JC Polyomavirus Attachment, Entry, and Trafficking: Unlocking the Keys to a Fatal Infection

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Abstract

The human JC polyomavirus (JCPyV) causes a lifelong persistent infection in the reno-urinary tract in the majority of the adult population worldwide. In healthy individuals infection is asymptomatic, while in immunocompromised individuals the virus can spread to the central nervous system and cause a fatal demyelinating disease known as progressive multifocal leukoencephalopathy (PML). There are currently very few treatment options for this rapidly progressing and devastating disease. Understanding the basic biology of JCPyV-host cell interactions is critical for the development of therapeutic strategies to prevent or treat PML. Research in our laboratory has focused on gaining a detailed mechanistic understanding of the initial steps in the JCPyV life cycle in order to define how JCPyV selectively targets cells in the kidney and brain. JCPyV requires sialic acids to attach to host cells and initiate infection, and JCPyV demonstrates specificity for the oligosaccharide lactoseries tetrasaccharide c (LSTc) with an α 2,6-linked sialic acid. Following viral attachment, JCPyV entry is facilitated by the 5-hydroxytryptamine (5-HT)₂ family of serotonin receptors via clathrin-dependent endocytosis. JCPyV then undergoes retrograde transport to the endoplasmic reticulum (ER) where viral disassembly begins. A novel retrograde transport inhibitor termed Retro-2^{cycl} prevents trafficking of JCPyV to the ER and inhibits both initial virus infection and infectious spread in cell culture. Understanding the molecular mechanisms by which JCPyV establishes infection will open up new avenues for the prevention or treatment of virus-induced disease.

Keywords

JC polyomavirus; PML; sialic acid; LSTc; serotonin receptor; Retro-2^{cycl}

Introduction

JC polyomavirus (JCPyV) infects greater than 50% of the adult population and establishes an asymptomatic lifelong, persistent infection in the kidney (Dorries, 1998; Egli et al, 2009).

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Conflict of Interest

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In immunosuppressed individuals, JCPyV can spread to the central nervous system (CNS) (Houff et al, 1988; Dorries et al, 1994; Dubois et al, 1996; Dubois et al, 1997) where it infects glial cells critical for myelination: astrocytes and oligodendrocytes (Silverman et al, 1965; Zurhein et al, 1965; Spiegel et al, 2006; Sorensen et al, 2008; Bradl et al, 2010). JCPyV infection of astrocytes and cytolytic destruction of oligodendrocytes causes the fatal, demyelinating disease progressive multifocal leukoencephalopathy (PML) (Astrom et al, 1958; Ferenczy et al, 2012). PML is a devastating disease that proves fatal in 90% of patients within 1 year of symptom onset (Brew et al, 2010). PML affects ~5% of HIV-1⁺ individuals, is considered an AIDS-defining illness, and is one of the most common CNS-related diseases in AIDS (Ferenczy et al, 2012). In the past decade, the incidence of PML has risen in individuals receiving immunomodulatory therapies for immune-related diseases, particularly those receiving the drug natalizumab (Tysabri®) for treatment of multiple sclerosis (Carson et al, 2009; Hellwig et al, 2011; Bloomgren et al, 2012). Patients with PML develop symptoms of hemiplegia, paralysis, vision loss, and loss of cognitive function (Astrom et al, 1958; Berger et al, 1998). There is currently no effective treatment for PML other than removal of immunosuppressive therapies and immune reconstitution. However, this can cause immune reconstitution inflammatory syndrome, which worsens the CNS condition and is often fatal (Steiner et al, 2012). Given the ubiquitous nature of JCPyV and the devastating disease caused in immunosuppressed individuals, a need for antiviral therapies is critical. Understanding the basic biology of virus-host cell interactions is essential for the development of effective therapeutics.

JCPyV is a member of the *Polyomaviridae* family, which is comprised of mouse polyomavirus (mPyV), simian virus 40 (SV40), and 13 human polyomaviruses including BK polyomavirus and the cancer-causing Merkel cell polyomavirus (Feng et al, 2008; DeCaprio et al, 2013; Rinaldo et al, 2013; Mishra et al, 2014). JCPyV has a nonenveloped, icosahedral capsid that encloses a circular 5130 bp dsDNA genome (Frisque et al, 1984; Shah et al, 1996). The viral capsid is ~40 nm in diameter and is formed by three structural proteins: viral proteins 1, 2, and 3 (VP1, VP2, and VP3). The surface of the capsid is comprised of 72 VP1 pentamers that are interconnected through C-terminal extensions (Liddington et al, 1991). Each VP1 pentamer also interacts with a VP2 or VP3 molecule in the interior of the capsid (Chen et al, 1998). VP2 has been shown to be critically important, as viruses with point mutations that disrupt the VP2 start site are non-viable (Gasparovic et al, 2006). VP1 serves as the viral attachment protein as residues on the external surface mediate direct interactions with cellular receptors (Liu et al, 1998a; Chen et al, 2002; Gee et al, 2004; Neu et al, 2010).

Sialic acid receptors are required for JCPyV infection

The initial step in the infectious life cycle of a virus is attachment to cell-surface receptors. The specificity of this interaction can influence the tropism of the virus. Therefore, viruses have evolved elegant strategies to achieve this critical recognition event. Interactions with viral receptors can facilitate attachment as well as internalization. In many cases viruses have multiple receptors to drive these distinct steps, providing another layer of selectivity in cellular tropism. Many viruses first bind to a carbohydrate receptor such as sialic acids (SA) in the initial attachment step in a low affinity, high avidity interaction (Grove et al, 2011).

