VIRAL AND HOST-CELL FACTORS CRITICAL FOR JC VIRUS INFECTION

BY

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B.A. BOSTON UNIVERSITY, 2003

A DISSERTATION IS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF DOCTOR OF PHILOSPHY IN THE DIVISION OF BIOLOGY AND MEDICINE AT BROWN UNIVERSITY

PROVIDENCE, RI

MAY 2009

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To my family and Andrew

Who encourage me, support me, and love me no matter what

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ACKNOWLEDGEMENTS

I would like to thank my advisor Dr. Walter Atwood for the opportunity to work in his lab. He took a chance on a dirt farmer from Colorado and helped me grow into a scientist. I am grateful for all his support and encouragement throughout the years, even for years when science was unkind. I greatly appreciate his belief that graduate students should be involved in the greater scientific community and, therefore, his support of our trips to many national and international conferences. I would also like to thank him for his humor and relaxed attitude that made the Atwood lab a fun place to work.

I would also like to thank my thesis committee: Dr. Tricia Serio, Dr. Richard Freiman and Dr. Arthur Salomon for their guidance and support during my graduate career. I am also very thankful to Dr. Daniel DiMaio for traveling to Brown and serving as my outside reader.

A huge thanks is due to all the past and present members of the Atwood lab. It was great to be a part of a group of angels and learn about the benefits of tight pants and stilettos. To former members Drs. Gretchen Gee and Syliva Eash: thanks for making the lab so much fun with long lunches, afternoon walks, and the occasional trip to the mall. Gretchen, thanks for letting me watch you do a mini-prep and not getting upset that I didn't know how to do anything when I first started. Dr. Aisling Dugan, thanks for all the wild adventures during conferences. Who knew so many crazy things happen in mini-vans or on the streets of Las Ramblas at 4am. Dr. Melissa Maginnis, I am so happy you joined our lab and became our "mini-PI." Your guidance has definitely helped me get out of grad school faster than I would have been able to do on my own. Also, thanks for being such a good friend and always being willing to listen to crazy stories. Thanks to

Joslynn Jordan and Bethany O'Hara for all the fun lunch times and conference memories. Stacy-Ann Allen, you have been so much fun to have in the lab. Keep smiling and don't lose your positive attitude – you'll need it when you become a 4th year!

I would like to thank the past and present administrative staff in both the MMI and MCB departments: Carol Reiss, Tammy Glass, Heather Forand, Wendy Virgadamo, Elaine Butler, Amanda Robinson, Lorie St. Pierre and Amy Bozek for all your help in negotiating the sometimes frustrating policies of Brown.

To my parents: thanks for all the years of encouragement and support, even when I moved so far away. I am so fortunate to have you, not just as parents, but also as friends. Thanks for understanding me and just letting me be Megan. To Zac, thanks for the many years of love and support you have given me, even after many bad teenage years!

Most importantly the biggest thanks goes to my husband, Andrew. You have been my biggest cheerleader throughout this process. You made me believe I could get through this; without you I would never have gotten to where I am today. Andrew, thanks for everything.

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ABBREVIATIONS

$5HT_{2A}$ R	5-hydroxytryptamine (serotonin) receptor
AIDS	Acquired Immunodeficiency Syndrome
BKV	BK Virus
CNS	Central Nervous System
CPE	Cytopathic Effect
DNA	Deoxyribonucleic acid
ER	Endoplasmic Reticulum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HAART	Highly active anti-retroviral therapy
HIV	Human Immunodeficiency Virus
Hsc70	Heat Shock chaperone 70
JCV	JC virus
KIPyV	KI polyoma virus
MCC	Merkel Cell Carcinoma
mPyV	Mouse Polyomavirus
NCCR	Non-coding control region
NFAT	Nuclear Factor of Activated T-cells
NFkB	Nuclear Factor kappa B
ND10	Nuclear Domain 10
NLS	Nuclear localzation signal
NMT	N' myristyl transferase
p53	Tumor suppressor protein 53
PARP	Poly (ADP-ribose) Polymerase
PCR	Polymerase Chain Reaction
PHFG	Primary Human Fetal Gial Cells
PKC	Protein kinase C
PML	Promyelocytic Leukemia Protein
PML	Progressive Multifocal Leukoencephalopathy
PP2A	Protein phosphatase 2A
Rb	Retinoblastoma protein
RT	Reverse Transcriptase
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SV40	Simian virus 40
SVG	SV40 T-Ag transformed Primary Human Fetal Glial Cells
T-Ag	Large T-Antigen
t-Ag	Small t-antigen
V-Ag	V-Antigen
VLP	Virus-like particles
Vp1	Viral protein 1
Vp2	Viral protein 2
Vp3	Viral protein 3
WT	Wild-type

CHAPTER 1

INTRODUCTION

Polyomaviruses and Disease

Progressive Multifocal Leukoencephalopathy

JC virus (JCV) is the causative agent of the fatal demyelinating disease Progressive Multifocal Leukoencephalopathy (74). This disease develops from a lytic infection of the myelin-producing oligodendrocytes in the central nervous system (CNS). JC virus has a limited tropism; it is restricted to oligodendrocytes, astrocytes, B-lymphocytes, tonsils, and kidney epithelial cells (66, 67). It is widespread in the human population; an estimated 70% of people are seropositive for JCV (73). The initial infection is thought to be subclinical and acquired during early childhood. JCV is generally latent but can become reactivated and traffic to the CNS upon immunosuppression, lytically infecting oligodendrocytes and causing Progressive Multifocal Leukoencephalopathy (4, 84). This disease, once considered rare, has become more common in recent years due to the acquired immune deficiency syndrome (AIDS) epidemic, where 4-6% of AIDS patients will develop Progressive Multifocal Leukoencephalopathy (60). Other groups at risk are patients undergoing chemotherapy, transplant recipients, and patients with multiple sclerosis (MS) or Crohn's disease being treated with natalizumab. Although other groups can contract Progressive Multifocal Leukoencephalopathy, 85% of cases are from human immunodeficiency virus (HIV)-positive patients (110).

Progressive Multifocal Leukoencephalopathy is characterized by multiple large areas of demyelination, large bizarre astrocytes, and nuclear inclusions in oligodendrocytes (Figure 1). It is diagnosed using magnetic resonance imaging (MRI) or polymerase chain reaction (PCR) of the cerebrospinal fluid (CSF). The lesions produced

are strikingly different than the lesions produced in MS. Progressive Multifocal Leukoencephalopathy lesions are usually diffuse and subcortical. They have ill determined boundaries, are irregularly shaped, and grow asymmetrically (110). Symptoms first appear as limb weakness and ataxia. Cognitive, speech, and visual deficits can also present during the progression of the disease, with death usually occurring within a year of onset (4, 84).

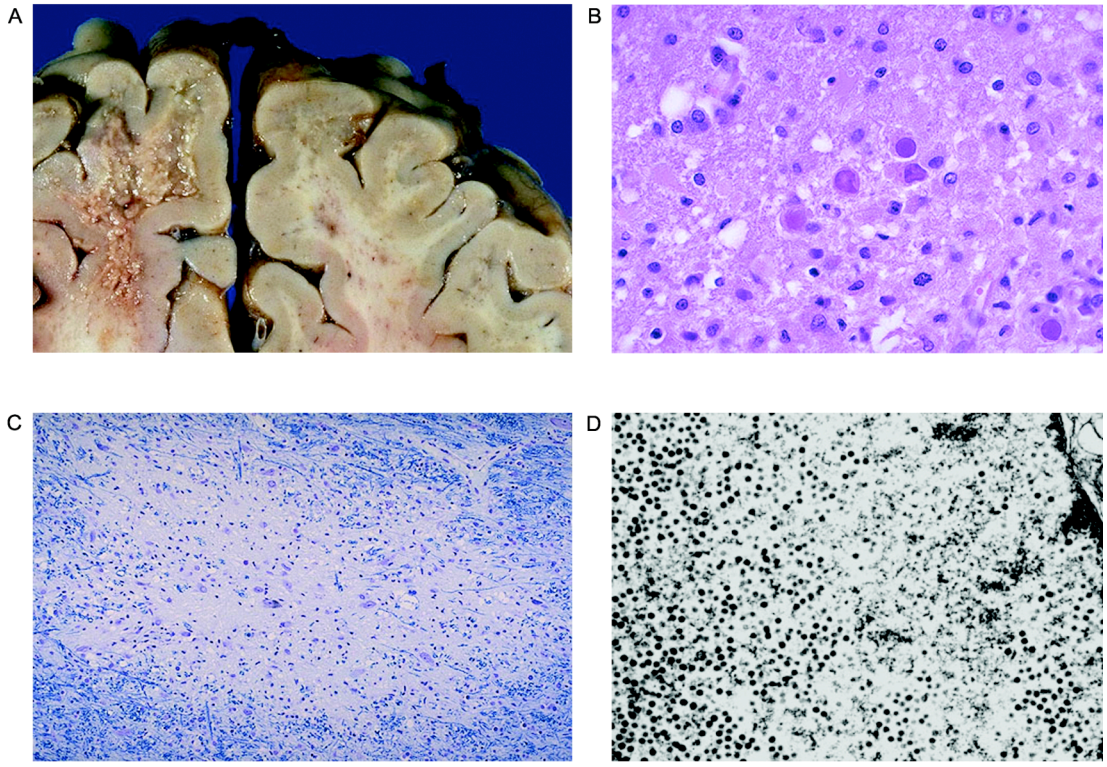

Figure 1: Progressive Multifocal Leukoencephalopathy brain sections. (A) Gross brain sections showing granularity in white matter. (B) Hemotyoxylin and eosin staining of lesions showing large, "bizarre" astrocytes and enlarged oligodendrocytes. (C) Luxol fast blue staining of oligodendrocytes showing demyelination. (D) Electron micrograph of oligodendrocytes infected with JC virus.
(All images are used with the written permission of Dr. Klatt from: http://library.med.utah.edu/WebPath/webpath.html)

There is no cure for Progressive Multifocal Leukoencephalopathy. Clinical trials have been performed using cytosine arabinoside, topotecam, cidofovir, and high dose of azidothymidine (also known as AZT). All of these failed to improve symptoms or were shown to be too toxic (45, 48). Currently the best treatment is highly active anti-retroviral therapy (HAART), which aims to treat the underlying immunosuppression and has been shown to slow the progression of the disease (84).

