antigen (purchased from ATCC), Pab 597 mouse anti-VP1 (obtained from Edward Harlow, Harvard Medical School), and Pab BH3-FG mouse anti-VP1 were cultured in DMEM plus 20% FBS and standard supplements.

To generate HeLa S3 cells expressing short hairpin RNAs (shRNAs) against GM3 synthase (shGM3) and glucosylceramide synthase (shGC), cells were infected with concentrated retroviral stocks of pSiren encoding the appropriate shRNAs (see below) and selected in DMEM10 and 1 μg/ml puromycin for 3 days. The cells were maintained in medium with 0.5 μg/ml puromycin.

**Reagents.** Monosialoganglioside GM1 from bovine brain was purchased from Sigma-Aldrich Corp. (St. Louis, MO). Alexa Fluor 488 doxygen anti-mouse and donkey anti-rabbit IgG (H+L) and Alexa Fluor 488 cholera toxin B (CTXB) were purchased from Invitrogen (Carlsbad, CA). Rabbit anti-VP1 antibodies were purchased from Abcam (Cambridge, MA). RNeasy kits and PCR purification kits were purchased from Qiagen (Valencia, CA). iScript cDNA synthesis and SYBR green Supermix kits were purchased from Bio-Rad (Hercules, CA). The GeneMorph II domain mutagenesis kit was purchased from Agilent (Santa Clara, CA). RQ Dnase was purchased from Promega (Madison, WI), and PCR grade proteinase K was obtained from Roche Applied Sciences (Indianapolis, IN).

**Viruses.** SV40 776 genome present in either the pBR322 or the pH1 vector was excised with BamHI and KpnI, respectively, and religated into circles. The SV40 genome in pHM1 vector is designated pSV776.1. The ligation reaction mixture was then ethanol precipitated and transfected into 293TT or CV-1 cells with either TransIT 293 transfection reagent (Mirus, Madison, WI) or Lipofectamine (Invitrogen). Primary virus stocks were generated by three rounds of freeze/thaw lysis at day 4 (293TT) or 7 (CV-1) posttransfection. Subsequent virus stocks were generated by infecting CV-1 or 293T cells with primary virus, and final high-titer virus stocks were prepared by using a modified Pava1 protocol described previously (17, 33). Mutations were introduced into VP1 of pSV776.1 by a standard quick-change mutagenesis protocol.

shRNAs against GM3 synthase and glucosylceramide synthase were designed using Invitrogen Block-it RNAi Designer and cloned into the pSiren retroviral vector (Clontech, Mountain View, CA). The sequences of the shRNAs targeting GM3 synthase and glucosylceramide synthase that were cloned between the BamHI and EcoRI sites of pSiren are as follows: shGM3-1, 5'-GCACAGTGTGAGGGATATTCCAC
GAATGAAATATCCCTCAGTGGTGCC-3'; shGM3-2, 5'-GCACTACCT CGACAGTCAATGCGAACATTGACTGTCGAAGTAGTGC-3'; shGC-1, 5'-GCACTACCTGGACAAGCCTTATCGAAATAAGGCTGTTTGTCAGT TGCC-3; shGC-2, 5'-GCTTGTACCATGNGTAATCCAGAATGGTCA CCAATGCTGATAGC-3'. The pSiren vector expressing a nonspecific shRNA (shControl) was used as a control. Retrovirus stocks were generated in 293T cells as described previously. Virus was harvested at 48 and 72 h posttransfection and concentrated with Peg-It (System Biosciences, Mountain View, CA) as described previously (28, 53).

**SV40 infection and flow cytometry of large T antigen.** Cells were plated in triplicate and infected with the indicated number of virions in DMEM with 10% FBS. At the indicated times postinfection, the cells were trypsinized and washed with PBS. The cells were then fixed by adding ice-cold methanol dropwise to the cell pellet while vortexing gently, and the fixation continued for at least 20 min on ice. After centrifugation, methanol was removed, and the cells were blocked for 5 min in phosphate-buffered saline (PBS) plus 0.5% bovine serum albumin (BSA). The cell pellet was then resuspended in 100 μl of a 1:1 mixture of 5% normal donkey serum in PBS (NDS-PBS) and PAb 108 monoclonal mouse anti-large T antigen supernatant and incubated for 1 h at 37°C. The cells were then washed twice with PBS plus 0.5% BSA and resuspended in 100 μl NDS-PBS containing a 1:500 dilution of Alexa Fluor 488 donkey antimouse IgG and incubated at 37°C for 30 min. Following two washes in PBS plus 0.5% BSA, the cell pellet was resuspended in 200 μl PBS and kept on ice until analysis. The fraction of cells expressing large T antigen was measured using a 488-nm excitation and 530-nm emission filter on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) and plotted on a 650-nm versus 530-nm emission filter two-dimensional (2D) density plot. The protocol used for measuring VP1 expression was identical to the protocol used for measuring large T antigen expression, except that the cells were fixed in 10% formaldehyde and permeabilized in 10% formaldehyde with 0.2% Triton X-100. PAb 597 and PAb BH3-FG mouse anti-VP1 were used to measure VP1 expression. For all infection assays, the percentages of infected cells for each condition were corrected for the possibility of multiple infections per cell using the Poisson distribution.

**GM1 supplementation assay.** HeLa S3 cells were plated in triplicate and were either left untreated or treated with 10 μM GM1 overnight in DMEM with 1% FBS. The cells were then washed with DMEM10 and infected with the indicated number of encapsidated WT and mutant genomes. Infection was measured at the indicated times postinfection as described above.

**GM1 neutralization assay.** Equal numbers of encapsidated WT and mutant genomes were treated with 7 μM GM1 in 1 ml of DMEM with 1% FBS for 30 min at 37°C. HeLa S3 or CV-1 cells plated in triplicate were then infected with the virus for 3 h. The cells were washed with PBS and fed with DMEM10, and infection was measured at the indicated times postinfection as described above.