The majority of polyomaviruses studied to date require SA to bind and infect host cells (Neu et al, 2009; Neu et al, 2010; Neu et al, 2013). Sialic acids are a diverse group of 9-carbon monosaccharides that are terminally attached to the glycan chains of N-or O-linked glycoproteins and glycolipids (Varki et al, 2009). These negatively charged SA are abundantly expressed on the surface of all eukaryotic cells and serve many physiological roles, including cellular adhesion and signaling (Varki et al, 2009). The potential for more than 50 natural modifications leads to a tremendous amount of diversity in SA structures (Altheide et al, 2006). The N-acetyl group located at the 5-carbon position is the most commonly found SA and serves as a precursor to the predominant SA in humans, 5-N-acetyl neuraminic acid (Neu5Ac) (Varki et al, 2009). Sialic acids gain further diversity through different alpha linkages on the 2-carbon position of the main sugar chain, with the most common linkages being 3-to the galactose (Gal) residue or 6-to the Gal or N-acetylglucosamine (GlcNAc) residue to generate α 2,3- or α 2,6-linked SA. Sialic acids can also attach to other internal sialic acids resulting in an α 2,8-linked SA (Varki et al, 2009). Cell-surface SA are commonly founds on gangliosides, a group of glycosphingolipids whose ceramide chain is embedded in the plasma membrane of eukaryotic cells with an extracellular oligosaccharide comprised of one or more SA that decorate the cell surface. Gangliosides are divided in 4 groups or “series” based on the number and complexity of their SA branching patterns (Maccioni et al, 2011). Many viruses that utilize SA receptors for infection interact with a specific SA linkage, oftentimes having strain-specific or species-specific differences in SA linkage utilization. For instance, mPyV exhibits strain-specific differences in its affinity for sialic acid receptors. While the large plaque (LP) and small plaque (SP) strains of mPyV both bind to straight chain oligosaccharides that terminate in α 2,3-SA (NeuNAc- α 2,3-Gal- β 1,3-GalNAc), the SP strain can also bind to a branched disialyl oligosaccharide with α 2,6-SA (NeuNAc- α 2,3-Gal- β 1,3-[NeuNAc- α 2,6]-GalNAc). The LP strain is more pathogenic and tumorigenic in mice than the SP strain (Freund et al, 1991). The differences in receptor usage and pathogenicity correlate with a single point mutation in the SA-binding site of the viral attachment protein VP1 (Freund et al, 1991). These findings have led to the hypothesis that binding to additional sialic acid structures or pseudoreceptors may prevent the virus from spreading efficiently and thereby reduces the pathogenicity of the SP virus. Thus, sialic acid receptors are common viral receptors and govern critical outcomes in viral infection and pathogenesis.

The original studies of JCPyV receptors used the Mad-1 laboratory prototype strain and indicated that JCPyV utilizes sialic acid receptors, specifically an N-linked glycoprotein with α 2,6-linked SA. These conclusions were based on experiments in which crude neuraminidase that removes both α 2,3- and α 2,6-linked cell-surface SA reduces the virus's ability to agglutinate red blood cells (RBCs) and to bind to permissive SVGA glial cells, but a neuraminidase specific for α 2,3-SA had no effect. Further, prevention of N-linked glycosylation by tunicamycin prevents infection while an inhibitor of O-linked glycosylation, benzylN-acetyl- α -D-galactosaminide, has no effect (Liu et al, 1998b). A structural homology model of JCPyV based on the X-ray crystal structure of mPyV in complex with sialic acid revealed a SA-binding pocket in the BC-, DE-, and HI-loops of JCPyV VP1 that is essential for viral propagation (Gee et al, 2004). Subsequent studies to further define the types of sialic acids utilized by JCPyV expanded these findings to suggest

that JCPyV interacts with either $\alpha 2,3$ - or $\alpha 2,6$ -SA. Dugan et al. demonstrated that JCPyV binding to glial cells is reduced by treatment of cells with neuraminidases that reduce either $\alpha 2,3$ - or $\alpha 2,6$ -SA but have a preference for removal of $\alpha 2,3$ -SA. Additionally, cells were stripped of the native glycome and restored with exogenous sialic acids using sialyltransferases that add $\alpha 2,3$ -SA or $\alpha 2,6$ -SA in specific orientations, and either $\alpha 2,3$ - or $\alpha 2,6$ -SA could restore binding and infection by JCPyV (Dugan et al, 2008).

The notion that JCPyV can engage multiple SA structures has been corroborated by experiments showing that virus-like particles (VLPs) comprised of VP1 bind to glycoproteins and glycolipids with oligosaccharides containing $\alpha 2,3$ -, $\alpha 2,6$ -, or $\alpha 2,8$ -SA as demonstrated by biochemical binding analyses. VLPs of the genotype 1 strain Mad-1 bind to oligosaccharides containing $\alpha 2,3$ -, $\alpha 2,6$ -, and $\alpha 2,8$ -SA including gangliosides GM3, GD2, GD3, GD1b, GT1b, and GQ1b, and bind weakly to GD1a, by indirect virus overlay assays (Komagome et al, 2002). VLPs with the VP1 of the genotype 3 strain called “WT3” bind to gangliosides with $\alpha 2,3$ - or $\alpha 2,8$ -SA, including asialo-GM1, GD1a, GD1b, GD2, GT1a, and GT1b, as measured by enzyme-linked immunosorbent assay (ELISA) (Gorelik et al, 2011). Additionally, WT3 VLPs have been reported to bind to gangliosides GM1 and GM2 with $\alpha 2,3$ -SA by glycan array (Leonid Gorelik, Consortium for Functional Glycomics [CFG], available online at <http://www.functionalglycomics.org>, according to CFG policy). While the Mad-1 VLPs were able to bind to a panel of $\alpha 2,3$ -, $\alpha 2,6$ -, and $\alpha 2,8$ -SA-containing structures in a virus overlay assay, the VLPs had the highest affinity for an $\alpha 2,6$ -SA that was expressed on lactoseries tetrasaccharide c (LSTc) conjugated to ovalbumin (OVA). LSTc is a linear pentasaccharide, which expresses a terminal $\alpha 2,6$ -SA on glycoproteins and glycolipids (Xu et al, 2009). VLPs exhibit a reduced binding affinity for LSTb, which has an internal $\alpha 2,6$ -SA, and for LSTa (terminal $\alpha 2,3$ -SA). Furthermore, pretreatment of VLPs with LSTc-OVA inhibits agglutination of RBCs and binding to SVGA and IMR-32 cells. Additionally, pretreatment of virus with LSTc-OVA inhibits binding to SVGA and IMR-32 cells and infection of IMR-32 cells as measured by agnoprotein production (Komagome et al, 2002). Taken together, these data indicate that JCPyV is able to engage oligosaccharides containing either $\alpha 2,3$ -, $\alpha 2,6$ -, or $\alpha 2,8$ -SA, but there may be strain-specific differences in sialic acid receptor engagement or differences in affinities for oligosaccharide receptors that influence binding to host cells.