Progressive Multifocal Leukoencephalopathy was first described in the 1950s (117). However, it took more than a decade before the viral nature of the disease was uncovered. Originally, the disease was thought to have arisen from complications of lymphoproliferative diseases, since many of the early patients also had lymphatic leukemias or Hodgkin's Disease. As the pathology became more recognized, it was discovered patients with very different conditions were able to contract Progressive Multifocal Leukoencephalopathy. The common thread between patients was their impaired immunity, suggesting to researchers a viral nature to the disease (53).

During the 1960s, papilloma-like viruses were seen in electron micrographs of brain matter from patients with Progressive Multifocal Leukoencephalopathy. At that time, there were no characterized human polyomaviruses. As better staining techniques were developed, it was determined that virions found in the brain tissue were polyoma and not papilloma (117). Throughout the 1960s, virions were found in all brain samples from patients with Progressive Multifocal Leukoencephalopathy. Also during this time, cell culture systems were introduced and shown to support growth of simian virus 40, another polyomavirus. Researchers had also begun to culture primary human fetal glial (PHFG) cells. In 1970, the characteristic polyoma virions were found in the brain biopsy

of a patient named John Cunningham. This brain section was different than others: it was larger and contained more virions (108). By 1971, these virions were isolated from Mr. Cunningham's brain matter and cultured in PHFG cells. Since these were the first confirmed infectious virions, the virus was named with John Cunningham's initials (115, 116).

Polyomaviruses

Polyomaviruses were originally classified as *Papovaviradae,* which included both polyomaviruses and papillomaviruses. In 2000, the International Committee on the Taxonomy of Viruses split the family into *Polyomaviridae* and *Papillomaviridae. Polyoma* stems from the Greek words "poly," meaning many, and "oma," meaning tumors, as many of these viruses have been found to cause tumors in non-native host species.

There are 15 known polyomaviruses. These viruses infect a diverse array of species, including humans, non-human primates, murine, bovine, and avian (Table 1). The most commonly studied polyomaviruses are simian virus 40 (SV40), mouse polyoma, and the human polyomaviruses JCV and BKV. Recently, new human polyomaviruses have been discovered through the screening of respiratory secretions and human tumor samples. The first new human polyomavirus was discovered from sequencing DNA in respiratory secretions and analyzing the found sequences using GenBank. This analysis yielded a circular DNA molecule with homology to polyomaviruses. The genes are closely related to the early genes of polyomaviruses JCV, BKV, and SV40. However, its late genes are quite divergent. It has been named "KIPyV" (or "KI") after its discovery at the Karolinska Institute. Its infectivity and effect on humans has not been characterized (1).

Around the same time as the discovery of KI, another virus was found in patients with respiratory syndromes. A virus was isolated from nasal secretions of patients suffering from respiratory distress. The viral genes were also similar to genes of SV40, BKV, and JCV, but there is a low homology to these known polyomaviruses (30-40%).

When this virus was compared to KI, it was found to have a much higher homology (~65%). This new virus was named "WU" after its discovery at Washington University. WU also seems to be ubiquitous in the human population, as it has been found on most continents and in patients with ages ranging from 3 - 53 years old. WU, like KI, has not been shown to be infectious or capable of replicating in respiratory cells. It is hypothesized WU and KI are a separate branch of the human polyomavirus family that are more closely related to murine or simian members (39).

The third newly identified family member came from analysis of Merkel Cell Carcinoma (MCC). MCC is an aggressive skin cancer mostly found in elderly and immunosupressed patients. The phenotype of this cancer was reminiscent of Kaposi's sarcoma, which are tumors caused by herpesviruses, prompting researchers to search for a viral component to the tumors. MCC samples were analyzed using digital transcriptome subtraction, a method developed by the researchers that identifies foreign transcripts using cDNA sequencing data. When MCC tumor samples were examined, a genome with homology to other polyomaviruses was found. There were sequences similar to large T, the viral capsid proteins, and the viral origin. The viral genomes were integrated into the tumor DNA. The new virus has been named "Merkel cell polyomavirus" and is hypothesized to be the cause of the tumorigenesis seen in MCC (35).

Host	Virus	Characteristics
Human	JC virus (JCV)	• Infects kidney epithelium in healthy patients • Infects oligodendrocytes in immuno-compromised patients, causing PML
	BK virus (BKV)	• Infects kidney epithelium in healthy patients • Causes PVN in transplant recipients
	WU	• Found in patients with respiratory syndromes
	KIPyV	• Found in respiratory secretions
	Merkel Cell polyomavirus	• Found in Merkel cell tumors
Monkey	simian virus 40 (SV40)	• Naturally occurring in kidneys of macaques • Causes PML-like illness in immuno-compromised macaques
	simian agent 12 (SA12)	• Found naturally in baboons
	lymphotrophic papovavirus (LPV)	• Found in lymphoblasts of African green monkeys
Cattle	bovine polyoma virus	• Common in cattle
Rabbit	rabbit kidney vacuolating virus	• Found in wild rabbits
Mouse	mouse polyoma virus	• Naturally occurring in kidneys of mice
	K virus	• Naturally occurring in lung epithelium of mice
Hamster	hamster papovavirus	• Found to produce tumors in hamsters
Rat	rat polyomavirus	• Found in parotid gland of athymic rat
Parakeet	Budgerigar Fledgling Disease virus (BFDV)	• Causes fatal illness in avian species

Table 1: ***Polyomaviridae***. All 15 known polyomavirus family members and their associated diseases.
(Adapted from Fields Virology, Fifth Edition Knipe and Howley, 2007)

Polyomaviruses and Cancer

Polyomaviruses are classified as DNA tumor viruses. JCV has been shown to induce tumors in non-native host species. Golden Syrian hamsters were inoculated intracerebrally with JCV and monitored over the course of six months. By month four, hamsters receiving the virus began exhibiting diseases of the central nervous system. Their symptoms included unsteady gait, decreased activity, and circling. Once the symptoms appeared, the animals died within ten days. Upon examination of their tissues, it was revealed that the majority of animals had brain tumors that were glial in nature. JC virus was isolated from a few tumors; other tumors had expression of large T, the JCV early regulatory protein (109). Since this initial discovery, JCV has been found to cause tumors in other animals, including transgenic mice, rats, and primates (56). Owl monkeys inoculated intracranially with JCV produced malignant brain tumors within 16 to 36 months from inoculation. These malignant brain tumors were found in 25% of the animals and were glial in nature. The JCV genome was integrated in the DNA of the tumors in several animals. BKV and SV40 were unable to produce tumors in owl monkeys (65).

Although the tumor inducing properties of JCV in small animals has been well defined, its role in human cancers is still widely debated. JCV has been found in cancer tissues using PCR and immunocytochemistry for early viral protein large T. JCV has been found in many types of brain tumors in people with and without Progressive Multifocal Leukoencephalopathy (25, 28, 29). It has also been found in colon cancer and esophageal cancer (57, 83). However, JCV DNA has also been found in many of these tissues even without the presence of cancer, which is not surprising since most of the

population is seropositive for JCV (18). However, the rate of tumor formation is low in comparison.

The other strongly debated issue of polyomaviruses is whether SV40 can cause tumors in humans. SV40 is not thought to be currently circulating in the human population. However, from 1955 to 1963, around one hundred million people were potentially exposed to SV40 through contamination of poliovirus vaccines created using rhesus monkey cells. Since the rhesus monkey is a natural host for SV40, many of these cells were infected. Researchers think both the Salk and Sabin forms of the vaccine were affected. Subsequently, people have been found to have antibodies against SV40. SV40 DNA has also been found in some human tumors, but the role it plays in these tumors is still unknown (111).

JC Virus

Viral Classification and Biology

The JCV virion is small, 40-45nm particle that does not contain a lipid envelope (Figure 2A & B). The capsid has an icosahedral shape with 5- and 6-fold symmetry. The outer capsid is solely composed of the major protein Vp1, arranged in pentamers (112).

The genome is ~5.1kb and contains reading frames for six proteins divided into early and late gene products. The regulatory region contains a bidirectional promoter and the viral origin of replication, which bridges these two regions. The early region contains small t, large T, T'_{135}, T'_{136} and T'_{165} . All of these products are produced from alternative splicing of the viral early mRNA (107). The late region contains agnoprotein and the viral capsid proteins Vp1, 2, and 3 (Figure 2C). The viral DNA is packaged with histones H2A, H2B, H3, and H4 and creates a mini-chromosome structure that is almost indistinguishable from the host's chromatin (68). The host cell-type specificity can be controlled through transcriptional blocks to infection as well as virus-receptor interactions (16, 40, 61). The JC virus promoter contains multiple binding sites for transcription factors. It was recently determined nuclear factor for activated T-cells (NFAT) is a transcription factor required for JCV transcription. NFAT is activated by calcium release, presumably triggered by virus-receptor interactions. Once activated, it moves to the nucleus where it is able to interact with JCV genome and drive transcription (61). Additionally, JCV has binding sites for NF-1, NFkB, and AP-1, which could influence the cell type specificity of the virus or act as competitors in viral gene regulation (2).