**Generation of mutant libraries.** The generation of the VP1 mutant library used in the first screen was described previously (32). The library contained approximately 23,000 members with an average of 1 nucleotide substitution per VP1 gene. Primary virus library stocks were generated by digesting 18 μg of the pSV776.1 library with KpnI to liberate SV40 genomes. Digestion products were then purified and transfected into four 25-cm plates of 293TT cells with 293 TransIT transfection reagent (Mirus) according to the manufacturer’s protocol. The number of infectious units present in primary virus stocks was then determined by infecting CV-1 cells with serial dilutions of virus and measuring the percentage of cells expressing large T antigen as described above.

For the library used in the second screen, mutations were introduced into A70L VP1 by using the GeneMorPh II domain mutagenesis kit according to the manufacturer’s protocol. Briefly, the A70L VP1 gene was amplified by error-prone PCR with the VP1 primers 5'-GTTTCAAGAGTTAAAACCTTGCAGAC-3' and 5'-TCACTGATCCATTGAGGGTGTTTGGTGTTTGGT TGTC-3'. The PCR conditions for this reaction were one cycle at 95°C for 2 min; 20 cycles at 95°C for 30 s, 50°C for 20 s, and 72°C for 90 s (amplification step); and one cycle at 72°C for 10 min. Parallel PCRs were performed with 25 and 30 cycles of the amplification step. The products of the PCR were pooled, electrophoresed on a 1% agarose gel, and purified. The PCR products were then digested with Accl and BamHII, PCR purified, and ligated into the BamHII/AccI sites of pSV776.1. Ligations were transformed intoSURE electropermeation–competent cells (Agilent) according to the manufacturer’s protocol. The resulting library consisted of approximately 380,000 bacterial transformants. The VP1 genes of 34 isolated clones were sequenced; 29 of 34 clones contained additional mutations with an average of 4.4 nucleotide changes per gene. Bacterial transformants were scraped in LB broth and pooled, and DNA was isolated by midiprep. Infectious virus was generated by digesting 60 μg of the A70L pSV776.1 library with KpnI to liberate SV40 genomes, religating these genomes into circles, and ethanol precipitation. DNA was then transfected into six 25-cm dishes of 293TT cells as described above. Primary virus stocks were generated by three rounds of freeze/thaw at day 4 posttransfection.

**Genetic screens.** To eliminate individual capsids containing multiple different VP1 molecules (“mixed capsids”), the primary library was cleared by infecting CV-1 cells with 2.1 × 10^6 infectious units (IU) of primary library at a low multiplicity of infection (MOI) (<0.3). At 67 h postinfection (p.i.), virus was harvested by freeze/thaw, and the titer was determined in CV-1 cells as described above. Three separate pools of 5 × 10^6 IU of virus were then treated with 5 μM GM1 in DMEM10 for 15 min at 37°C. The cells were infected with the virus overnight. At 3 days postinfection, virus was harvested by freeze/thaw. A fraction of this virus was also treated with GM1 and used to infect CV-1 cells as described above. This process was repeated two times for a total of three rounds of selection. To isolate virus from these pools, a fraction of virus was again treated with GM1 and used to infect CV-1 cells for 6 days. Viral genomes were then harvested by using the Hirt preparation protocol. Briefly, infected CV-1 cells were treated with 2 ml of Hirt solution (0.6% SDS, 10 mM EDTA) for 30 min at room temperature. The cells were then scraped in Hirt solution and treated with 600 μl of 5 M NaCl at 4°C overnight. Cellular DNA was removed by centrifugation at 15,000 rpm for 20 min. The supernatant was collected, supplemented with 100 μl of 10% SDS, and treated with 50 μg/ml of proteinase K for 6 h at 37°C. Viral genomes were then isolated by sequential phenol extraction, phenol-chloroform extraction, isopropanol precipitation, and ethanol precipitation. VP1 genes were excised from Hirt genomes by digestion with Accl and BamHII. VP1 fragments were gel purified and ligated into pSV776.1, and the ligations were then transformed into Sure cells.

In the second screen, 293TT and CV-1 cells were infected with 3.5 × 10^5 infectious units of the library at an MOI of 0.3 to clear the library. At 2.5 (CV-1) and 3 (293TT) days postinfection, virus was harvested by freeze/thaw and pooled; 5.6 × 10^5 IU of virus was then treated with 12 μM GM1 at 37°C for 30 min or left untreated and used to infect two separate dishes of 293TT cells. At 4.5 days postinfection, virus was harvested by freeze/thaw. A fraction of this virus was treated with GM1 or left untreated and used to infect 293TT cells as described above. This process was repeated two times for a total of three rounds of selection. To harvest virus genomes, viruses isolated after three rounds of selection was treated with 5 μM GM1 or left untreated and added to 293TT cells. At 5 days postinfection, virus genomes were extracted by the Hirt protocol, and the VP1 gene was cloned into pSV776.1 as described above.

**Titering virus by qPCR.** The protocol used for titering encapsidated genomes was adapted from reference 48. Briefly, 5 μl of virus stocks was treated with 4 μl of RQ1 DNase (Promega) in 100 μl of DNase buffer (50 mM Tris-HCl, pH 7.6, 10 mM MgCl2) for 1 h at 37°C. After inactivating the DNase at 75°C for 30 min, samples were supplemented with 13 μl of 10× PK buffer (100 mM Tris-HCl, pH 8.0, 100 mM EDTA, 2.5% SDS) and treated with 50 μg of proteinase K (Roche) for 1 h at 37°C. DNA was isolated from the samples by using a PCR purification kit (Qiagen). The extracted DNA was then diluted 1/100 in H2O. The number of encapsidated genomes isolated was measured by quantitative PCR (qPCR).
Briefly, PCR assays were set up in triplicate using 10 μl iQ SYBR green Supermix, 8.2 μl of H₂O, 0.8 μl of 5 μM primer set, and 1 μl of diluted viral DNA, and reactions were run on a Bio-Rad MyiQ Single-Color Real-Time PCR detection system. The primers used for quantitative PCR (5'-GAGGAGGTTAGGGTTTATGAGGA-3' and 5'-CATCAATGTTCTCTGGA-3') annealed to a region of VP1 with no mutations. The number of genomes present in each virus stock was calculated by comparing the critical threshold values of virus stocks to critical threshold values of 10-fold serial dilutions (10⁹ to 10³ genomes/μl) of pSV776.1 run on the same plate.

qRT-PCR. Quantitative reverse transcriptase PCR (qRT-PCR) and primer design were performed as described previously (19). Briefly, total RNA was harvested using the RNeasy kit with DNase treatment. A microgram of RNA was used as a template for cDNA synthesis using an iScript cDNA synthesis kit. qRT-PCR was performed using iQ SYBR green Supermix with 40 ng cDNA per 20-μl reaction mixture and the Bio-Rad MyiQ Single-Color Real-Time PCR detection system. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts were detected using primers 5'-CAGCCTCAGATCTACAGCA-3' and 5'-TGTTGTCATGTCCTGTTCCA-3'. GM3 synthease transcripts were detected using the primers 5'-CTGCGCTTGGACATCTCCAGT-3' and 5'-CGATTTGTCGGAGGAGTTAGGGTTTATGAGGA-3'.