LSTc is the preferred sialic acid receptor motif for JCPyV

While it is clearly established that JCPyV infection depends on interactions between VP1 and sialic acid receptors, the nature of the oligosaccharide receptor and the relative specificity of this receptor interaction for a productive JCPyV infection remained unclear. To better define the oligosaccharide receptors in JCPyV infection, we analyzed binding of purified recombinant VP1 pentamers using glycan microarrays containing 81 lipid-linked sialylated oligosaccharides differing in backbone structures, chain lengths, and branching patterns (Neu et al, 2010). The glycan arrays represented carbohydrates found on N- and O-linked glycoproteins, as well as glycolipids and all known gangliosides. JCPyV VP1 pentamers bound specifically to only one glycan on the array; $\alpha 2,6$ -SA linked LSTc (Neu et al, 2010). Interestingly, this was the same $\alpha 2,6$ -SA ligand used by Komagome et al., who serendipitously selected to utilize LSTc-OVA as a representative $\alpha 2,6$ -SA (Komagome et al,

2002). Although the glycan array contained several carbohydrates that closely resembled LSTc including LSTa, LSTb, and all known gangliosides, VP1 pentamers showed no interaction with these carbohydrates. Furthermore, Gorelik and colleagues demonstrated that VLPs of the Mad-1 strain bind only to LSTc on a glycan array, indicating that VLPs with 72 copies of VP1, which have enhanced avidity for receptor structures, retain the specificity of binding to LSTc (data from CFG). The biological significance of LSTc as a functional receptor motif for JCPyV was demonstrated by showing that incubation with soluble LSTc pentasaccharide blocks infection, while LSTb does not. The X-ray crystal structure of the JCPyV VP1 pentamer in complex with LSTc reveals that LSTc binds on top of the JCPyV VP1 pentamer, on the outer surface of the virion, in a binding site formed by residues from the BC-, DE-, and HI-loops from one monomer, as well as the BC-loop from its clockwise neighbor (Neu et al, 2010) (Fig. 1). These loops also engage the sialic-acid-containing-ganglioside receptors in BKPyV and SV40. Notably, these are the only parts of VP1 that differ significantly among the polyomaviruses, despite only having ~75% amino acid identity (Neu et al, 2008; Neu et al, 2010; Neu et al, 2013). LSTc, with the linear sequence NeuNAc- α 2,6-Gal- β 1,4-GlcNAc- β 1,3-Gal- β 1,4-Glc is capable of rotating freely in solution but binds to JCPyV VP1 in a specific conformation (Breg et al, 1989; Neu et al, 2010). JCPyV engages a single, terminal SA that is connected to remaining oligosaccharide chains via an α 2,6-linkage through parallel hydrogen bonds mediated by Ser266 and Ser268 in VP1. Additional contacts occur between Asn123 of VP1 and the GlcNAc of LSTc, resulting in a unique L-shaped conformation. Furthermore, the specific contacts between VP1 and LSTc were demonstrated to be critical for binding and infection, as mutagenesis of VP1 residues that disrupt SA binding results in viruses that are not infectious and VP1 pentamers that are unable to bind to glial cells (Neu et al, 2010). The X-ray crystal structure of VP1 with and without LSTc reveals that VP1 undergoes induced fit movements to accommodate LSTc binding, with significant rearrangements at residue Asn123. This suggests that both sialic acid contacts and the L-shaped binding conformation of LSTc are required for JCPyV attachment (Neu et al, 2010). The simian polyomavirus SV40 binds to α 2,3-SA-containing ganglioside GM1, while the human BKPyV utilizes the α 2,8-SA-containing b-series gangliosides (Neu et al, 2008; Neu et al, 2013). Comparison of the X-ray crystal structures of JCPyV, BKPyV, and SV40 in complex with their sialic acid receptors demonstrates that although the VP1 proteins have a high degree of sequence and structural similarity, each virus retains significant specificity for engagement of the appropriate sialic acid receptor. All three viruses bind to the terminal SA motif through conserved interactions, yet also require additional contacts outside of the SA binding pocket to confer specificity to their appropriate receptors (Neu et al, 2008; Neu et al, 2010; Neu et al, 2013). A single point mutation in BKPyV VP1 to the SV40 sequence redirects BKPyV to bind to GM1 and infect GM1-expressing cells, demonstrating that minor differences in amino acid sequence can influence receptor utilization and cellular tropism (Neu et al, 2013). JCPyV has a very narrow host range and tissue tropism within the human host as viral infection is mostly limited to the kidney and glial cells (Silverman et al, 1965; Zurhein et al, 1965; Yogo et al, 1990). Receptor expression is only one factor that contributes to tissue tropism, but expression of SA receptors *in vivo* correlates with JCPyV tropism and infection (Eash et al, 2004). Staining of human tissues with the Maackia amurensis lectin specific for α 2,3-SA and the Sambucus nigra lectin (SNA) specific for α 2,6-SA reveals that α 2,6-SA is present