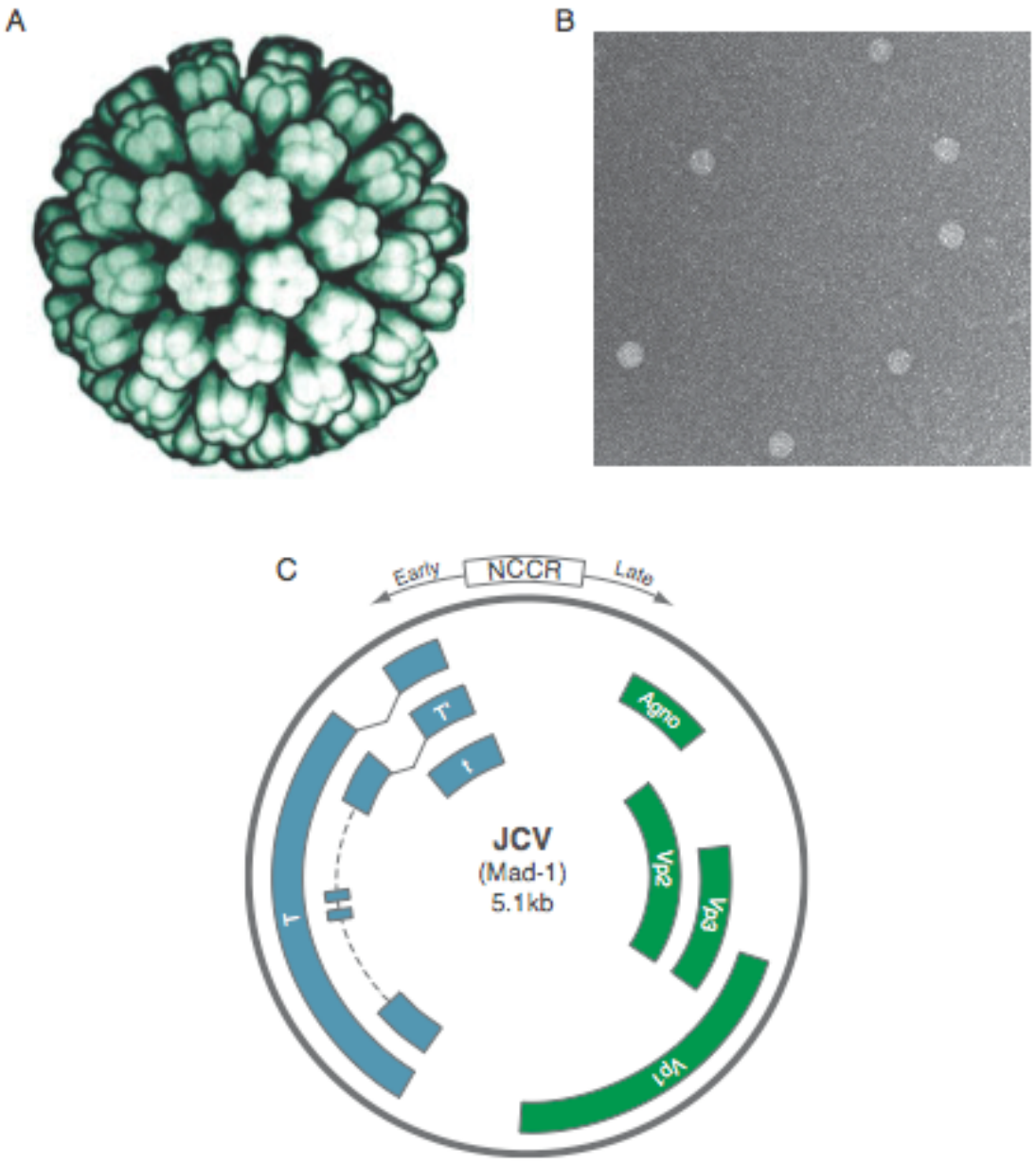

Figure 2: JC Virus Structure and Genome. (A) CryoEM of SV40. JCV is predicted to have the same capsid shape as SV40, but it has not been solved at this time. (B) EM of JCV virus particles. Virus is negatively stained with uranyl acetate and visualized at 112,000X. (C) JCV genome. The genome is divided into three regions: early, late, and the non-coding control region (NCCR). The early region produces the five regulatory proteins small t, T'_{135}, T'_{136}, T'_{165}, and large T antigen. The late region produces viral capsid proteins Vp1, 2, and 3 and the regulatory protein agno. The NCCR contains promoter elements and the viral origin of replication.

Regulatory Proteins

The T-antigens are produced as alternatively spliced products from a common pre-cursor pre-mRNA. They are classified as large T, small t, T'_{135}, T'_{136}, and T'_{165} based on their size. Large T antigen is a regulatory protein involved in deregulation of the cell cycle and viral DNA replication. It is composed of a variety of domains that interact with cellular factors to promote these actions (64). Small t and the T' proteins also have regulatory function, but their roles have not been as fully characterized as large T.

The N' terminal region of all T antigens has been described as a J domain, due to its homology to bacterial DnaJ chaperone. This domain has been shown to stimulate the ATPase activity of Hsp70 (DnaK) and is able to functionally substitute for the bacterial DnaJ. This functional substitution was a key experiment in showing large T itself has intrinsic chaperone activity (54). Additionally, human DnaJ homologues can substitute for the SV40 J domain (12). The J domain of SV40 binds and stimulates Hsc70, assisting in the release of bound cell cycle regulators. Hsc70 only interacts with cell cycle regulators when it is bound by SV40 large T. Hsc70 binding to large T is critical for the viral lifecycle. When this interaction is lost, large T is unable to cause replication of viral DNA (12).

Large T (LT) is able to interact with retinoblastoma (Rb) family members through its LXCXE motif and with p53 in its C' terminal ATPase domain (Figure 3) (103). These physical interactions have been demonstrated by immunoprecipitation assays of virally infected cells. In addition, these contacts are required for the transforming ability of large T, demonstrated by soft agar assays (77). Interactions of LT with p53 and Rb allow for cell cycle progression. Rb negatively regulates the E2F transcription factor; large T

breaks this association. The released E2F is competent to bind to DNA, stimulate the transcription of its products, and cause cell cycle progression (104, 113). This is a key step in the lifecycle of polyomaviruses: without cell cycle progression, the genome is not replicated and the capsid proteins are not produced. The dissociation of E2F requires the binding of Rb and the LT J domain activity to work in *cis* (94).

Figure 3: LT interacting domains. Large T interacts with Hsc70, Rb, and p53 to regulate the cell cycle, allowing viral gene production.

Recently, microRNAs (miRNAs) were found during the late phase of the JCV lifecycle. Polyomavirus miRNAs were first discovered in SV40, using an algorithm aimed at recognizing pre-miRNA in small genomes. A pre-miRNA was identified in the SV40 genome that produced a hairpin capable of being processed by RNA-induced silencing complex (RISC). This hairpin was able to produce two miRNAs targeting the early mRNA of SV40 (105). Similar analysis has since been performed for JCV. This analysis showed JCV also contains a homologous miRNA that targets the early mRNAs. This miRNA is unique in that both cleavage products target the same early transcript. The miRNA for JCV downregulates large T antigen late during the viral lifecycle. SV40 was still infectious in the absence of the pre-miRNA *in vitro*. However, it is hypothesized the viral miRNAs are important for downregulating large T to evade immune response *in vivo (93)*.

The role small t plays in JCV infection has not been extensively studied. We can gain insights into its role through its known functions in SV40 infection. SV40 small t has been shown to interact with the protein phosphatase 2A (PP2A) (99). PP2A is a cellular phosphatase with roles in both cell growth and transformation. SV40 small t interacts with PP2A and blocks its inhibition of protein kinase C. This release of inhibition stimulates extracellular signal-regulated kinase (ERK) and mitogen-activated protein kinases (MAPK) pathways, leading to increases in NFκB gene expression (100). Recently, small t in JCV has also been shown to interact with PP2A. However, these studies showed this interaction blocked the effect of PP2A on the late viral protein agno. The authors suggest this regulation of agno is critical for proper capsid maturation (90).

The T' proteins were discovered in 1995 and originally thought to be degradation products of large T (107). They all share the N' terminal J domain, but their sequences diverge at their C' terminus. This difference is thought to change their phosphorylation status and influence their interactions with the Rb family members p107 and p130 (10). All three are hypothesized to be important to tightly control the changes in the cell cycle needed for viral replication and transcription (9, 76).

Viral Origin and Promoter Region

The non-coding control region (NCCR) divides the two transcriptional regions of the virus. The NCCR contains a bi-directional promoter and enhancer for transcription of early and late gene products and the viral origin of replication. The initial strain of JC virus isolated from the brain of a patient in Madison, WI and was named Mad-1. The Mad-1 JCV regulatory region is made up of two tandem 98bp repeats. The promoter regions have binding sites for many transcriptional regulators, including NFAT, NFkB, AP-1, and NF-1.

At low levels, large T will stimulate its own transcription. However, it suppresses its transcription and stimulates viral replication once large quantities accumulate (19). This repression is hypothesized to be necessary for the replication of the genome. It is thought to repress transcription through inhibiting TATA initiation complexes rather than direct binding to the DNA. The NCCR also contains large T binding sites, which facilitate replication machinery assembly. One of the main factors recruited by LT is DNA polymerase α-primase. The α-primase is also thought to be an important factor regulating species specificity of the viruses as the mouse primase is unable to bind to large T of the human polyomaviruses (98, 101).