VP1 pentamer expression and purification. Recombinant WT and mutant VP1 proteins were truncated after amino acid position 306 and expressed from the pGEX-4T2 plasmid (GE Healthcare, Piscataway, NJ) as glutathione S-transferase (GST) fusion proteins in Escherichia coli BL21 (DE3) cells (Agilent). Protein expression and purification were performed as described previously (5), with some modifications. Briefly, cells were grown at 37°C in 2× yeast extract and tryptone (YT) broth supplemented with ampicillin (100 μg/ml) to an optical density at 600 nm of ~0.2. The culture was cooled to 25°C, protein expression was induced with the addition of 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and the culture was incubated overnight at 25°C (>16 h). Cell pellets were resuspended in buffer L (50 mM Tris, pH 8.0, 200 mM NaCl, 1 mM EDTA, 5% glycerol, 5 mM dithiothreitol [DTT]) supplemented with protease inhibitors (Complete Protease; Roche). The cells were lysed by incubating them with 0.1% deoxycholate and 0.5 mg/ml lysozyme for 30 min on ice, followed by DNase I (40 units supplemented with 5 mM MnCl2) treatment for 1 h, and finally passed through a French press two times at 1,000 lb/in². The cell lysate was clarified by centrifugation, and the supernatant was applied to a glutathione-Sepharose column (GE Healthcare) equilibrated with buffer L. The flowthrough was collected by gravity filtration and reapplied to the column. The column was then washed with 5 to 10 volumes of buffer L until no protein was detectable in the wash. The protein was cleaved from the GST tag, still immobilized on the glutathione-Sepharose column, by overnight incubation at 4°C with thrombin. Peak fractions were collected, and protein concentrations were determined by a Bradford assay. The eluted protein was further purified and concentrated on a phosphocellulose cation-exchange column. Protein purity was confirmed by SDS-polyacrylamide gel electrophoresis and Coomassie blue staining, and pentamer formation was confirmed by negative staining with uranyl acetate and electron microscopy.

VP1 pentamer binding. HeLa S3 cells were treated with 20 μM GM1 or left untreated as described above. The cells were then washed with DMEM10 and treated with 0.25% trypsin for 15 min at 37°C. To neutralize the trypsin, the cells were washed with DMEM10 and chilled on ice for 10 min in PBS. The cells were then treated with 1 μg of pentamers in DMEM without serum for 1 h on ice. The cells were washed with ice-cold PBS and fixed with 1% paraformaldehyde diluted in PBS. The cell pellet was then resuspended in 100 μl of PBS containing a 1:400 dilution of rabbit anti-VP1 and incubated at room temperature for 1 h. The cells were washed two times with PBS and resuspended in 100 μl of PBS containing a 1:500 dilution of Alexa Fluor 488 donkey anti-rabbit IgG for 30 min. Following two washes in PBS, the cell pellet was resuspended in 200 μl of PBS and kept on ice until analysis. VP1 binding was measured using a 488-nm excitation and 530-nm emission filter on a FACS Calibur flow cytometer.

Cholera toxin B binding. Cells were mock treated or treated with GM1 as described above. After removing unincorporated GM1, the cells were trypsinized, neutralized, and washed once with ice-cold PBS. The cells were then resuspended in 100 μl of ice-cold DMEM with 1 μg/ml Alexa Fluor 488-labeled CTXB and incubated at 4°C for 30 min. The cells were then washed with cold PBS, resuspended in PBS, and kept on ice until analysis. CTX binding was analyzed using a 488-nm excitation and 530-nm emission filter on a FACS Calibur flow cytometer.

Virus production assay. WT and A70L/F75L/H129Q viruses were excised from their vector with KpnI and religated into circles overnight. The ligation was then ethanol precipitated and transfected into CV-1 cells in triplicate as described above. At 80 h posttransfection, virus was harvested by three rounds of freeze/thaw. The amount of virus produced was determined by qPCR as described above.

Molecular modeling. The structure of SV40 VP1 in complex with GM1 was uploaded into COOT (11), and residues A70, F75, H129, and N138 were mutated accordingly. All possible rotamers were evaluated, and in each case, the most plausible rotamer was chosen. In addition, energy minimization calculations were carried out for each of the two most likely rotamers of leucine, valine, and threonine in the mutants A70L, A70V, and A70T, as well as the most abundant rotamers for F75L and N138Y. Water, small molecules, and carbohydrate ligands were deleted from the structures, which were then subjected to simulated annealing in PHENIX (1) from 500 to 300 K in 20 K steps. To assess the effects of the mutations, all residues within a distance of 7 Å from the mutated residues in the resulting Protein Data Bank (PDB) files were superimposed over the ones before energy minimization in COOT. The root mean square deviation (RMSD) values of the superimpositions were between 0.21 and 0.32 for all atoms of one binding site, indicating only very small structural changes in the area of the binding pocket surrounding residue 70 upon mutation. Images were created using PYMOL (43).

Statistical analysis. A two-tailed t test was used for all statistical analysis of data.