on the surface of B lymphocytes of the tonsils and spleen, kidney cells, and astrocytes and oligodendrocytes. This contrasts with lower levels of $\alpha 2,3$ -SA expression in these tissues (Eash et al, 2004). JCPyV has been detected in neuronal cells of individuals with PML including cortical neurons within demyelinating PML lesions in the cerebral cortex and in granule cell neurons in the granule cell layer (Du Pasquier et al, 2003; Wuthrich et al, 2009; Wuthrich et al, 2012). However, only very low-level SNA lectin staining is detected in neurons (Eash et al, 2004), suggesting that infection of neuronal cells may rely on other sialic acid structures or that infection occurs through an alternate receptor or unique entry mechanism such as cell-to-cell spread. Infection in neurons is more often characterized by expression of viral T antigen rather than VP1 (Wuthrich et al, 2009; Wuthrich et al, 2012), suggesting that JCPyV does not effectively replicate in neurons. Therefore, JCPyV infection of neuronal cells is distinct from glial cell infection. Further studies are needed to characterize JCPyV neuronal infection as it may contribute to PML pathogenesis.

PML-associated mutations change JCPyV receptor specificity

The mechanisms by which JCPyV spreads to the CNS are not clear, but it is thought to spread from the site of persistence in the kidney via a hematogenous route, possibly involving B lymphocytes, to astrocytes and oligodendrocytes in the CNS (Atwood et al, 1992; Tornatore et al, 1992; Chapagain et al, 2010). Virus that persistently infects the kidney, called the archetype strain, is non-pathogenic and can be detected in the urine of ~30% of healthy individuals (Yogo et al, 1990; Agostini et al, 1996; Daniel et al, 1996; Yogo et al, 2008). Viruses found in the cerebral spinal fluid (CSF), brain tissue, and blood, contain polymorphic changes in non-coding control region (NCCR) and are referred to as PML-type strains (Ferenczy et al, 2012). The NCCR contains the viral origin of replication and binding sites for transcription factors (Frisque, 1983a; Martin et al, 1985; Kenney et al, 1986a; Kenney et al, 1986b; Sock et al, 1996). Rearrangements in PML-type strains, including duplication of enhancer elements, convert the virus to the neuropathogenic form within the infected host (Frisque, 1983b; Krebs et al, 1995; Sock et al, 1996). NCCR rearrangements are necessary for JCPyV growth in the CNS, yet there are likely other viral, cellular, and individual host factors that influence PML pathogenesis as the incidence of PML is rare when considering the high rates of seropositivity in the population (Ferenczy et al, 2012).

Interestingly, a number of recent studies have reported that as many as 80–90% of viral isolates from the blood and CSF of individuals with PML exhibit mutations in VP1 in addition to NCCR rearrangements. While the majority of the isolates from individuals with PML exhibit mutations in one or more sites in VP1, the most frequently arising mutations are L54F, S266F, and S268F/Y (Zheng et al, 2005; Delbue et al, 2009; Sunyaev et al, 2009; Gorelik et al, 2011; Reid et al, 2011). These mutations termed “PML-associated mutations” are located in the sialic-acid binding pocket of VP1 (Neu et al, 2010). PML-associated mutations are only found in viral isolates from the CSF and blood of individuals with PML, but have never been detected in urine isolates of individuals with or without PML (Delbue et al, 2009; Sunyaev et al, 2009; Gorelik et al, 2011; Reid et al, 2011). Genotypic analysis of viral isolates collected over time from a patient infected with a single JCPyV genotype demonstrate that virus isolated from the blood and CSF contained PML-associated

mutations in VP1 (L54F, N264S, and S266F), while these mutations were not present in urine isolates (Reid et al, 2011). This finding suggests that VP1 mutations can arise within the JCPyV-infected individual due to either host-or virus-specific factors. It is hypothesized that viruses with PML-associated mutations are the pathogenic form of the virus (Sunyaev et al, 2009; Gorelik et al, 2011), and recent studies have sought to determine their binding properties and pathogenicity. Binding analysis of WT3 VLPs with PML-associated mutations demonstrate an altered capacity to engage sialic-acid containing receptors, as these VLPs have reduced hemagglutination activity, bind to glial cells in a neuraminidase-insensitive manner, and have altered binding to gangliosides as measured by ELISA (Sunyaev et al, 2009; Gorelik et al, 2011). However, when these mutations were introduced into the infectious genomic clone of JCPyV in a Type 1 background, with the VP1 of the laboratory prototype strain Mad-1, the mutations were deleterious for viral propagation (Maginnis et al, 2013). Mutations at L54F, S266F, and S268F/Y significantly reduced infection and spread in SVGA cells. Introduction of PML-associated mutations into recombinant VP1 pentamers revealed that VP1 pentamers with these mutations no longer bind to SVGA cells. X-ray crystallography showed that VP1 pentamers with PML-associated mutations do not bind to LSTc oligosaccharide, as these mutations block the sialic acid binding site (Maginnis et al, 2013). Furthermore, introduction of PML-associated mutations into replication-defective pseudoviruses demonstrate that these mutations reduce the hemagglutination ability and the capacity of the virus to infect a panel of brain cell types, including glial cells (Maginnis et al, 2013). The differences in results obtained in the studies described above may also be attributable to the different genetic backgrounds of the viruses utilized as the infectivity of viruses with PML-associated mutations in the WT3 background has not been reported (Gorelik et al, 2011). Therefore, while these viruses are commonly found in individuals with PML, the mechanism by which they arise and their specific cellular tropism remains undefined. One hypothesis suggests that the mutations in VP1 sialic-acid binding sites allow the virus to spread more readily to the brain due to reduced non-specific attachment to sialic acid pseudoreceptors in the periphery resulting in enhanced neurotropism. This is similar to the results observed with mPyV in which a point mutation in the sialic acid binding pocket, a site orthologous to the Ser268 point mutation found in individuals with PML, enhances neurotropism in mice (Freund et al, 1991). However, it is also possible that high levels of viral replication results in defective particles with PML-associated mutations and that these isolates are readily available in biofluids due to their lack of sialic acid binding. Another possibility is that they are immune escape variants. The nature of viruses with PML-associated mutations warrants further investigation to understand whether these mutations are advantageous or detrimental for the virus and host.