Large T is also an activator of the late viral genes and will assist in their production after the completion of genome replication (47). Large T can be differentially regulated through its phosphorylation status. These post-translational modifications will affect its binding to both DNA and cell cycle regulators.

Late Proteins

The late region contains agno, Vp1, 2, and 3. The V antigens (VAg) make up the virus capsid consisting of 360 molecules of the major coat protein Vp1 arranged in 72 pentamers creating the icosahedral shape (102). One of the minor coat proteins, Vp2 or Vp3, lies in the center of each pentamer (Figure 4D) (17). The pentamers are tied together by N' terminal regions of Vp1 that invade the next protein. The C' terminal tails of Vp1 interact with adjacent pentamers, tying the virion together (Figure 4B). JCV proteins are very similar to SV40 and BKV (Table 2). Although the crystal structure for JCV has not been solved at this time, the structures of SV40 and mouse polyoma provide insight into the structure of JCV. From these structures, we can see that Vp1 has a β-barrel structure with three large exterior loops (Figure 4A) (112). Vp1 is the sole component of the outer capsid and is the viral component that interacts with the cellular receptor (Figure 4C). Using site-directed mutagenesis, the amino acids required to interact with the cellular receptor have been mapped. From these mutations, a molecular model has been created showing JCV has a deep groove on its surface to accommodate receptor binding (41). The restricted tropism of JCV is also due to Vp1 binding with cellular receptors. In chimeric viruses that express SV40 early proteins, but JCV late proteins, the host cell range of JCV is maintained. This suggests viral regulation happens both at the gene level and the cell receptor level (16).

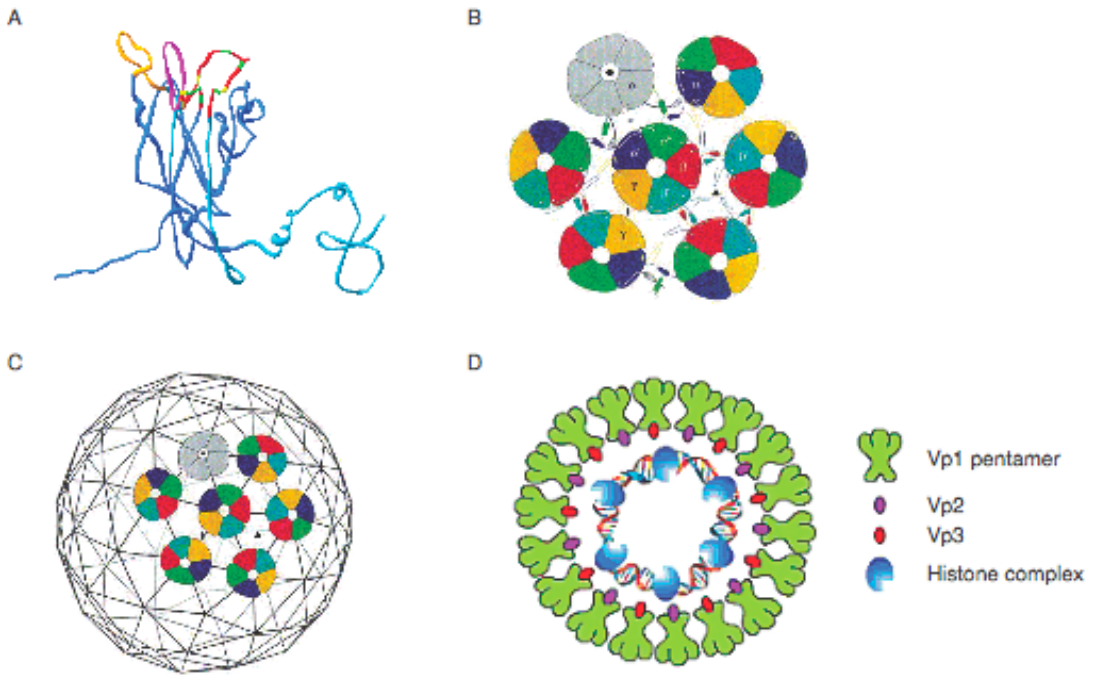

Figure 4: Vp1 pentamer and virus capsid. (A) JCV Vp1 pentamer created using SwissModel software. The structure was created using known SV40 Vp1 structure as a model. The molecule has a β-barrel structure with the outer, receptor interacting loops shown in yellow, purple, and red. (B) Vp1 molecules form a pentamer, each color indicates a unique monomer. The N' and C' terminal tails from each molecule are used to tie the virion together. The N' terminal tails interact within the pentamer, the C' terminal tails tie adjacent pentamers together. *Stehle et al, Structure 1996, used with written permission from Cell Press.* (C) Arrangement of the pentamers on the viral capsid surface. The virion has 5- and 6-fold symmetry. *Stehle et al, Structure 1996, used with written permission from Cell Press.* (D) Cross-section of the virion. Inside each pentamer there is a minor protein, Vp2 or Vp3. The minor proteins also interact with the viral DNA, which is complexed with histones and act as a bridge for the entire structure. Created by Kate Manley.

Vp3 is identical to the C' terminal two-thirds of Vp2 where this shared domain is comprised of the nuclear localization signal (NLS), the DNA binding domain, and Vp1 interacting domain (6, 20, 21, 42). Vp2 is also modified by a myristoylation moiety on its N' terminus (Figure 5). Myristoylation is process of adding a fatty acid co-translationally to a protein. Myristoyl proteins may be either cytoplasmic or membrane-associated. The

myristoyl group is transferred by the enzyme N-myristoyl transferase (NMT) after the methionine is removed and an N' terminal glycine residue is recognized (81). Myristoylation itself is not strong enough to anchor a protein within a membrane and therefore requires a basic region adjacent to the insertion point or an additional anchor such as a palmitylation. This membrane association can also be modulated through modifications. If a membrane-associated protein becomes phosphorylated, the negative charge will act to repel it out of the membrane. The exposure of the myristoylation site can also be modulated by conformational changes: it can be sequestered in a hydrophobic pocket until a stimulus allows it to be exposed (81). Many viral proteins contain myristoylation sites. These proteins are important for viral uncoating and release from membrane-bound compartments.

Previous work evaluating the role of minor coat proteins in SV40 determined Vp2 was dispensable but Vp3 was necessary for infection. They also suggested the importance of Vp3 could be its activation of poly (ADP-Ribose) polymerase (PARP) (43). This over-activation of PARP is thought to deplete intracellular ATP and cause cellular necrosis, releasing the virus (44). Recent work has shown both minor proteins are required for SV40 infection and that these proteins contain the ability to lyse bacteria. This could be an additional tool the virus uses to release itself from the cell (23). A new minor protein, Vp4, has also been discovered. Vp4 is not found in the virion itself, but is found in cellular lysates during late time points of infection. It has been postulated Vp4 is a lytic factor produced in order to complete the viral lifecycle (24).

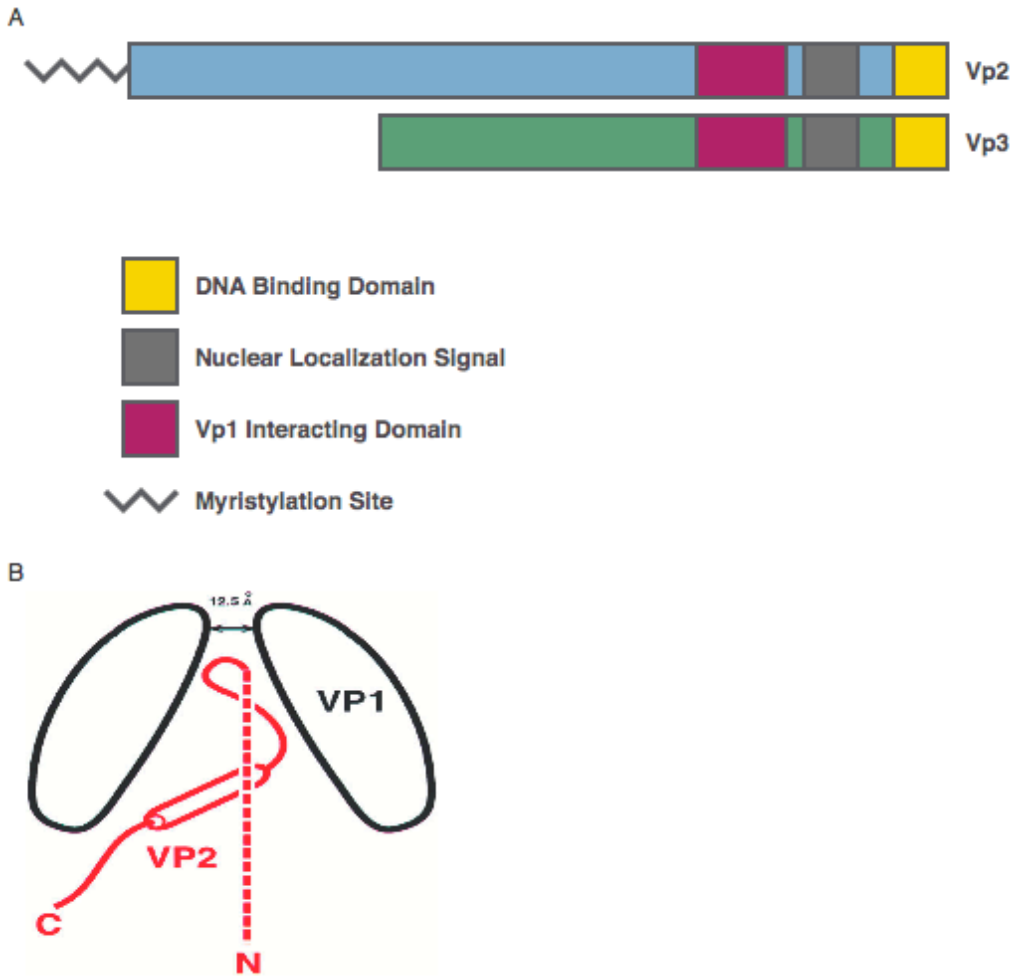

Figure 5: Minor protein domains. (A) Vp3 is identical to two-thirds of Vp2. This shared region is comprised of the DNA binding domains, the nuclear localization signal, and the Vp1 interacting domain. Vp2 is modified N' terminally with a myristoylation moiety. (B) Possible orientation of Vp2/3 within the Vp1 pentamer as determined from X-ray crystallography. *Chen et al, EMBO 1998, used with written permission from Nature publishing Group.*

