Modeling a Sialic Acid Binding Pocket in the External Loops of JC Virus VP1*

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JC virus (JCV) is a common human polyomavirus that infects over 70% of the population worldwide. JCV has a restricted cell tropism that is caused partly by the initial interaction between the virus and sialic acid-containing host cell receptors. To identify the molecular interactions between the virus and its cellular receptor, we used a combined approach of site-directed mutagenesis and homology-based molecular modeling. A model of the major viral capsid protein VP1 based on sequence alignment with other closely related polyomaviruses allowed us to target specific amino acids in the extracellular loops of VP1 for mutagenesis. An analysis of the growth rates of 17 point mutants led to the identification of VP1 amino acids that are critical in virus-host cell receptor interactions. Molecular dynamics simulations were then used to build and confirm a model of the interaction between VP1 and the sialic acid component of the JCV receptor.

JC virus (JCV)‡ is a small, non-enveloped human polyoma-virus that causes the fatal demyelinating disease progressive multifocal leukoencephalopathy. Progressive multifocal leukoencephalopathy is caused by JCV-induced lytic destruction of oligodendrocytes, the myelin-producing cells within the central nervous system. Although JCV is a very common virus, progressive multifocal leukoencephalopathy only occurs in immunosuppressed individuals, and approximately 6% of AIDS patients will eventually develop the disease (1). JCV has a very restricted cellular tropism and is efficiently replicated only in glial cells, although it can also infect B-cells, hematopoietic progenitor cells, and tonsillar stromal cells (2). JCV preferentially binds N-linked glycoproteins containing terminal α(2,6)-linked sialic acid, although the protein component of the cellular receptor has not been determined (3, 4). The receptor for murine polyomavirus (mPyV) is known to contain the viral DNA in bacteria. The SVG-A cell line is a subclone of the original SVGA cell line established by the transformation of primary human fetal glial cells with an origin-defective SV40 mutant (16). Cells were grown in Eagle’s minimum essential medium (Mediatech) supplemented with 10% heat-inactivated fetal bovine serum (Mediatech) and 1% penicillin/streptomycin (HyClone) and maintained at 37 °C in a humidified atmosphere of 5% CO₂. JC12, a subclone of the Mad1-SVEΔ hybrid strain of JCV described previously (17), was linearized at the BamHI site and subcloned into the pUC19 vector for propagation of viral DNA in bacteria.

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§ The abbreviations used are: JCV, JC virus; mPyV, murine polyoma-virus; PBS, phosphate-buffered saline.

Experimental Procedures

Homology Modeling of VP1—A homology model of JCV VP1 was built using SWISS-MODEL and the structures of other polyomavirus proteins within the Protein Data Bank. The C terminus of JCV (residues 290–354) that facilitates capsid formation was not included in the homology model. The model of the sialic acid-JCV complex was created using the crystal structure of the recombinant polyomavirus VP1 complexed with a branched disialylated hexaxasaccharide receptor (Protein Data Bank entry 1VPS). The JCV homology model was superimposed onto VP1, and the branched sugar from the crystal structure was replaced with NeuNAc-(α(2,6)-Gal-(α(1,3)-GlcNAc, a sialyloligosaccharide with terminal α(2,6)-linked sialic acid (see Fig. 5o), given the important role of this carbohydrate in binding to JCV (3).

Computer Simulations of the Sialic Acid-JCV Complex—The homology-based JCV VP1-sugar complex was subjected to several cycles of energy minimization to remove initial strain, using the AMBER force field within the InsightII (Molecular Simulations, Inc.) program. The complex was then placed centrally in a cube of 12 nm and soaked with water (37,700 water molecules), and energy was minimized using a steepest descent algorithm. Extensive molecular dynamics simulations (at 300 K, with an integration time step of 2 fs and a constant pressure of 1 bar) were carried out with the complex using the GROMACS program (15) operating on Pentium III processors, running Linux software. Each of the mutations that were examined experimentally in this study was introduced, and additional energy minimization and molecular dynamics simulations were carried out. To generate alternative topologies of the sugar binding distance, restraints were applied between charged amino acids of JCV and atoms of the sugar (e.g. among the complexes Arg-75-GlcNAc, Arg-56-NeuNAc, and Arg-273-NeuNAc) during the molecular dynamics simulations. Large force constants (5000 kJ mol⁻¹ nm⁻²) were initially applied to induce structural changes and were then reduced during the remainder of the simulation.