Serotonin receptor is required for JCPyV entry

JCPyV entry into host cells is sensitive to chlorpromazine, an inhibitor of clathrin-dependent endocytosis (Pho et al, 2000; Assetta et al, 2013). Chlorpromazine is also an antagonist of serotonin 5-hydroxytryptamine receptors (5-HTRs) *in vitro* (Suzuki et al, 2012), and this led to the hypothesis that serotonin receptors may play a role in JCPyV infection. The Atwood laboratory identified the serotonin 5-HT_{2A} receptor (5-HT_{2A}R) as the proteinaceous receptor required for JCPyV infection (Elphick et al, 2004). 5-HT_{2A}R is a seven transmembrane-

spanning G-protein-coupled receptor that belongs to the family of serotonin receptors. These receptors are expressed in the CNS and are most notable for regulation of physiologic functions such as mood and psychiatric disorders such as depression. 5-HT₂Rs are abundantly expressed on multiple cell types in the brain including glial cells and also in the kidney; the major sites of JCPyV infection (Bonhaus et al, 1995; Bockaert et al, 2006). Additionally, 5-HT₂Rs are expressed in neurons, with 5-HT_{2A}R being highly expressed in the cerebral cortex (Willins et al, 1997), a site in which JCPyV has been detected in demyelinating lesions in individuals with PML (Du Pasquier et al, 2003; Wuthrich et al, 2012). Given that receptor expression is consistent with JCPyV tropism, it is reasonable that 5-HT_{2A}R expression is a key host-cell determinant of infection. Antibodies specific to either 5-HT_{2A}R or 5-HT_{2C}R block JCPyV infection in glial cells as well as stem cell-derived oligodendrocyte progenitor cells (Elphick et al, 2004; Schaumburg et al, 2008). Serotonin inhibitors including chlorpromazine, clozapine, ketanserin, ritanserin, mianserin, and mirtazapine reduce JCPyV infection in glial cells (Elphick et al, 2004; O'Hara et al, 2008). Additionally, expression of 5-HT_{2A}R in the poorly permissive HeLa or human embryonic kidney (HEK293A) cell lines enhances JCPyV infection (Elphick et al, 2004; Maginnis et al, 2010). Furthermore, JCPyV has been shown to colocalize with 5-HT_{2A}R-GFP within 5 and 30 minutes following infection by confocal microscopy (Elphick et al, 2004), suggesting that JCPyV and 5-HT_{2A}R internalize into a similar endocytic compartment during infection.

The observations that JCPyV utilizes an N-linked glycoprotein with α 2,3-SA (Liu et al, 1998b) or α 2,6-SA (Dugan et al, 2008) and 5-HT_{2A}R (Elphick et al, 2004) to drive infection led us to question whether serotonin receptor glycosylation was important for viral attachment to host cells. 5-HT_{2A}R contains five potential N-linked glycosylation sites that are predicted to be expressed in the extracellular amino terminal region, where they could be accessible to the virus (Backstrom et al, 1995). Therefore, it was hypothesized that the sialic acids necessary for JCPyV infection may in fact be expressed on the 5-HT_{2A}R (Focosi et al, 2008). To determine whether potential N-linked glycosylation sites expressed in 5-HT_{2A}R are required for JCPyV infection, Asn residues were mutated to Ala residues in a 5-HT_{2A}R-YFP construct and expressed in poorly permissive HEK293A cells. We found that N-linked glycosylation of 5-HT_{2A}R was critical for proper receptor expression at the cell surface, but was not necessary for JCPyV infection (Maginnis et al, 2010). Mutation of individual N-linked glycosylation sites had no effect on infection. However, when glycosylation was prevented by treatment of cells with tunicamycin or by mutating all glycosylation sites at once, the 5-HT_{2A}R expression at the cell surface was significantly reduced, and thereby infection was decreased. These data indicate that JCPyV infection requires N-linked glycosylation for the proper expression of the 5-HT_{2A}R, but treatment of cells with PNGase F, which removes sialic acids from N-linked glycoproteins at the cell surface, indicates that N-linked glycosylation is not directly required for infection (Maginnis et al, 2010). These results led us to conclude that the sialic acid receptor is likely not expressed on 5-HT_{2A}R. Furthermore, JCPyV is able to bind to a broad range of cell types yet retains a narrow cell tropism suggesting that multiple receptors contribute to JCPyV cellular tropism (Wei et al, 2000; Schweighardt et al, 2001).