Similar work on minor proteins has also been performed for mouse polyoma (mPy), with Vp2, Vp3, and Vp2 myristoylation mutants being produced. It was determined that Vp2 and Vp3 were essential for early and late events in the viral lifecycle. The myristoylation site has been changed to alanine, glutamate, glutamine and

histidine using site-directed mutagenesis. The alanine change showed delayed early kinetics, with viral replication and Vp1 production occurring later than wild type. This mutant did not show any virion stability defects. The change to glutamate or glutamine did not exhibit delayed entry kinetics during a single round of infection. However, in a longer time course they showed reduced reinfection, which was attributed to interactions with host cell structures. The glutamate change also showed a change in virion morphology. The histidine change showed an inability to enter and release from the cells, similar to the Vp2 and Vp3 mutants (55, 62, 88).

JCV requires the presence of both its minor proteins Vp2 and Vp3 for viral propagation. It also required the myristoylation site on Vp2, where unlike mouse polyoma, large bulky groups were not able to rescue the loss of the myristoylation site. JCV also requires Vp2, Vp3, and the myristoylation site to properly protect its genome from degradation by DNase (38).

	Large T	**Vp1**	**Vp2**	**Vp3**	**Agno**
SV40	72%	78%	79%	75%	62%
BKV	82%	75%	72%	66%	79%

Table 2: Sequence similarity to JCV. Comparison of the sequence similarity between SV40 and BKV to JCV early and late proteins.

Agnoprotein is also produced during the late viral lifecycle, but it is not packaged within virions. Its role in JCV infection remains elusive, but recent studies have begun to clarify its properties. Much of what is known about agno comes from studies of SV40.

All late transcripts are produced in a polycistronic manner. The agno reading frame is the leader sequence for all the late gene products (46). However, researchers did not know for many years the leader sequence actually produced a protein product. The SV40 agnoprotein was discovered in the early 1980s. It is a small protein (~61 a.a.), is highly basic, and has a very short half life (~ 2 hours), which is thought to make it a regulatory protein (51). These basic properties give it an affinity for binding to DNA. Agno has been found associated with replicating DNA and with DNA in partially assembled virions (50).

When agnoprotein is removed, the virus exhibits growth defects. This removal does not impact the production of early genes, DNA replication, or late gene transcription or translation. Virions are still made, but the rate of assembly is decreased and there is a decrease in the release of virions (82).

Agnoprotein has been characterized as localizing to the cytoplasm and perinuclear space by indirect immunofluorescence (71, 87). It contains multiple potential phosphorylation sites that, when removed, cause decreases in viral growth. Agno has been shown to be a substrate for PKC; it is believed changes in agno phosphorylation also change its cellular localization (89). Recently, agno has been described as a substrate for PP2A. Small t proteins' interaction with PP2A is believed to regulate the dephosphorylation of agno (90). With the highly basic nature of agno, it has been suggested that these changes in phosphorylation status of agnoprotein regulate its ability to bind to DNA (86).

JCV Lifecycle

Virus Entry, Trafficking, and Uncoating

We have determined through work in our laboratory that JCV binding and entry into the cell requires both an N' linked glycoprotein with an α(2-6)- or α(2-3)-linked sialic acid and the serotonin receptor 5-HT_{2A} (30, 32, 58). It is currently unknown whether the sialic acid is on the serotonin receptor itself. Following binding to the cell surface receptors, the virus is internalized by the ligand inducible clathrin-dependent pathway (78). The virus is initially trafficked to early endosomes and uses a Rab-5-dependent pathway to access the caveosome, from which it traffics to the endoplasmic reticulum (ER) (Figure 6) (79).

Virus trafficking is pH dependent, as demonstrated by an increase in endosomal pH causing a reduction in virus infection (3). Many viruses require low pH for one of three main reasons: viral membrane fusion, protease activation, or vesicular trafficking. Influenza has three proteins in its envelope, hemagglutinin (HA), neuraminidase (NA) and M2 the proton channel. Upon entering the endosome, the acidic pH causes HA to undergo conformational rearrangement, exposing a membrane penetrating form of the HA protein (11). In the endosome, M2 will pump protons into the viral particle, thereby releasing viral-genome complexes from the envelope (75). Ebola virus also requires acidification of the endosomes, but low pH does not allow for membrane fusion. Low pH activates cathepsins, which are endosomal cysteine proteases. Ebola requires both cathepsin B and L to create a viral peptide which is then able to induce endosomal membrane fusion (14, 92). Reoviruses also require low pH and cathepsin B and L for efficient disassembly and membrane penetration. This is confirmed by observation that

when reoviruses are digested prior to infection to generate their infectious subvirion particle (ISVP), they are able to overcome the requirement for low pH and cathepsins (5, 31).

One of the rate-limiting steps in the viral lifecycle is the uncoating of the viral genome and its delivery to the nucleus. Recently, a role for ER chaperones has been discovered for both SV40 and mouse polyoma. For mouse polyoma, interactions with ERp29, a protein disulfide isomerase (PDI) family member, cause conformational changes within the viral capsid (59). These conformational changes allowed the virus to interact with lipid membranes so it could deliver its genome to the cytoplasm. Furthermore, *in vitro* studies of mouse polyoma reveal Vp2 is capable of binding to and penetrating into the lipid membrane of the ER (80). After the genome has reached the cytoplasm, it is able to import into the nucleus using the traditional nuclear pore pathway. SV40 localizes and exposes its minor proteins in the ER (72). SV40 has been shown to interact with and require ER chaperones. It is hypothesized that upon delivery of the SV40 genome to the ER, chaperones uncoat the virus, where it becomes a candidate for the ER-associated degradation (ERAD) pathway (91). The ERAD pathway then pulls the partially assembled virus into the cytoplasm. SV40 is unable to undergo this retrotranslocation when the proteasome and membrane protein Derlin-1 are inhibited, which further support the role of an ERAD pathway in SV40 infection.

JCV traffics throughout the cell on a series of filamentous networks. Treating cells with nocodazole, cytochalsin D, and acrylamide disrupts these networks and JCV is no longer infectious. This indicates that JCV infection requires microtubules, microfilaments, and intermediate filaments during its lifecycle (3). The current model

suggests actin is important during early points of infection, either by directly interacting with virus-containing vesicles or indirectly affecting clathrin-dependent endocytosis. Microfilaments and microtubules are used for subsequent steps in the lifecycle as the virus continues to be transported in vesicles to the caveosome and then the ER.