RESULTS

Design of a screen for SV40 mutants that are resistant to GM1 neutralization. Because GM1 binds to the major SV40 capsid protein, VP1, we reasoned that mutations in VP1 might eliminate the requirement for GM1 in infection. Therefore, we used libraries of VP1 mutants to isolate viruses that no longer used GM1 for infection. We previously constructed a library of viable SV40 mutants with VP1 mutagenized by error-prone PCR and used this library to identify the VP1 epitope recognized by neutralizing monoclonal antibodies (31). The library contains approximately 23,000 members, with an average of one nucleotide substitution per mutant. To identify SV40 mutants that no longer required GM1 for infection, we took advantage of the ability of purified GM1 to neutralize WT SV40. Treatment of SV40 with GM1 at 37°C prior to infection of CV-1 cells, a monkey cell line permissive for SV40 infection, inhibits infection (49). To identify viruses that use a different receptor, we selected mutant viruses that escaped neutralization by GM1. We note that the experiments described here used NeuNAGc-GM1 (designated GM1) purchased from a commercial source because NeuNGc-GM1 is not readily available.

The design of this genetic screen is shown in Fig. 1A. CV-1 cells were first infected with primary stocks of the virus library at an MOI of <0.3 infectious units/cell to minimize the number of coinfected cells that produced capsids comprised of more than one type of VP1 (“mixed capsids”). After one round of infection, replication, and assembly, the resulting virus was harvested by freeze/thaw and used for the subsequent screen. We treated three
separate pools of mutant virus stocks with NeuNAc-GM1 for 15 min at 37°C and then infected CV-1 cells. After 3 days, virus was harvested, similarly treated with GM1, and used to infect CV-1 cells as before. This process was repeated two times for a total of three rounds of selection. To test whether the selection enriched for viruses that were resistant to GM1 neutralization, WT SV40 and mutant viruses harvested after three rounds of selection were treated with GM1 or left untreated. CV-1 cells were then infected with these virus preparations, and at 24 h p.i., the efficiency of infection was determined by flow cytometry for large T antigen. The results of a typical experiment are presented and represent the average of triplicate samples. Similar results were obtained in at least five independent experiments. The error bars indicate standard deviations.

Viruses with mutations at residue 70 of VP1 are resistant to GM1 neutralization. In order to isolate individual GM1-resistant mutant viruses, viruses harvested after three rounds of screening were treated with GM1 and used to infect CV-1 cells. Six days postinfection, SV40 genomes were isolated, and the AccI-to-BamHI fragment of the VP1 gene was cloned into the WT SV40 backbone in the plasmid pSV776.1. At least 10 clones from each pool were sequenced (Table 1). Two WT genomes and five different mutants were identified from 31 complete VP1 sequences. All of the mutant viruses contained either an alanine-to-valine (27/31) or an alanine-to-threonine (2/31) substitution at residue 70 of VP1; most mutants also contained mutations at other positions. We focused subsequent studies on mutant viruses containing the single substitutions A70V and A70T.

To determine whether these mutants were resistant to GM1 neutralization, full-length mutant virus genomes were excised from the vector and transfected into CV-1 cells. After 7 days, virus was harvested and treated with DNase to digest DNA that was not packaged into virus particles. Encapsidated viral genomes were quantitated by quantitative PCR (qPCR) to assess the relative numbers of virions in the preparation. Equal numbers of WT, A70V, A70T, and H136Y (32) virions were treated with GM1 or left untreated and added to CV-1 cells. At 24 h p.i., the efficiency of infection was determined by flow cytometry for large T antigen (Fig. 1B). Infection by WT SV40 was reduced 20-fold by GM1 treatment. Strikingly, infection by the A70V mutant virus not only was resistant to neutralization, but was actually slightly stimulated by GM1. Infection by the A70T and H136Y mutant viruses was reduced by 5-fold and 2.5-fold, respectively, by GM1, indicating that the mutants were partially resistant to GM1 neutralization. Because only the VP1 fragment from the mutant genomes was cloned into the WT SV40 genome, the phenotype of the mutants can unequivocally be assigned to the mutations present in VP1. To assess the consequences of other hydrophobic amino acids at residue 70, leucine and isoleucine substitutions were introduced at this position, and mutant viruses were generated. These substitutions also resulted in a complete block to GM1 neutralization, similar to the A70V substitution (Fig. 1B). Examination of the structure of the WT SV40 VP1 pentamer bound to GM1 (Fig. 2) revealed that the side chain of the alanine at position 70 directly interacts with the terminal galactose of GM1 (a distance of 4 Å). Larger side chains at position 70 would result in unfavorable contacts with the galactose (see Fig. 1A). Taken together, these data suggested that inserting a larger amino acid at residue 70 of VP1 altered the ability of SV40 to interact with GM1 and therefore decreased the ability of GM1 to neutralize infection.

**Mutations at A70 reduce the ability of SV40 to use GM1 for infection.** We next tested the ability of the A70 mutant viruses (e.g., A70V, A70T, A70L, and A70I) to use GM1 for infection by

![FIG 1 Design of a screen to select for SV40 mutant viruses that are resistant to GM1 neutralization. (A) Schematic outline of the screen to isolate GM1 neutralization-resistant mutant viruses. (B) The WT and indicated mutant viruses were left untreated (black bars) or treated with 7 μM GM1 for 30 min at 37°C (gray bars). CV-1 cells were then infected with 1,000 virions/cell for 3 h. The fraction of cells expressing large T antigen was measured by immunostaining and flow cytometry at 24 h p.i. and is displayed as percent infection relative to cells infected with the respective untreated virus. The results of a typical experiment are presented and represent the average of triplicate samples. Similar results were obtained in at least five independent experiments. The error bars indicate standard deviations.](http://jvi.asm.org/)

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**TABLE 1 Accl-to-BamHI fragments of VP1 excised from harvested genomes, cloned, and sequenced**
conducting a GM1 supplementation assay in HeLa S3 cells. These cells have significantly lower levels of cell surface GM1 than CV-1 cells (as assessed by cell surface binding of fluorescently labeled cholera toxin B [CTXB], a bacterial toxin subunit that binds to GM1 [7]) (Fig. 3A). HeLa S3 cells were incubated with GM1 overnight to allow incorporation of GM1 into the plasma membrane (Fig. 3A). The cells were then washed to remove unincorporated GM1 and infected with equal numbers of WT and mutant virions. Infection efficiency was determined at 48 h p.i. by flow cytometry. Addition of GM1 to HeLa S3 cells stimulated infection by WT virus 4.5-fold, but infection by the A70 mutant viruses increased only 2- to 3-fold relative to untreated cells, suggesting that these mutants have a partial defect in the ability to use GM1 for infection (Fig. 3B). The A70V, A70I, and A70L mutant viruses, which were completely resistant to GM1 neutralization, displayed the least stimulation by GM1. The fact that addition of GM1 to cells stimulates infection by the A70 mutant viruses suggested that these mutants can still interact with GM1.