Although 5-HT_{2A}R was found to be required for JCPyV infection, the role of 5-HT_{2A}R in the infectious cycle of JCPyV was unclear. The original report indicated that antibodies and

inhibitors to 5-HT_{2A}R and 5-HT_{2C}R blocked JCPyV infection, suggesting that multiple serotonin subtypes may function in JCPyV infection, possibly serving redundant roles (Elphick et al, 2004). Serotonin receptors belong to the family comprised of 15 isoforms divided into seven subfamilies based on their overall sequence similarity (Bockaert et al, 2006). To clarify the role of serotonin receptors in JCPyV infection, Assetta et al. tested the ability of 14 isoforms of the 5-HTRs to support JCPyV infection in the HEK293A cells, which express low levels of each 5-HTR isoform (Assetta et al, unpublished results) and are poorly permissive for infection (Maginnis et al, 2010; Assetta et al, 2013). Upon exogenous expression of the 5-HTRs of the 2-subfamily, infection is rescued in HEK293A cells, while none of the other serotonin isoforms can support infection (Assetta et al, 2013). Importantly, 5-HTRs of all subfamilies are widely distributed in the brain, and 5-HT₂Rs are heterogeneously expressed in the kidneys (Bonhaus et al, 1995). Some groups have reported that 5-HT_{2A}R is not required for JCPyV infection as some cell types such as human brain microvascular endothelial cells, which have reportedly low levels of 5-HT_{2A}R, are still infected by JCPyV (Chapagain et al, 2007). The clarification that all 5-HTRs of the 2-subfamily can support infection may explain why JCPyV can infect cells with low levels of 5-HT_{2A}R and why selective serotonin receptor inhibitors exhibit modest reductions in JCPyV infection in some cell types (Chapagain et al, 2007; O'Hara et al, 2008). It was also demonstrated that attachment of JCPyV to HEK293A cells over-expressing 5-HT₂Rs is equivalent to HEK293A cells alone, indicating that 5-HT₂Rs do not contribute to virus binding. In addition, expression of 5-HT₂Rs does not change the expression of α 2,6-SA on the cell surface, and soluble LSTc pentasaccharide blocks infection in 5-HT₂R-expressing HEK293A cells, suggesting that LSTc mediates viral attachment in these cells. However, HEK293A cells over-expressing 5-HT₂R-YFP have enhanced viral internalization in comparison to control cells, demonstrating that 5-HT₂Rs mediate JCPyV entry (Assetta et al, 2013). Further experiments are necessary to determine whether JCPyV directly interacts with 5-HT₂Rs to define whether 5-HT₂Rs serve as entry receptors or entry factors.

Intracellular transport of JCPyV

JCPyV undergoes intracellular transport and is ultimately delivered to the host cell nucleus to initiate viral replication (Schematic diagram, Fig. 2). Through a series of detailed cellular and molecular studies, the cellular factors involved in JCPyV entry and trafficking have become increasingly understood. Following engagement of receptors on the cell surface, JCPyV enters cells by clathrin-dependent endocytosis through a mechanism involving 5-HT₂Rs and epidermal growth factor receptor pathway substrate clone 15 (Eps-15) (Pho et al, 2000; Querbes et al, 2004; Assetta et al, 2013). Glial cells and HEK293A cells over-expressing 5-HT₂Rs are both sensitive to chlorpromazine, indicating that viral entry is mediated by clathrin-dependent uptake in both brain and kidney cell types (Pho et al, 2000; Assetta et al, 2013). Following clathrin-mediated endocytosis, JCPyV enters the endocytic system and colocalizes with Rab5-GFP as soon as 15 minutes post infection (Querbes et al, 2006). Rab proteins are small GTPases that serve as central regulators of membrane transport through their recruitment of effector proteins (Stenmark, 2009). Rab-GFP fusion proteins and dominant-negative (DN) Rab constructs have been useful in determining the specific endocytic requirements for JCPyV entry. Rab5 plays a critical role in formation,

transport, and fusion of vesicles to early endosomes (Zerial et al, 2001; Stenmark, 2009). Expression of Rab5-DN mutants inhibits JCPyV infection and demonstrates the importance of delivery of JCPyV to the endocytic system for infection (Querbes et al, 2006). Expression of the constitutively active form of Rab5 also decreases infection, further demonstrating that modulation of Rab5 function is refractory to JCPyV infection. It is likely that internalized 5-HT_{2A}R and JCPyV associate in early endosomes, as JCPyV and 5-HT_{2A}R colocalize in endocytic organelles as early as 5 minutes post infection (Elphick et al, 2004).