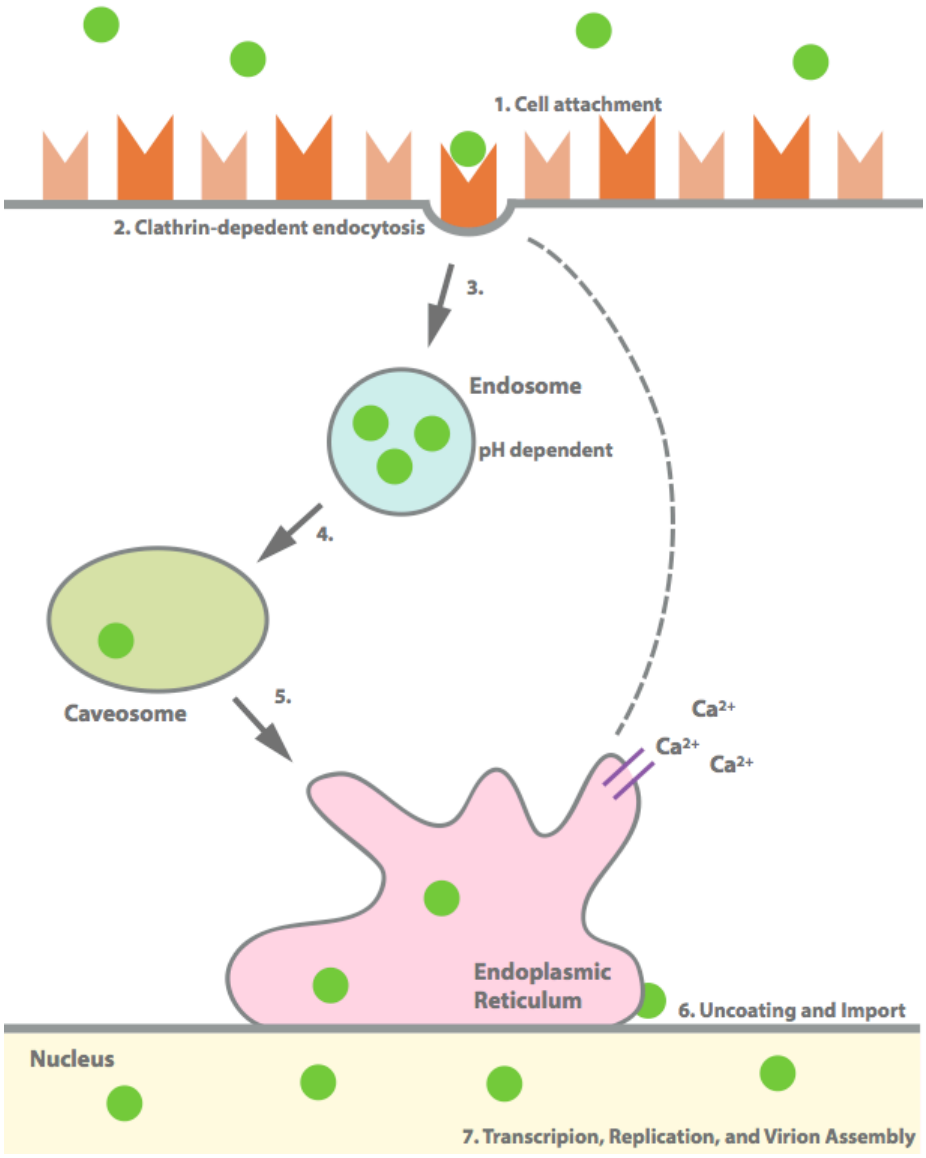

Figure 6: JC virus lifecycle. JC virus binds to cells using an α(2-6)- or α(2-3)-linked sialic acid and the serotonin receptor 5-HT_{2A}R (1). After binding, JCV is internalized using clathrin-dependent endocytosis where it traffics to early endosomes (2 and 3). JCV requires pH at early times during infection to complete its trafficking to the caveosome and the ER (4 and 5). Uncoating is hypothesized to occur in the ER. The virus is then delivered to the cytoplasm where it can import into the nucleus using nuclear pores (6). Once inside the nucleus, the virus transcribes its early genes, replicates its genomes, and transcribes late genes. Virus assembly also takes place in the nucleus (7).

Nuclear Domains

Upon entering the nucleus, DNA viruses can localize to distinct regions in the nuclear space. This provides a way to better regulate genome production and efficiently package the resulting virions. Nuclear domains are heterogeneous groups of proteins located within the nuclear matrix. They are connected to each other through a scaffolding of microtubules and microfilaments that allows them to dynamically change their localizations, protein contents, and responses to stimuli. These domains can be distinctly identified through their morphology and antigenic responses. Examples of the domains include the cajal bodies, the nucleolus, and the promyelocytic leukemia domain (PML, also known as ND-10). Each domain performs a unique function, such as ribosome biogenesis, storage, or responses to cellular stresses (Figure 7) (114).

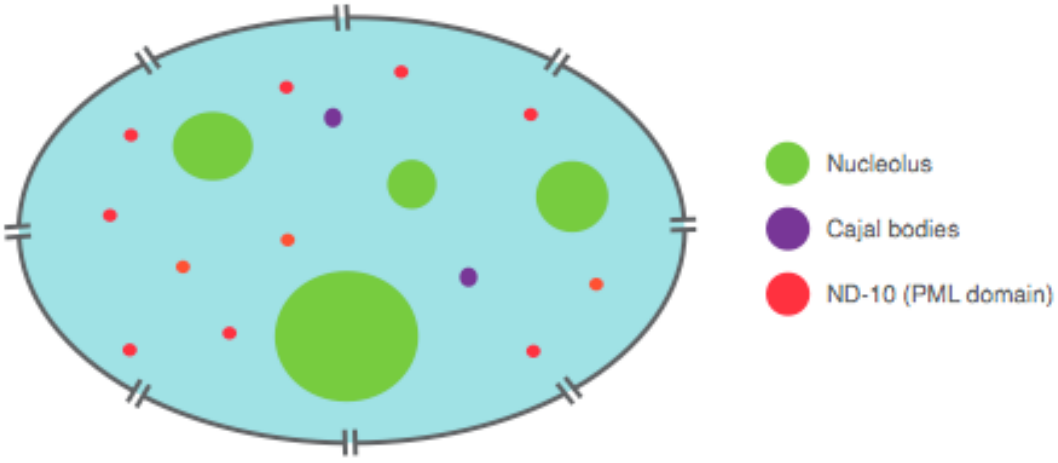

Figure 7: Nuclear structures. The nucleus is composed of domains used to regulate ribosome biogenesis, storage, and responses to cellular stress. Examples of these domains are the nucleolus, the cajal bodies, and the ND-10 or PML domains.

ND-10 domains appear in a speckled pattern, but the size and number of the domains vary by cell type. The PML domains are mobile structures whose movements

are ATP- and myosin-dependent. Proteins associated with this domain include Daxx, Sp100, and CREB binding protein. The PML protein seems to provide the scaffolding and allow for recruitment of other proteins. Daxx is a pro-apoptotic, Fas-interacting protein, recruited to this domain through its C' terminal region in association with SUMOylated PML. Daxx is not a permanent resident and can be recruited to heterochromatic domains by ATRX (49). Sp100 has a DNA binding domain and has been implicated in transcriptional modulation (70). Many transcription factors, such as CREB binding protein (CBP), are also localized to ND-10. Cells can survive without the presence of PML, suggesting possible redundancies in its role (114). The loss of PML is not without consequences. Acute promyelocytic leukemia (APL) has been linked to a chromosomal translocation that causes a fusion between PML and the retinoic receptor RARα. This, in turn, causes PML to be relocalized to the cytoplasm, where it is unavailable to respond to cell stress (114).

Multiple PML isoforms interact with p53 under stress conditions. In this state, p53, in complex with CBP, is recruited to the ND-10 domains. This recruitment will sequester p53 and cause cell cycle arrest. Additionally, when cells are lacking PML, they become resistant to radiation-induced cell death similar to p53 null cells. This suggests the ND-10 domain is also involved in apoptotic response pathways (27). PML mRNA can be upregulated in a p53-dependent manner where this regulation and expression are needed to induce senescence (27). Additional cancers can be formed when PML or p53 are missing. PML can also be regulated through MAPK signaling to promote premature senescence in a *ras*-dependant pathway (114).

Both PML and Sp100 are known to contain small ubiquitin-like modifiers (SUMO)

modifications, which are correlated with their ND-10 localization. When PML is de-SUMOylated, it becomes diffusely localized in the nucleus. This causes the ND-10 domain to disperse, further implying its role as a scaffold. Recently, a cysteine protease SuPR-1 was found to be able to remove the SUMO modification from PML. The removal of SUMO from PML allowed PML to activate transcription factors such as c-jun. This removal of SUMO-1 also caused relocalization of ND-10 components, such as CBP (8).

Many viruses have antagonistic relationships with the PML domain. Adenovirus type 5 immediate protein pIX has recently been shown to form inclusion bodies in the nucleus. These inclusion bodies consist of PML protein surrounded by pIX as a way to modulate PML function and enhance adenovirus infectivity (85). Herpesviruses also target the PML protein early in infection. HSV-1 immediate early protein ICP0 disrupts the ND-10 domain and causes PML to be degraded. In the absence of this degradation, HSV-1 is not able to complete its lifecycle (Table 3) (33, 63).

Papillomaviruses, have been shown to localize to ND-10 domains. Papillomaviruses contain one minor capsid protein, L2, which can localize to ND-10 domains within 24 hours of infection (26). L2 is required for L1, the major capsid protein, and E2, an early regulatory protein, to also localize to ND-10 (36). However, these associations are seen in undifferentiated cells. Papillomaviruses infect squamous-stratified epithelium and initially only express early genes. Late genes L1 and L2 are not expressed until cells become terminally differentiated (34). When terminally differentiated kerotinocytes were evaluated, it was seen that PML was not expressed. This indicates that PML is not required for HPV replication and transcription (7, 69). Other polyomavirus family members, SV40 and BKV, have been shown to replicate

adjacent to PML nuclear bodies (52). This replication is dependent on having a complete viral origin with LT binding sites and the expression of the LT protein itself (Table 3) (106).

JC virus minor proteins have been shown to accumulate in PML nuclear bodies. Assembled virus has been shown to reside in PML structures in post-mortem brains of patients with Progressive Multifocal Leukoencephalopathy (96, 97).

Virus Family	Virus	Interactions with PML domains
Herpesviruses	HSV-1	• ICP0 degrades PML in proteasome dependent manner
	HCMV	• Pp71 degrades hDaxx during early infection • Pp71 stimulates production of viral protein IE72 which disrupts PML by removing SUMO
Rhabdoviruses	VSV	• Increases in PML decrease mRNA and protein expression • Increased infection in PML-null mice • P protein sequesters PML and changes nuclear domains
Picornavirus	Polio	• Causes post-translational modifications of PML which recruits pro-viral factors to nuclear domains
Adenoviruses	Type 5	• pIX sequesters PML and enhances infection
Papillomaviruses	HPV	• Localizes adjacent to PML domains during early and late times in lifecycle • Does not require PML
Polyomaviruses	SV40	• Replicate adjacent to PML domains • Requires LT and origin of replication for localization
	BKV	• Replicate adjacent to PML domains
	JCV	• Localizes adjacent to PML domains • Does not require PML

Table 3: Viruses and PML domains. Representative list of viruses known to both positively and negatively interact with PML.