Cells, Virus, and Plasmid—The SVG-A cell line is a subclone of the original SVGA cell line established by the transformation of primary human fetal glial cells with an origin-defective SV40 mutant (16). Cells were grown in Eagle’s minimum essential medium (Mediatech) supplemented with 10% heat-inactivated fetal bovine serum (Mediatech) and 1% penicillin/streptomycin (HyClone) and maintained at 37 °C in a humidified atmosphere of 5% CO₂. JC12, a subclone of the Mad1-SVEΔ hybrid strain of JCV described previously (17), was linearized at the BamHI site and subcloned into the pUC19 vector for propagation of viral DNA in bacteria.

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Transfection—The viral DNA was isolated from plasmid DNA by endonuclease digestion with BamHI, separated by agarose gel electrophoresis, and purified using the Wizard® SV Gel and PCR Clean-Up system (Promega). Linear viral DNA was transfected into SVG-A cells grown on coverslips or in 75-cm² flasks by lipofection using LipofectAMINE™ (Invitrogen) and Plus™ (Invitrogen) reagents according to the manufacturer’s instructions.

Site-directed Mutagenesis—Site-directed mutagenesis was performed using the GeneEditor™ in vitro site-directed mutagenesis system (Promega) according to the manufacturer’s directions. Primers used for mutagenesis were (5'<H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H11032 H1103213

Indirect Immunofluorescence Assay—Cells grown on coverslips were washed in PBS and fixed for 10 min in acetone. The cells were incubated with the anti-V antigen monoclonal antibody PAB597 (undiluted hybridoma supernatant) for 45 min at 37 °C. The cells were then washed three times with PBS and incubated with Alexa-Fluor-488-labeled goat anti-mouse secondary antibody for 45 min at 37 °C. Cells were again washed three times with PBS, counterstained with PBS containing 0.02% Evan’s Blue, and washed again in PBS. Coverslips were mounted on slides and visualized on a Nikon E800 epifluorescence microscope, and images were captured by a Hamamatsu digital camera and processed in Photoshop. Positively stained JCV-infected cells were counted every 3 days starting on the fourth day following transfection to generate a growth curve and to determine the ability of transfected viral DNA to produce viable virus.
Electron Microscopy—Four days after transfection, SVG-A cells transfected with linear viral DNA grown on Lab-Tek chamber slides were fixed with 2.5% glutaraldehyde in 0.15M sodium cacodylate buffer. Cells were rinsed in buffer, scraped from slides, and post-fixed with 1% osmium tetroxide for 40 min at 4 °C. Following centrifugation, cells were rinsed several times with buffer. A cell pellet was prepared in which filtered fetal bovine serum was mixed with the cells. To bind the pellet together, the cells were pelleted, and the supernatant was replaced with 2.5% glutaraldehyde and allowed to fix for several days. The pellet was then rinsed, dehydrated through a graded ethanol series, infiltrated, and embedded in Spurr’s epoxy resin. Using a Reichert Ultracut S microtome, ultrathin sections were prepared and placed on 300 mesh, thin bar copper grids. The sections were stained with 5% uranyl acetate in methanol followed by lead citrate. These sections were examined using a Philips 300 electron microscope.

RESULTS

JCV VP1 Model Based on Sequence Alignment—Based on the sequence alignment of VP1 with other polyomaviruses, we created a model of a JCV VP1 monomer using the SWISS-MODEL protein modeling server (Fig. 1) (12–14). We then used the crystal structure of mPyV in complex with a sialic acid receptor fragment as a guide to identify homologous amino acids on the JCV VP1 surface that were involved in binding sialic acid. To verify whether these amino acids were critical for the receptor binding pocket of JCV VP1, we made several single amino acid point mutations in the JCV sequence at Arg-56, Gly-57, Lys-60, Asp-66, Glu-69, Asp-71, Arg-75, and Asp-76 on the BC loop, as well as Arg-266 and Arg-273 on the DE loop (Fig. 1). The mutated linear DNA was then transfected into the human glial cell line SVG-A to produce virus that could be evaluated in comparison with the wild type.