Rab7 sorts cargo from early endosomes and plays an important role in endosomal maturation (Stenmark, 2009; Girard et al, 2014). However, JCPyV does not colocalize with Rab7-GFP during entry and a Rab7-DN mutant does not inhibit infection, indicating that JCPyV does not enter maturing endosomes, late endosomes, or endolysosomes (Querbes et al, 2006; Qian et al, 2009; Qian et al, 2010; Engel et al, 2011). In addition, expression of a Rab11-DN mutant has no effect on JCPyV infectivity, suggesting that recycling endosomes do not play a role in infection. Following transport to early endosomes, JCPyV associates with Caveolin-1 (Cav-1) positive vesicles at 2 hours post infection (hpi). Knockdown of Cav-1 by shRNA inhibits JCPyV infection, and addition of the cholesterol-sequestering drug methyl- β -cyclodextrin blocks infection when added at time points up to 1 hpi. These results demonstrate that Cav-1, present in cholesterol-rich membrane domains, plays an important role in infection (Querbes et al, 2006). The association of JCPyV with cholesterol-rich domains was also demonstrated by sucrose flotation assays, showing that JCPyV, unlike transferrin, readily associated with markers of cholesterol-rich domains. Intracellular transport of JCPyV appears to differ from that of other polyomaviruses. SV40 and mPyV, which bind to the gangliosides GM1 and GD1a, respectively, undergo endocytosis by caveolar- or lipid-dependent mechanisms to enter early endosomes following receptor engagement (Pelkmans et al, 2001; Campanero-Rhodes et al, 2007; Qian et al, 2009; Ewers et al, 2010; Qian et al, 2010). SV40 is then transported to Cav-1- and Rab7-positive late endosomes, while mPyV is transported to Rab7-positive endosomes. However, there are conflicting reports as to the role of Rab7-positive endosomes in SV40 and mPyV entry (Mannová et al, 2003; Tsai et al, 2003; Damm et al, 2005; Gilbert et al, 2005; Liebl et al, 2006; Qian et al, 2009; Hayer et al, 2010; Engel et al, 2011). It is likely that these differing results are due to modifications in experimental conditions. Further studies are needed to determine whether the association between JCPyV and Cav-1 occurs in distinct endosomal compartments or in Cav-1-positive late endosomes. However, any differences in endosomal accumulation between JCPyV and other polyomaviruses are likely due to differences in receptor usage. From the endocytic system, JCPyV undergoes retrograde transport to the endoplasmic reticulum (ER) (Querbes et al, 2006; Nelson et al, 2012). It is likely that JCPyV leaves the endosomal system from early endosomes, but further studies are needed to definitively identify the site of endosomal egress. Many polyomaviruses, including SV40, mPyV, BKPyV, and even JCPyV, bind gangliosides and this interaction is critical for sorting of mPyV to the ER (Komagome et al, 2002; Campanero-Rhodes et al, 2007; Qian et al, 2009; Gorelik et al, 2011; Neu et al, 2013). Under physiologically normal conditions, gangliosides undergo endocytosis and are either rapidly recycled to the plasma membrane or are transported to lysosomes for degradation (Riboni et al, 1991; Tettamanti, 2004). In pathologic conditions where gangliosides are not degraded, accumulation of gangliosides in

the ER is seen and results in cellular toxicity (Tessitore et al, 2004). mPyV binding to gangliosides appears to retarget gangliosides towards the ER, resulting in transport of virions to the ER (Qian et al, 2009). However, it is unclear whether JCPyV binds gangliosides for productive infection, and JCPyV may utilize cellular transport factors differently from other polyomaviruses to promote ER transport.

Whereas JCPyV rapidly accumulates in endosomes, ER accumulation of virions is not seen until 6 hpi, which is similar to the transport kinetics of SV40 (Schelhaas et al, 2007; Nelson et al, 2012). This is in contrast to cholera toxin, which is delivered to the ER within 80 minutes post intoxication (Fujinaga et al, 2003). These differences in transport kinetics are likely due to differences in receptor usage or particle size. Transport to the ER is critical for JCPyV infectivity, as pharmacological treatments that inhibit retrograde transport or ER function decrease JCPyV infectivity (Querbes et al, 2006; Nelson et al, 2012). Previous studies have demonstrated that SV40 and mPyV interact with ER resident chaperones for productive infection. These chaperones, including PDI and ERp57, act to isomerize the interpentameric disulfide bonds of mPyV and SV40, resulting in partial disassembly of the viral capsid and exposure of the minor capsid protein VP2 (Gilbert et al, 2006; Schelhaas et al, 2007; Walczak et al, 2011). Knockdown of the ER chaperones PDI and ERp57 inhibits JCPyV infectivity, suggesting that JCPyV also engages the quality control machinery present in the ER to undergo partial disassembly, leading to exposure of VP2 (Nelson et al, 2012; Nelson et al, 2013). VP2 and VP3 have been suggested to play a critical role in egress of SV40 and mPyV from the ER (Magnuson et al, 2005; Daniels et al, 2006; Geiger et al, 2011). JCPyV mutants that lack VP2 or VP3 are noninfectious, demonstrating that these proteins are also important for JCPyV infection (Gasparovic et al, 2006). JCPyV is then transported from the ER to the host cell nucleus for viral replication.

A small molecule inhibitor of retrograde transport inhibits JCPyV infection

In recent years, increased interest has been directed towards development of antiviral compounds that target host cell proteins rather than viral proteins (Provencher et al, 2004; Bonavia et al, 2011; Krumm et al, 2011). As the host is not under selective pressure following antiviral treatment, targeting cellular proteins decreases the likelihood that escape mutants that are resistant to antiviral treatment will arise. Small molecules have been identified that target cellular proteins to inhibit JCPyV infection, including the Arf1 GTPase inhibitor brefeldin A1 (BFA). BFA inhibits retrograde transport of JCPyV to the ER (Querbes et al, 2006; Nelson et al, 2012), but also demonstrates numerous cytotoxic effects and alters cellular morphology (Misumi et al, 1986; Lippincott-Schwartz et al, 1989; Low et al, 1991; Huotari et al, 2011). As a result, BFA is not a promising candidate for pharmaceutical optimization (Barbier et al, 2012). The small molecule 2-[(5-methyl-2-thienyl)methylene]amino-N-phenylbenzamide (Retro-2) has been described to inhibit intoxication of cells by the AB toxins: ricin, Shiga-like, and cholera toxin (Stechmann et al, 2010). Retro-2 rapidly converts to a cyclic compound in solution, termed Retro-2^{cycl}, and acts on an unidentified host factor to inhibit transport of toxins from endosomes to the Golgi (Stechmann et al, 2010; Park et al, 2012; Nelson et al, 2013). Both AB toxins and JCPyV target the ER during cellular entry, and we therefore sought to determine whether Retro-2^{cycl} would inhibit replication of JCPyV (Stechmann et al, 2010; Nelson et al, 2013).