Virus Assembly and Release

Viral proteins are translated in the cytoplasm and must be imported back into to the nucleus to complete assembly. Unlike SV40, JCV Vp1 has a weak monopartite NLS. JCV Vp1 needs Vp2/3 to achieve proper import, suggesting a role for assembly of the pentamers in the cytoplasm. If the JCV monopartite NLS is changed to the bipartite SV40 NLS, it becomes exclusively localized to the nucleus and no longer requires assistance (95). Using insect cells to produce mouse polyoma capsid proteins in varying combinations, it was determined that not only were Vp2 or Vp3 needed for efficient nuclear import of Vp1 but that Vp2 also affected the post-translational modification of Vp1. This was observed using 2D gel analysis, which showed three of the six isoforms of Vp1 were missing when the minor proteins were removed. Additionally, the loss of the myristoylation moiety in Vp2, also affected the amount of the acidic Vp1 isoform (21, 37). Once inside the nucleus, the process of assembly is unclear. The minor proteins have DNA binding signals and interact with Vp1.

Virus-like particles (VLPs) composed of Vp1 alone are capable of self-assembly. VLPs created *in vitro* using bacoluvirus systems are capable of packaging DNA by manipulating the calcium and disulfide bonds in the system. These systems allow the capsid structure to be removed, DNA to be added, and the structure to be reformed upon reconstituting the disulfide bonds. The VLPs are able to take up this DNA and deliver it to another cell. The DNA incorporated is random and not due to interactions between Vp1 and the DNA. It is not clear whether these particles follow the same pathways as whole virus when they deliver their DNA (15).

Agnoprotein is also a likely factor assisting in virus assembly. It has a highly basic nature and an affinity for binding both single- and double-stranded DNA (50). When agno is removed during SV40 infection, Vp1 is less efficiently imported to the nucleus (13). In JCV infection, virus is still released when agno is altered. However, the DNA content of the capsids is diminished (90).

The manner of polyomavirus release is still unknown. Polyomaviruses known to be released in a lytic manner and not through apoptosis (44). For SV40, two mechanisms have been proposed, as described earlier. Capsid protein Vp3 is thought to stimulate PARP activities, depleting intracellular energy stores and causing necrosis (44). The late proteins Vp2 and Vp3 have also been shown to have lytic properties, which could contribute to membrane insertion and viral release (22). An additional lytic factor could be Vp4, which is only made late during viral infection and has been proposed to be the necessary factor for SV40 release (24). However, the lytic properties of the JCV minor proteins have not yet been characterized.

Specific Aims

The main focus of my research was to examine viral and host-cell factors that influence JC virus infection. JCV binds to the surface of cells and must traverse the cytoplasm to deliver its genome to the nucleus, where it will be replicated. We hypothesized that virus exposure to specific microenvironments within the cell would expose minor capsid proteins that could contribute to successful nuclear entry of the viral genome. Based on additional preliminary data we hypothesized that specific domains within the nucleus would influence virus gene expression and ultimately virus production from infected cells.

We therefore examined the environments JCV encounters early during infection. We characterized the role of pH, proteases, and the proteasome. Next we examined the role of nuclear domain (ND-10) on JCV gene expression. Lastly, we investigated the role of JCV minor capsid proteins in infection. Our specific aims were to:

1. characterize the role of pH and pH dependent proteases in virus infection
2. ask what role specific nuclear domains play in virus infection
3. define the role of the minor proteins Vp2, Vp3, and the myristoylation site in virus infection

The experimental findings of these aims and a discussion of their implications are presented in five chapters:

Chapter 1: This chapter provides appropriate background on JC virus and its associated disease Progressive Multifocal Leukoencephalopathy. Additionally, information is provided about the role of intracellular trafficking and nuclear domains in viral gene expression.

Chapter 2: This is an unpublished manuscript describing the role of pH, pH-dependent proteases, and the proteasome in viral entry and uncoating.

Chapter 3: This is a manuscript in preparation detailing the role of PML domains in regulating viral gene expression.

Chapter 4: This is a published manuscript detailing the requirements for the minor proteins Vp2 and Vp3 and the myristoylation site of Vp2 in the JCV lifecycle.

Chapter 5: This chapter describes the conclusions drawn from this work and its significance to the field.

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CHAPTER 2

CELLULAR FACTORS CRITICAL FOR JCV TRAFFICKING

Cellular Factors Critical for JCV Trafficking

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ABSTRACT

JC virus (JCV) is a human polyomavirus for which 70% of the world is seropositive. It binds to cells using an α(2-3)- or α(2-6)-linked sialic acid and the serotonin receptor 5-HT_{2A}R. JCV enters cells using clathrin-dependent endocytosis and traffics to the early endosome. JCV requires low endosomal pH for efficient infection. We show exposure of JCV to low pH does not allow for membrane fusion. Additionally, the low pH environment is not required for cathepsin protease activation. JCV exits the endosome to continue trafficking to the caveosome and the endoplasmic reticulum (ER), which is likely the site of viral uncoating. The ER is a calcium rich environment; we demonstrate that depleting ER calcium decreases JCV infection. However, we demonstrate that we can enhance JCV infection by loosening the capsid through trypsin digestion. This enhancement is likely due to an increase in the rate of uncoating.

INTRODUCTION

JC virus (JCV) is a human polyomavirus that infects most of the world's population during childhood. Currently 70% of people worldwide are seropositive for JCV (28). JCV is the etiological agent of the fatal demyelinating disease Progressive Multifocal Leukoencephalopathy. This disease is caused when JCV infects and lytically destroys the myelin-producing oligodendrocytes in the central nervous system (CNS) (29). JCV is normally latent in as yet unidentified peripheral sites, possibly the kidneys or B-lymphocytes. The virus becomes activated upon immunosupression and migrates to the CNS. 85% of Progressive Multifocal Leukoencephalopathy cases are in HIV positive patients, but patients undergoing chemotherapy, transplant recipients, or multiple sclerosis (MS) and Crohn's disease patients taking natulizumab can also become susceptible to this disease (2, 24, 36).

JCV is a small, non-enveloped, double-stranded DNA virus (26). It produces five early regulatory proteins, small t, large T, T'_{135}, T'_{136}, and T'_{165}, which are all created from alternative splicing of the early mRNA (40). JCV has three capsid proteins, Vp1, 2, and 3. Vp1 is the major capsid protein and makes up the exterior of the virion. There are 360 molecules of Vp1 that form 72 pentamers to create the icosahedral capsid (39, 41). Vp2 and Vp3 are on the interior of the capsid. There is one Vp2 or Vp3 molecule associated with each pentamer (9).

JCV binds to cells using an α(2-3)- or α(2-6)-linked sialic acid and the serotonin receptor 5-HT_{2A}R (15, 17, 22). JCV enters cells using clathrin-dependent endocytosis, where it traffics to early endosomes and then the caveosome using a Rab5 dependent pathway (30, 32). From the caveosome, JCV moves into the endoplasmic reticulum (ER)

(33). The ER is the likely site of uncoating for JCV, as it has been characterized as the location for SV40 and mouse polyoma. Mouse polyoma interacts with ERp29, a PDI family member, causing conformational change in the capsid (23). This conformational change promotes membrane association and penetration by the minor protein Vp2 (34). SV40 also requires assistance of chaperones for its uncoating. However, SV40 also requires the proteasome, presumably through the ER-associated degradation (ERAD) pathway, which helps deliver the uncoated capsid to the cytoplasm (37).

JCV has been previously shown to require low pH for its early entry events (1). Viruses require low pH for conformational changes, activation of pH dependent proteases, or vesicular trafficking (Fig. 1). Influenza has three proteins in its envelope, hemagglutinin (HA), neuraminidase (NA), and M2, the proton channel. Upon entering the endosome, the acidic pH causes HA to undergo conformational rearrangement, exposing a membrane-penetrating form of the HA protein (5). In the endosome, M2 pumps protons into the viral particle, releasing viral-genome complexes from the envelope (31). Ebola virus also requires acidification of the endosomes, but low pH does not permit membrane fusion. Low pH activates cathepsins, which are endosomal cysteine proteases. Ebola requires both cathepsin B and L to create a viral peptide, which then induces endosomal membrane fusion (7, 38). Reoviruses also require low pH and cathepsin B and L for efficient disassembly and membrane penetration. This is confirmed by the observation that when reoviruses are digested prior to infection to generate their infectious subvirion particle (ISVP), they are able to overcome the requirement for low pH and cathepsins (4, 16).

In our studies, we determined low pH was only required during the first two to three hours of infection. However, low pH does not allow membrane fusion to occur. Additionally, cathepsins, low pH activated proteases, did not play a role in JCV infection. When JCV was capsid underwent a change due to trypsin digestion, it became more infectious. However, these digested particles still required low pH within the endosomes, indicating low pH was needed for continued vesicular trafficking from the endosome to the caveosome and the ER. Additionally, JCV requires the high calcium environment of the ER prior to its gene expression. These results help clarify the path JCV takes during its entry into cells and its reliance on cellular factors.

MATERIALS AND METHODS

Cells, viruses, antibodies, and reagents. SVG-A cells are a subclone of the original SVG human glial cell line established by transformation of human fetal glial cells by an origin-defective SV40 mutant (25). SVG-A cells were maintained in a humidified 37°C CO_2 incubator in Eagle's minimal essential medium (EMEM) (Mediatech Inc., Herndon, VA), supplemented with 10% heat-inactivated fetal bovine serum (Mediatech Inc.). The PAB597 hybridoma produces a monoclonal antibody against the SV40 major capsid protein Vp1 and was a generous gift from Ed Harlow. This antibody has been previously shown to cross-react with JCV Vp1 (3). The PAB962 hybridoma produces a monoclonal antibody against the JCV large T antigen and was a generous gift from the Tevethia lab. The generation and propagation of the Mad-1/SVEΔ strain of JCV, which is the Mad-1 genome with SV40 regulatory elements, has been previously described (20, 21). Rabbit serum containing anti-JCV neutralizing antibody was used to neutralize infection and blot for Vp1. The Vp2/3 antibody was a generous gift from the Oppenheim lab. Reagents: purified Trypsin (Calbiochem, Gibbstown, NJ), Soybean Trypsin Inhibitor (Calbiochem), Thapsigargin (Calbiochem), FYdmk (Calbiochem), CA-074 (Calbiochem).