Expression and Nuclear Localization of Wild Type and Mutant VP1—SVG-A cells were plated on coverslips and transfected with either wild type or mutant linear DNA. Following transfection of viral DNA with mutations coding for alanine, glycine, or arginine substitutions at Arg-56, Gly-57, Lys-60, Asp-66, Glu-69, Asp-71, Arg-75, Asp-76, Arg-266, and Arg-273, VP1 expression and cellular localization were examined. Neither conservative nor non-conservative substitutions at the RG motif at positions 56 and 57 disrupted normal localization of VP1 to the nucleus (Fig. 2, b–d). Likewise, replacing the uncharged Gly at amino acid 57 with a positively charged Arg (Fig. 2e) did not disrupt VP1 expression or localization. Other non-conservative mutations were made by substituting the positively charged residues with uncharged amino acids in the K60G, K60A, R75G, R75A, R266G, and R273A mutants (Fig. 2, f–m, and p), Viral protein produced in each of these mutants localized to the nucleus in the same manner as wild type JCV VP1 (Fig. 2a). The identification of mutant VP1 molecules in the nucleus indicates that the mutations did not disrupt the normal targeting and expression of viral protein to the site where viral assembly occurs.

Infectivity and Growth of Mutant Viruses—To determine
whether the transfection of mutated viral DNA could result in the production of viable virus, we monitored the spread of V antigen-positive cells in culture over time and measured viral growth based on the average number of infected cells/ microscopic field at each time point (Fig. 3). Mutations that interfere with the ability of the virus to reinfect cells in culture result in an abortive infection that is evident 7–10 days after transfection. We found that the R56G, R56A, G57R, D66G, D71G, D71A, R75G, R75A, and R273A mutant viruses all failed to propagate (Fig. 3). The mutants D66A and E69A propagated more slowly in culture than did the wild type, with fewer infected nuclei at each time point, resulting, on average, in approximately half as many infected cells by day 13 (Fig. 3).

The R56K, D76G, K60G, K60A, E69G, and R266G mutants all propagated at a rate that was similar to wild type JCV (Fig. 3).

**DISCUSSION**

**A Model of the JCV VP1 Sialic Binding Pocket**—We targeted our mutations in the external loops of JCV VP1 based on the homology model built using the sialic acid binding pocket in mPyV. Extensive molecular modeling of the JCV VP1-sialic acid interactions was then carried out using the data from our mutagenesis studies. To model the interaction, we used the sialyloligosaccharide NeuNAc-(α2,6)-Gal-(β1,3)-GlcNAc because terminal α(2,6)-linked sialyloligosaccharide is known to be an essential component of the receptor that JCV uses on cells (3) and can bind JCV with higher affinity than other

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**FIG. 5. A potential carbohydrate-binding site is identified based on the biological characteristics of mutant viruses.**

*a*, the structure of sialyloligosaccharide receptor fragment used to demonstrate binding site is shown. The carbons of each ring are numbered on the oligosaccharide containing α(2,6)-linked sialic acid (NeuNAc-(α2,6)-Gal-(β1,3)-GlcNAc). Gal represents the galactose ring. The two oxygens of the carboxylate group on carbon 1 of NeuNAc are labeled O1A and O1B.  

*b*, a top view is shown of how NeuNAc-(α2,6)-Gal-(β1,3)-GlcNAc fits into the groove between the HI and BC2 loops of the JCV VP1 molecule.  