We next measured the effects of these mutations on the efficiency of infection. CV-1 cells were infected with equal numbers of WT and mutant virions. At 24 h p.i., infection efficiency was measured by flow cytometry for large T antigen. Figure 3C shows that the A70V, A70I, and A70L mutant viruses had a 3- to 4-fold reduction in their ability to infect CV-1 cells compared with the WT, while A70T and H136Y mutants showed a modest reduction in activity. These data indicated that more A70 mutant virus particles are needed to infect the same number of CV-1 cells as WT virus. Since these mutants are resistant to GM1 neutralization and are impaired in their ability to be stimulated by the addition of GM1 to cells, this decrease in infectivity likely represents a decrease in the ability of the mutant viruses to use GM1 for infection of CV-1 cells.

A screen for SV40 mutants that no longer use GM1. The results reported above described the isolation of SV40 mutants re-
Treat A70L library virus with GM1 or leave untreated

\[ \text{GM1} \]

↓

2x

Infect 293TT cells

↓

5 days

Harvest newly synthesized virus

↓

Infect 293TT cells

↓

4.5 days

Harvest viral genomes

FIG 4 Design of a screen to isolate mutant viruses that no longer use GM1. (A) Outline of screen conducted to isolate SV40 mutant viruses that do not use GM1 for infection. (B) Equal numbers of WT SV40 or the indicated mutant virions were left untreated (black bars) or treated with 7 μM GM1 (gray bars). HeLa S3 cells were then infected with 25,000 virions/cell, and infection was measured 48 h p.i. as in Fig. 1B. The results of a typical experiment are presented and represent the average of triplicate samples. Similar results were obtained in six independent experiments. The asterisks indicate a significant difference in the fold change in infection of cells with GM1-treated virus relative to cells infected with the respective untreated virus (*, $P < 0.04$; **, $P < 0.002$). The error bars indicate standard deviations. (C) HeLa S3 cells were treated with 25,000 virions/cell, and infection was measured and is presented as in Fig. 3B. The results of a typical experiment are presented and represent the average of triplicate samples. Similar results were obtained in five independent experiments. The asterisks indicate a significant difference in the fold change in infection of cells with GM1 relative to untreated cells (*, $P < 0.03$; ***, $P < 0.0004$).

TABLE 2 Accl-to-BamHI fragments of VP1 excised from harvested genomes, cloned, and sequenced

<table>
<thead>
<tr>
<th>Sequence</th>
<th>No. of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pool 1 (no GM1)</td>
</tr>
<tr>
<td>A70L</td>
<td>2</td>
</tr>
<tr>
<td>A70L/T273P</td>
<td>2</td>
</tr>
<tr>
<td>A70L/F75L/H129Q</td>
<td>1</td>
</tr>
<tr>
<td>A70L/T242A/T273P</td>
<td>1</td>
</tr>
<tr>
<td>A70L/Q74R</td>
<td>1</td>
</tr>
<tr>
<td>A70L/Q54R/V184G</td>
<td>1</td>
</tr>
<tr>
<td>A70L/Q54R/V184G/Y285S</td>
<td>1</td>
</tr>
<tr>
<td>A70L/N138Y</td>
<td>1</td>
</tr>
<tr>
<td>A70L/E83A/N138K</td>
<td>1</td>
</tr>
<tr>
<td>A70L/Q117H</td>
<td>1</td>
</tr>
<tr>
<td>A70L/A13V/N138Y</td>
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</tr>
<tr>
<td>A70L/E160D/V184F</td>
<td>0</td>
</tr>
<tr>
<td>A70L/N138K</td>
<td>0</td>
</tr>
</tbody>
</table>

Total 13 12
chain is in the vicinity of the GM1 binding site but does not form direct contacts with the ganglioside. Because the A70L/N138Y and A70L/F75L/H129Q mutant viruses contained mutations at residues that directly interact with GM1, we investigated their phenotypes further.

A70L/N138Y and A70L/F75L/H129Q mutant viruses are neither neutralized nor stimulated by GM1. To determine whether the mutant viruses isolated from this screen were still resistant to GM1 neutralization, we treated virus with GM1 or left virus untreated, as described above, and then infected HeLa S3 cells. The A70L/N138Y and A70L/F75L/H129Q viruses were resistant to GM1 neutralization and, unlike the A70L virus, treating these mutant viruses with GM1 did not stimulate infection (Fig. 4B). When GM1 was added to HeLa S3 cells, infection with the WT and A70L viruses was stimulated, whereas infection by the A70L/N138Y and A70L/F75L/H129Q mutant viruses was not (Fig. 4C). Similar results were obtained when GM1 was added to 293TT cells (data not shown). These data demonstrated that the A70L/N138Y and A70L/F75L/H129Q mutant viruses no longer used GM1 for infection. Infection by the other mutant viruses isolated from this screen was stimulated by the addition of GM1 to cells to an extent comparable to that of A70L mutant virus (data not shown), implying that they still used GM1 for infection. These mutants were not studied further.