Pretreatment of SVGA cells with Retro-2^{cycl} inhibits JCPyV infectivity in a dose-dependent manner with a half maximal inhibitory concentration of 28.4 μ M (Nelson et al, 2013). Retro-2^{cycl} also inhibits JCPyV pseudovirus transduction in a variety of glial and kidney cells, demonstrating that Retro-2^{cycl} targets cellular transport machinery factors that are conserved in multiple cell lines. BKPyV and SV40 infection is also inhibited by Retro-2^{cycl}, suggesting that this compound targets cellular transport machinery necessary for replication of multiple polyomaviruses. Human papillomavirus 16 (HPV16) has recently been described to undergo retrograde transport to the Golgi apparatus for productive infection, and treatment of HeLa cells with Retro-2^{cycl} inhibits both retrograde transport of HPV16 to the Golgi apparatus, as well as infection (Lipovsky et al, 2013). Retro-2^{cycl} appears to block a similar stage in cellular entry of JCPyV and Shiga-like toxins, despite differences in Golgi accumulation during cellular entry (Stechmann et al, 2010; Nelson et al, 2012; Nelson et al, 2013). Retro-2^{cycl} does not block JCPyV attachment or entry, and studies varying the time of addition demonstrate that Retro-2^{cycl} blocks infection at time points that coincide with arrival of virions to the ER. Colocalization experiments using proximity ligation assays demonstrate that Retro-2^{cycl} treatment reduces ER transport of JCPyV resulting in decreased exposure of VP2 in the ER (Nelson et al, 2013). Taken together, these data demonstrate that Retro-2^{cycl} is an effective small molecule retrograde transport inhibitor of JCPyV. It should be noted that Retro-2^{cycl} is a non-optimized lead compound, and further medicinal chemistry studies of Retro-2^{cycl} may lead to the development of more efficacious compounds. Indeed, recent studies have demonstrated that analogs of Retro-2^{cycl} can be generated that inhibit intoxication of Shiga toxin at 100-fold lower concentrations, and these compounds will likely be efficacious against polyomaviruses as well (Noel et al, 2013).

Conclusions

JCPyV requires sialic acids to attach to host cells and initiate infection. Although a number of SA structures have been reported to function in JCPyV binding and infection (Liu et al, 1998b; Komagome et al, 2002; Gee et al, 2006; Dugan et al, 2008; Gorelik et al, 2011), JCPyV demonstrates specificity toward the linear pentasaccharide α 2,6-LSTc (Neu et al, 2010; Maginnis et al, 2013). Binding to LSTc is mediated by specific contacts in VP1 and requires attachment to the terminal sialic acid as well as the GlcNAc, resulting in a kinked L-shaped conformation for LSTc (Neu et al, 2010; Maginnis et al, 2013). Following viral attachment, JCPyV entry is facilitated by the 5-HT₂ family of receptors via clathrin-dependent endocytosis in an Eps-15-dependent fashion (Querbes et al, 2004; Assetta et al, 2013). JCPyV then enters the endocytic pathway and is transported to early endosomes followed by further retrograde transport through Cav-1-positive vesicles to eventually arrive at the ER (Querbes et al, 2006; Nelson et al, 2012). JCPyV retrograde transport to the ER is blocked by Retro-2^{cycl}, highlighting this compound as a possible candidate for anti-JCPyV therapy (Nelson et al, 2013). Future research defining the molecular requirements for JCPyV infection will aid in identifying risk factors for JCPyV infection as well as identifying new targets for the development of antiviral therapies.

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Figure 1. JCPyV VP1 in Complex with LSTc

(a) Ribbon trace diagram of the X-ray crystal structure of the JCPyV VP1 pentamer in complex with LSTc at 2.0 Å. A side-view of a VP1 pentamer is shown in cartoon representation with one VP1 monomer highlighted in pink and the other monomers in gray. LSTc is shown as a stick model and colored according to atom type (blue = nitrogens, red = oxygens, and orange = carbons). Black box indicates one of the five binding sites on the pentamer. (b) Close-up view of VP1 (surface representation) in complex with LSTc (stick diagram). Residues that contribute to binding (Leu54, Asn123, Ser266, and Ser268) are labeled in black and side chains are highlighted. Key hydrogen bonds are demonstrated by black dashed lines. VP1 binds to LSTc in an “L-shaped conformation” with contacts clustering around the terminal α2,6-SA (NeuNAc) on the short leg of the L and GlcNAc on the long leg of the L. Figures reprinted from Cell Host & Microbe 8(4), Neu and Maginnis et al., Structure-function analysis of the human JC polyomavirus establishes LSTc pentasaccharide as a functional receptor motif, 309-19, ©2010, with permission from Elsevier

Figure 2. JCPyV Attachment, Entry, and Trafficking

JCPyV binds to the $\alpha 2,6$ -SA on LSTc to mediate viral attachment. Viral entry occurs via the family of 5-HT₂Rs but it is not clear whether they directly interact. JCPyV is internalized by clathrin-dependent endocytosis and then traffics to the early endosome, then to Caveolin-1-positive vesicles before retrograde transport to the ER. The virus undergoes partial uncoating in the ER (indicated by red virions) and VP2 is exposed in the ER. Retro-2^{cycl} blocks retrograde transport to the ER and efficiently inhibits viral replication. JCPyV eventually arrives at the nucleus through a retrotranslocation step through an unknown mechanism. Viral transcription, replication, and packaging of new virions (green) takes place within the nucleus. Figure modified and reprinted with permission from © American Society for Microbiology, Clin Microbiol Rev. 25(3), 2012, 471-506. doi: 10.1128/CMR.05031-11