*c*, a hypothetical model shows the interaction between NeuNAc-(α2,6)-Gal-(β1,3)-GlcNAc and the various amino acids of VP1 that are predicted to have a role in receptor binding. The amino acids that have been mutated to generate biological data are labeled in black, and amino acids predicted to have a role based on theoretical data are labeled in green. For the amino acid side chains, green represents carbon, red represents oxygen, and blue represents nitrogen. Curved arrows represent the location of the neighboring clockwise (CW) and counterclockwise (CCW) VP1 monomers in the intact pentamer.
linkages of sialic acid (4). The model resulting from the simulations of the JCV complex reflects many of the same sialic acid-protein interactions seen in the interactions between mPyV VP1 and NeuNAC-1 in the solved crystal structure of the mPyV VP1 pentamer with a disialylated hexasaccharide receptor fragment (11). The binding pocket of the NeuNAC ring is illustrated in Fig. 5b. Because of the conserved nature of the RG motif between JCV and mPyV on the BC loop and its importance to mPyV in sialic acid binding (5), the RG motif was initially suspected to have a similar role for JCV. The positions of these residues (Arg-56 and Gly-57) in relation to the sugar in our model, however, indicate an alternative orientation for the JCV VP1-sialic acid interaction. As shown in Fig. 5c, the BC loop creates a binding pocket because the BC1 and BC2 loops are oriented in a “boat” shape relative to each other. In our model, the amino acid Gly-57 resides at the point of the “twist” between the BC1/BC2 loops and faces toward the JCV core. Substitution of Gly-57 with a long, bulky arginine side chain would hinder the bend of the BC loop at this position (without a steric clash with other side chains of the virus), in accordance with our biological results (Fig. 3). The arginine in the RG motif is most likely important in binding the sugar. Although the conservative R56K mutant grows almost as well as wild type virus, both the R56G and R56A mutants are non-viable. Within the model (Fig. 5c), the positively charged Arg-56 creates a salt bridge with the carboxylate group (O1B) of sialic acid, stabilizing the negative charge of the bound sugar molecule. Consequently, removing the positive charge at position 56 would destabilize the energetically favorable conformation of sialic acid within the binding groove of JCV and result in virus that is able to assemble properly but is unable to bind the cellular receptor and initiate another round of infection.

The opposite side of the binding groove is provided by the HI loop. The model suggests a number of interactions for Arg-273 and may explain the non-viability of the R273A mutant. The charged Arg-273 forms hydrogen bonds with the hydroxyl oxygen on C7 (Fig. 5a) of NeuNAC and the carbonyl group of Gln-271 (not shown). The side chain of Arg-273 stabilizes a hydrophobic pocket with Leu-118 at the base of the DE loop, whereas the guanidyl moiety provides charge-ring interaction with the aromatic ring of the phenylalanine at position 263 (Phe-263). Our data indicate that the mutation of Arg-75, located on the BC2 loop, results in non-viable virus and that this residue is presumably involved in the recognition of sialic acid. In the resulting model, the positively charged Arg-75 is within 4 Å from the terminal oxygen on C6 (O-6) of the GlcNAc group. We would predict a salt bridge between Arg-75 and Asp-76 (Fig. 5c), although it is obviously not vital for sialic binding because the D76G mutant displayed only a slight retardation in viral growth (Fig. 3). According to the model, no direct intermolecular contacts are evident between Asp-76 and the GlcNAc ring because the side chain of the negatively charged aspartic acid faces away from the sugar.

Based on the homology model developed here, residues Asp-66, Glu-69, and Asp-71 at the end of the BC2 loop do not appear to be directly involved in binding of α(2,6)-linked sialic acid. Indeed, given their negative charge, these residues would not be expected to be in close proximity to the like-charged sialic acid. The slightly retarded growth rates for D66A, E69A, E69G, D71A, and D71G (Fig. 3) may therefore be indicative of important structural roles for these amino acids and may possibly enhance solubility or surface exposure. The homology model also provides some suggestions for the higher affinity for terminal α(2,6)-linked sialic acid over affinities for branched α(2,6) or α(2,3)-linked sialic acid. The terminal α(2,6) linkage of sialic acid bends the overall sugar structure (Fig. 5a), allowing the negatively charged sialic acid to enter deeply into the binding groove and come into contact with Arg-273 (Fig. 5). A branched α(2,6)-linked sialic acid from C6 of the GlcNAc group would be sterically hindered by the conformation of the BC2 loop (Fig. 5c), providing an explanation for the preferential binding of JCV VP1 to terminal rather than branched sialic acid. Likewise, because the side of the binding groove provided by the HI loop would directly interfere with the NeuNAC ring linked to C3 of the galactose ring in α(2,3)-linked sialic acid, the model also illustrates why JCV has a binding affinity that is stronger for the α(2,6) linkage than the α(2,3)-linked sialic acid.

The work described here has given us a better understanding of the nature of the role of sialic acid in binding JCV and helps to explain why terminal α(2,6)-linked sialic acid is an essential part of the JCV cellular receptor. In addition, the model of the interaction between VP1 and sialic acid has important implications in the design of drugs and small molecule inhibitors used to treat progressive multifocal leukoencephalopathy. We are currently performing additional experiments to confirm and further refine the model presented here.

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