Mutant viruses with an A70L mutation cannot bind to GM1 on the cell surface. Because mutant viruses with amino acid substitutions at residue 70 are resistant to GM1 neutralization and have a defect in their ability to be stimulated by adding GM1 to cells, we hypothesized that these mutants were unable to bind to GM1. To measure the binding of VP1 to GM1 on the cell surface, we purified recombinant VP1 pentamers with a C-terminal truncation after position 306, which prevents formation of higher-order VP1 structures, and confirmed pentamer formation by electron microscopy (data not shown). HeLa S3 cells were treated with GM1 overnight or left untreated. After removal of unincorporated GM1, the cells were harvested with trypsin, which eliminates background pentamer binding without affecting GM1 surface levels (data not shown), and then incubated with WT, A70L, A70L/N138Y, and A70L/F75L/H129Q recombinant VP1 pentamers on ice for 1 h. The cells were then washed to remove unbound pentamers, and bound VP1 was detected by immunostaining and flow cytometry. In the absence of GM1, neither WT nor mutant VP1 pentamers bound cells (Fig. 5, black lines). Addition of GM1 resulted in a dramatic increase in the binding of WT pentamers to cells (Fig. 5, blue lines) but did not stimulate binding by the mutant pentamers. GM1 addition also stimulated binding of intact WT capsids but did not stimulate binding of the any of the three types of mutant capsids (data not shown). Thus, the introduction of the A70L substitution prevented VP1 pentamers or capsids from binding to GM1 on the cell surface, as measured by flow cytometry, consistent with the biological evidence that these viruses are impaired in their interaction with GM1.

The A70L/F75L/H129Q VP1 mutant virus has altered cell tropism. Although GM1 binding was eliminated by the VP1 mutations, the mutant viruses were still able to infect cells. To test whether the tropism of these viruses was changed, we infected a panel of cell lines with an equivalent number of WT and mutant virions. Infection efficiency was then assessed by flow cytometry for large T antigen (in CV-1 and HeLa S3 cells and HFFs) or VP1 (in 293TT cells, which constitutively express T antigen). The A70L mutant virus infected all cell lines 2- to 3-fold less efficiently than WT virus (Fig. 6A). Like A70L, the double- and triple-mutant viruses infected monkey CV-1 cells 3-fold less than WT virus. In contrast, infection by the A70L/F75L/H129Q mutant virus was increased 3.5- to 4-fold compared with WT virus in 293TT and HeLa S3 cells, both of which display lower levels of cell surface GM1 than CV-1 cells. Infection of HeLa S3 and 293TT cells by the A70L/N138Y mutant virus was restored to the level seen for WT virus in these cells. These results indicated that the substitutions altered the cell tropism of SV40 and allowed the mutant virus to infect cells with low levels of GM1 more efficiently than WT virus. This change in cell tropism appeared to be cell type specific rather than species specific, because the mutant viruses failed to infect primary HFFs. In addition, neither the WT nor the mutant viruses were able to infect primary human keratinocytes (data not shown).

To further analyze this difference in tropism, we compared the abilities of WT and mutant viruses to infect additional cell lines, including kidney cells, a cell type infected by SV40 in vivo. HEK 293 (human), VERO (African green monkey kidney), and LLC-MK2 (rhesus macaque kidney) cells were infected with equal numbers of virions, and infection efficiency was measured by flow cytometry. As expected, infection by the A70L mutant was reduced in all three cell lines compared with the WT (Fig. 6B). However, although the A70L/N138Y and A70L/F75L/H129Q mutant...
viruses did not infect HEK 293 cells and infection was reduced 2.5-fold in Vero cells compared with WT virus, the mutants infected LLC-MK2 cells almost twice as well as WT virus. Taken together, these data demonstrated that altering the GM1 binding site of SV40 changed the tropism of the A70L/F75L/H129Q mutant virus, presumably by allowing it to utilize a receptor other than GM1.

All three substitutions in A70L/F75L/H129Q VP1 are required for the change in cell tropism. One or both of the additional substitutions in A70L/F75L/H129Q VP1 must contribute to the altered receptor specificity and change in tropism. In order to determine which amino acid substitution(s) altered receptor usage, we introduced F75L or H129Q separately into A70L VP1 and measured how these individual changes affected GM1 usage. Like the A70L/F75L/H129Q virus, both double-mutant viruses were completely resistant to GM1 neutralization (data not shown). Infections by the A70L and A70L/F75L mutant viruses were stimulated to similar extents by the addition of GM1 to HeLa S3 cells, while the A70L/H129Q mutant virus was not stimulated (Fig. 7A). These data suggest that H129Q further interferes with the ability of SV40 to use GM1 for infection. We next measured the effects of these mutations on the tropism of SV40. CV-1 and HeLa S3 cells were infected with equal numbers of virions, and infection efficiency was measured by flow cytometry for large T antigen. All of the mutants infected CV-1 cells less efficiently than WT virus. However, although the A70L mutant displayed a reduced ability to infect HeLa S3 cells, the A70L/F75L and A70L/H129Q viruses infected these cells as efficiently as WT virus, and infection by the triple-mutant virus was increased 3.5-fold compared with the WT virus. Although the F75L and H129Q substitutions can alter the tropism of the A70L virus, both of these substitutions appear to be needed for optimal interactions with a novel receptor and maximum infection of HeLa S3 cells.

Mutants with altered cell tropism still require gangliosides for infection. Because the A70L/F75L/H129Q and A70L/N138Y mutant viruses are no longer stimulated by GM1 and the A70L/F75L/H129Q virus can infect cell lines with low levels of GM1 more efficiently than WT virus, we hypothesized that these mutants were using an alternative molecule for infection. Since most polyomaviruses use gangliosides, we tested whether these mutant viruses utilized gangliosides other than GM1. We used RNA interference to repress levels of GM3 synthase mRNA, an enzyme that is essential for the synthesis of GM3, a simple ganglioside from which the larger gangliosides, including GM1, GD1b, and GT1b, are derived (54). A control shRNA (shControl) or an shRNA against GM3 synthase was stably expressed in HeLa S3 cells using retrovirus transduction, and repression of GM3 synthase mRNA levels in these cells was confirmed by qRT-PCR (Fig. 8A). As expected, cells knocked down for GM3 synthase displayed significantly lower levels of surface GM1 than control cells, as assessed by CTXB binding (data not shown). We infected these cells with equal numbers of WT and mutant virions and measured...
infection by flow cytometry. Interestingly, the ability of WT and mutant viruses to infect cells expressing shGM3-2 was inhibited greater than 15-fold compared with the cells expressing shControl (Fig. 8B). A second shRNA against GM3 synthase also inhibited WT and mutant infection, as did shRNAs against glucosylceramide synthase, another enzyme essential for ganglioside synthesis (data not shown). These results implied that the mutant viruses utilized a ganglioside other than GM1. The shRNA against GM3 synthase had no effect on infection by human papillomavirus type 16 pseudovirus (data not shown), which does not use gangliosides for infection.

To confirm that the block to infection was the result of repression of ganglioside synthesis, we conducted a rescue experiment. Incubation of cells expressing shGM3-2 with GM1 overnight not only restored WT and A70L SV40 infection, but also stimulated infection 7-fold and 2-fold, respectively, compared with cells expressing control shRNA (Fig. 8B). These data indicated that the block to WT and A70L SV40 infection was specific to inhibition of ganglioside synthesis and not the result of an off-target effect of the shRNA. In contrast, even after GM1 treatment, the ability of A70L/N138Y and A70L/F75L/H129Q mutants to infect knockdown cells was still dramatically reduced relative to cells expressing shControl, as expected for viruses that do not interact with GM1. Taken together, these data demonstrated that WT SV40 requires endogenous gangliosides for infection and that the A70L/N138Y and A70L/F75L/H129Q mutant viruses require gangliosides other than GM1.

Substitutions at A70 in VP1 inhibit the ability of SV40 to induce vacuolization in CV-1 cells. One of the hallmarks of SV40 infection of some strains of permissive monkey cells is marked vacuolization at late times after infection. We previously reported that following infection by the H136Y mutant, the number of infected cells with vacuoles was significantly reduced compared with cells infected with WT SV40 (32). In order to determine whether there was a consistent correlation between the ability of SV40 to use GM1 and form vacuoles, we infected CV-1 cells with a panel of mutant viruses at an MOI of 0.25 and examined the cells 4 days later. Figure 9A shows that the GM1 neutralization-resistant mutant viruses also had a significant defect in the ability to induce the formation of vacuoles. We observed an occasional cell containing vacuoles following infection with A70T, but no vacuolization occurred in cells infected with A70V, A70I, or any mutant virus containing the A70L substitution, including A70L/F75L/H129Q (Fig. 9A and data not shown). These results suggested that the more resistant a virus is to GM1 neutralization, the greater the defect in vacuolization. Although little vacuolization is seen in CV-1 cells infected with the defective mutants, the cells eventually lysed, and cells transfected with mutant virus DNA produced at least as many virions as cells transfected with the WT viral genome (Fig. 9B and data not shown). Taken together, these data indicated that there is a correlation between the ability of SV40 to interact with GM1 and its ability to induce the formation of vacuoles.

DISCUSSION

Altering the receptors used by viruses for cell attachment and entry can have dramatic effects on virus tropism, pathogenicity, and virulence. In this report, we demonstrated that amino acid substitutions in the receptor-binding site of SV40 VP1 altered receptor usage and tropism. We first selected viruses that were resistant to GM1 neutralization by treating a library of viable SV40 VP1 mutants with GM1 prior to infection of CV-1 cells. Mutant viruses in which the alanine at residue 70 was replaced with larger hydrophobic amino acids were resistant to GM1 neutralization. VP1 pentamers containing the A70L mutation failed to bind to GM1 on the surfaces of cells, directly demonstrating that the mutation interfered with GM1 binding. Additional biophysical studies.
showed that the A70L mutation diminishes but does not eliminate GM1 binding (unpublished results), consistent with the ability of GM1 addition to weakly stimulate infection by the mutant virus. In the wild-type VP1 pentamer, A70 directly interacts with the terminal galactose moiety of GM1 via van der Waals interactions (36). Molecular modeling and energy minimization calculations predict that each of the allowed conformations of the mutant leucine side chain would result in steric clashes with the terminal galactose of GM1 (Fig. 10A), accounting for the binding defect and the phenotype of the mutant. The calculations further show that mutation of A70, N138, and F75 leads only to minor structural changes in the area surrounding these residues.

It is interesting that the mutant viruses that are fully resistant to GM1 neutralization can still be stimulated by GM1, although to a lesser extent than WT SV40. In fact, treatment of A70V, A70I, and A70L viruses with GM1 prior to infection actually increased infection, presumably because excess free GM1 is incorporated into cell membranes, where it can serve, albeit poorly, as a cell surface SV40 receptor. This stimulatory effect might be explained by the high valency of the virus bound to multiple molecules of GM1 in the membrane. An analysis of binding of CTXB, a bacterial toxin, to GM1 supports this suggestion. The avidity of CTXB for membrane-associated GM1 is 3 orders of magnitude higher than its affinity for monomeric GM1 in solution (12, 22, 42, 50). Thus, high avidity for cell surface GM1 may account for the ability of GM1 addition to stimulate infection by mutant viruses that display lower affinity for monomeric GM1. A similar scenario appears to occur with poliovirus mutants that are resistant to neutralization by soluble receptor (8).

To isolate mutants that were incapable of using GM1, a library constructed in an A70L mutant backbone was screened in 293TT cells, a cell line with low levels of cell surface GM1. Two mutants recovered from this screen, A70L/N138Y and A70L/F75L/H129Q, were not stimulated when GM1 was added to the cells and remained defective for GM1 neutralization and binding to GM1 on the cell surface, indicating that these mutants were no longer using
GM1 for infection. Moreover, A70L/F75L/H129Q virus exhibited a striking change in cell tropism, infecting HeLa S3 and 293TT cells, cell lines with low levels of GM1, up to 4-fold better than the WT. Since A70L/F75L/H129Q virus no longer used GM1 for infection, it appears that the mutant used another molecule as a receptor. Because genetic repression of ganglioside synthesis inhibited infection of the mutant virus, it is likely that the receptor used by this mutant is a ganglioside. Preliminary data suggest that this putative ganglioside receptor is not GT1b, GD1b, GD1a, or GM2, because supplementation of HeLa S3 cells with those gangliosides failed to stimulate infection by the mutant (unpublished results). Future experiments are required to identify the novel receptor utilized by the GM1 binding-defective SV40 mutants. We hypothesize that the triple-mutant virus infects certain cells better than WT SV40 because this receptor is more abundant on these cells than GM1. This change in tropism is not species specific, because the mutant viruses fail to infect other human cells.

A70L/F75L/H129Q and A70L/N138Y mutants both contained additional substitutions at residues that directly interact with GM1. Like A70, N138 directly interacts with the terminal galactose of GM1 via van der Waals forces. Modeling and energy minimization predict that the bulky tyrosine side chain of the mutant will approach the hydroxyl groups of the terminal galactose moiety and impinge upon the binding pocket (Fig. 10A). H129 does not directly interact with GM1, but it lies adjacent to A70 in the quaternary pentamer structure (Cβ-Cβ distance, 4.8 Å) (Fig. 2). The H129Q mutation presumably perturbs the position of A70, which directly contacts GM1, thus further impairing GM1 usage. F75, which is invariant in BKV and JCV VP1 proteins (Fig. 11), is one of several residues that line a deep hydrophobic cavity that accommodates the acetyl group of NeuNAc (Fig. 10B) (36). This pocket is substantially larger in SV40 pentamers than in the VP1 pentamers of other polyomaviruses (34, 36, 45), a difference that may explain the preferential binding of SV40 to the monkey NeuNGc-GM1, which has a hydroxyl group attached to the methyl group and is believed to be the natural receptor for SV40 (6). Modeling and energy minimization reveal that the replacement of the aromatic side chain at position 75 with leucine enlarges this hydrophobic cavity (Fig. 10B). We hypothesize that the cavity may now be able to accommodate a larger carbohydrate moiety, thereby expanding the repertoire of molecules that can serve as receptors. F75L and H129Q each individually increased the infectivity of the A70L mutant in HeLa S3 cells, but the most pronounced change in tropism was exhibited by the triple mutant, suggesting that both of these substitutions are required for optimal usage of an as-yet-unidentified receptor. The requirement for all three substitutions in the A70L/F75L/H129Q mutant virus for its change in tropism highlights the power of the genetic approach used here. Because the phenylalanine at position 75 is invariant and the histidine at position 129 is not directly located in the receptor-binding site, it was not possible to predict the constellation of substitutions that would alter ganglioside usage and SV40 tropism.

Structural and biological studies by Benjamin and colleagues demonstrated that amino acid substitutions in the glycan binding region of mPyV VP1 were sufficient to alter receptor specificity and cell tropism. By preventing recognition of branched-chain oligosaccharide pseudoreceptors without affecting binding to the functional straight-chain receptor, the G91E substitution in mPyV VP1 increases virus spread and broadens virus tropism in the murine host, which results in increased pathogenicity (4, 14). Moreover, the V296A substitution, which is found in the LID laboratory strain of mPyV that also contains the G91E mutation, further increases virus spread and expands tropism by reducing avidity for the functional receptor, resulting in an extremely virulent virus (3). In contrast, the A70L/F75L/H129Q substitutions in SV40 VP1 appear to alter cell tropism by reducing affinity for the functional GM1 receptor and by allowing recognition of a new receptor. Thus, a genetic analysis of the receptor usage of SV40, which is more closely related to clinically relevant BKV and JCV than is mPyV, highlights an alternative mechanism by which polyomavirus tropism can be altered by substitutions in the glycan binding site.

Most of the amino acid differences among the VP1 sequences from SV40, BKV, and JCV are located in the BC and DE loops, two of three regions in VP1 that constitute the oligosaccharide binding site (Fig. 11). Our results show that a limited number of amino acid substitutions at the GM1 binding site are sufficient to deter-
mine specific receptor recognition by SV40 and to alter cell tropism. Furthermore, the differences we detect in ganglioside binding can be readily explained by the known structure of the wild-type VP1/GM1 complex and molecular modeling of the mutants. These results imply that the specificity of oligosaccharide binding by SV40 VP1 is modular, in the sense that it is not determined by amino acid differences throughout VP1, but rather by the amino acids in the glycan binding site. These findings, taken together with the relatively low level of sequence conservation in the BC and DE loops and research conducted on mPyV variants, strongly suggest that evolution of VP1 during polyomavirus divergence from a common ancestor was driven primarily by the need to accommodate different oligosaccharides receptors.

In addition to the effects of mutations in the receptor binding region of mPyV VP1 noted above (3, 4), similar mutations have previously been implicated in changes in human polyomavirus receptor usage and pathogenicity. BKV isolated from patients with viremia and nephropathy had a high frequency of substitutions in the BC loop region (27). JCV variants from patients with PML often carry substitutions in the LSTc binding site. JCV virus-like particles (VLPs) containing these mutations typically displayed significantly reduced ability to bind sialic acid and gangliosides (18, 46), and similar mutations at the same positions inhibited VP1 pentamer binding to glial cells and virus infectivity (34). Although the effects of these mutations on JCV tropism and pathogenesis remain to be determined, our results demonstrate that mutations that alter ganglioside usage can affect SV40 tropism. One mutation in the binding site allowed JCV VLPs to bind to two novel oligosaccharides in vitro (18), consistent with our conclusion that the A70L/F75L/H129Q SV40 mutant described here utilizes a different ganglioside receptor. Studying the pathogenicity of the SV40 mutants discussed here in the established hamster tumor system (10, 29) may provide insight into how changes in the receptor-binding sites of JCV and BKV alter tropism and pathogenicity in humans.

Further investigation of these SV40 mutants and their interactions with their receptors will provide insight into the broader question of how changes in cell surface receptors affect pathogenicity. Changes in receptor specificity have contributed to two of the most recent viral pandemics. Two amino acid substitutions in the receptor-binding domain of severe acute respiratory syndrome (SARS) coronavirus were sufficient to change the receptor specificity from the palm civet angiotensin-converting enzyme 2 (ACE2), the cell surface receptor used by the virus, to human ACE2 (23). Amino acid substitutions in the receptor-binding region of the pandemic influenza A (H1N1) virus may have altered cell surface receptor usage and contributed to its increased pathogenicity (25, 30, 51). Therefore, understanding the interaction of...
Since its discovery, SV40 has become one of the most intensely studied animal viruses, but little is known about how and why SV40 induces vacuolization. Previous studies showed that the H136Y SV40 mutant failed to induce robust vacuolization in CV-1 cells (31). This mutant was also resistant to GM1 neutralization. Moreover, there appears to be a gradation in the interaction of VP1 with GM1 in cells is required for vacuolization, but the molecular basis for this effect is unknown. Thus, more than 50 years later, there is much to be learned about SV40.

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REFERENCES