Indirect immunofluorescence. SVG-A cells were grown to 50% confluence on coverslips. Cells were treated with drugs at indicated time points. JCV infection was assayed 48 hrs post-infection for LT or 72 hrs for Vp1. Cells were fixed in 2% paraformaldehyde (PFA) for 20 mins and washed several times in phosphate-buffered saline (PBS) (137 mM NaCl, 2.682 mM KCl, 8.1 mM Na2HPO4, 1.47 mM KH2PO4, pH 7.2). Cells were permeabilized in 0.5% Triton-X 100 for 15 mins at room temperature,

then incubated with a 1:10 dilution of the PAB597 or PAB962 monoclonal antibody in PBS at 37°C for 1h. Cells were washed three times in PBS, incubated with a 1:500 dilution of goat anti-mouse Alexa Fluor 488 (Molecular Probes, Carlsbad, CA) at 37°C for 45 min, and rinsed in PBS. Coverslips were mounted onto slides with mounting medium containing DAPI (Vector Labs, Burlingame,Calif.). JCV-positive cells were visualized on a Nikon epifluorescence microscope Eclipse E800 (Nikon Inc., Melville, N.Y.) and scored by counting. At least ten visual fields were counted for each sample.

CsCl Purification. SVG-A cells were grown to 50% confluence in 20 150-cm^2 flasks. Cells were infected with JCV for 1 hr at 37^{o}C. After incubation, the virus was maintained and media replaced. Virus was cultured for two weeks or until exhibiting cytopathic effects. Cells were scraped down and pelleted by centrifugation at 2,000rpm for 10 mins. Pellets were resuspended in 10% of the supernatant volume and subjected to three rounds of freeze/thaw. After final thaw, 2.5% deoxycholate was added to bring the final concentration to 0.025% deoxycholate. Samples were spun at 10,000rpm for 30 mins. Supernatant containing virus was layered onto 20% sucrose in buffer A (50mM NaCl, 10mM Tris, 0.01mM$CaCl_2$) and spun at 35,000rpm for 3 hrs in a SW-41 swinging bucket rotor (Beckman, Fullerton, CA). Supernatant was removed and pellets were resuspended in buffer A with 0.01% Triton-X and layered on top of a CsCl gradient containing 0.5mL each 1.35g/mL, 1.32g/mL, 1.29g/mL, 1.26g/mL, and 1.23g/mL CsCl. Samples were spun at 33,000rpm for 16 hrs in a SW55Ti swinging bucket rotor (Beckman). Virus was removed and dialyzed against buffer A for 48 hrs.

Hemolysis. Equal volumes of CsCl JCV were incubated with PBS pH 7.5, 7, 6.5, 6, 5.5, and 4.5 at 37^{o}C for 30 mins. Samples were added to 3% calf red blood cells and incubated at 37^{o}C or 4^{o}C for 30 mins. Samples were pelleted by centrifugation and 25uL of supernatants were plated into a 96-well plate and diluted 50% in PBS. Absorbance was read at 415nm. % hemolysis= $[(A_{sample}-A_{blank})/(A_{Detergent}-A_{blank})]*100$

DNase Sensitivity Assay. CsCl JCV protein level was determined with a Bradford assay. Equal amounts of CsCl JCV were digested with 250ug/mL of trypsin or PBS for 1 hr at 4^{o}C. Reaction was stopped by adding 3-fold excess soybean trypsin inhibitor on ice for 15 mins. Equal amounts of digested and undigested CsCl JCV were treated with 20-fold excess DNaseI or mock treated with water at RT for 10 mins. DNase was inactivated with equivalent amount of 25mM EDTA and incubated at 65^{o}C for 10 mins. The capsids were digested with Proteinase K for 15 mins at 56^{o}C. The DNA was purified using a Blood Kit (Qiagen, Germantown, MD) per manufacturer's directions. Purified DNA was amplified by PCR (1ug of each primer using Promega GoTaq System (Promega, Madison, WI) (Primers (5'-3') JCV_{for}401: GTG AAG ACA GTG TAG ACG G and JCV_{rev}1070: GAA TTT CCT GAG AGG TTA AGC) and visualized in a 1.5% agarose gel with ethidium bromide.

Electron Microscopy. CsCl JCV was digested with 250ug/mL of trypsin for 1 hr at 4^{o}C. Reaction was stopped by adding 3-fold excess soybean trypsin inhibitor on ice for 15 mins. 3uL of digested and undigested CsCl JCV were bound to carbon-coated formvar grids for 20 sec. Excess was removed with blotting. Samples were washed with water for

15 sec. Excess was removed with blotting. Samples were stained with 2% uranyl acetate for 15 sec. Excess was removed with blotting. Virus was visualized using a Philips 410 transmission electron microscope.

Western blot analysis. CsCl JCV was digested with 250ug/mL of trypsin for 1 hr at 4^{o}C. Reaction was stopped by adding 3-fold excess soybean trypsin inhibitor on ice for 15 mins. OR CsCl JCV was digested with 50ug/mL of proteinase K for 20 min at 37^{o}C. Reaction was stopped by adding 3-fold excess phenylmethanesulphonylfluoride (PMSF) on ice for 15 mins. Digested and undigested samples were loaded onto a 4-15% Tris-HCl polyacrylamide gel (Bio-Rad, Hercules, CA), run at 30mA, transferred to nitrocellulose membranes using a mini-*trans* blot apparatus (Bio-Rad), and blocked with 5% milk in PBS containing 0.05% Tween 20 (PBS-T). Blots were probed with the respective antibodies all diluted in 5% milk in PBS-T, washed in PBS-T, and then incubated with goat anti-rabbit Alexa Fluor 680 (Molecular Probes, Carlsbad, CA) antibody diluted 1:5,000 in 5% milk in PBS-T. This was followed by further washes with PBS-Tween 20 and one in PBS. Blots were viewed using an infrared scanner (LI-COR, Lincoln, NE) and analyzed using Odyssey software (LI-COR).

RESULTS

Low pH is required for JCV infection but does not cause membrane fusion. We have previously shown that pre-treating permissive SVG-A cells with 25mM ammonium chloride (NH_4Cl) will decrease JCV infection (1). To determine when low pH is important, SVG-A cells were pre-treated with ammonium chloride, treated at the time of infection, or treated four hours post-infection. It takes two to three hours to raise the pH of the endosomes after NH_4Cl treatment. JCV infection was assayed 48 hours later with an indirect immunofluorescence assay of large T (Fig. 2). This determined NH_4Cl is only able to repress JCV infection when cells are pre-treated. There is no effect when samples are treated at the time of infection, indicating low pH is important very early during JCV infection.

To determine whether pH is able to cause a change in JCV, allowing it to interact and lyse membranes, a hemolysis assay was performed. Hemolysis tests the ability of red blood cells (RBCs) to be lysed and release their hemoglobin. Intact JCV virions are able to bind RBCs as shown by hemmaglutination assays, but they are not capable of lysing them. However, JCV does contain a myristoylated Vp2, which could become accessible following a conformational change (6, 27). Vp2 and Vp3 of SV40 and mouse polyoma are capable of both lysing and inserting into membranes (11, 12, 23, 34). Additionally, the reovirus μ1 protein contains an N'terminal myristoylation site that becomes exposed and allows the virus to penetrate the endosomal membrane and be released into the cytoplasm. To test whether low pH exposes lytic peptides in JCV, the virus was incubated with a range of acidic PBS solutions at both 37^{o}C and 4^{o}C. After incubation, JCV was combined with RBCs and the released hemoglobin was measured with a

spectrophotometer (Fig. 3A). There was no hemoglobin release in any of the JCV samples, indicating it does not gain the ability to lyse membranes upon exposure to low pH. To determine the effects of low pH on the JCV capsid, purified JCV was incubated with PBS solutions (pH 7.5 to 4.5), subjected to limited proteolysis, and analyzed by Western blot analysis (Fig 3B). JC virus can be digested with proteinase K (PK), but lowering the pH does not cause further cleavage of the capsid. Additionally, incubation of purified JCV with different pH PBS did not change bis-ANS binding (data not shown). Bis-ANS binds exposed hydrophobic groups suggesting internal parts of the capsid are not exposed when the pH is lowered.

Cathepsins are not required for JCV infection. To determine whether cathepsins, pH-dependent cysteine proteases, are involved in digesting virions during infection, SVG-A cells were treated with inhibitors of both cathepsin L and cathepsin B. The inhibitor CA074 was used to specifically inhibit cathepsin B. FYdmk was used at low concentrations to inhibit cathepsin L and at high concentrations to inhibit both cathepsin B and L as previously described (7). Both inhibitors reduced Ebola and reovirus infections (7, 16). SVG-A cells were treated with cathepsin inhibitors prior to infection or during early times post-infection. Viral protein expression was assayed 48 hours post-infection by indirect immunofluorescence of large T (Fig. 4). Inhibiting cathepsin L or B did not affect JCV infection, indicating they do not contribute to early events in the JCV lifecycle. Additional treatment of cells with E64 a broad range cysteine protease inhibitor showed no affect on JCV infection (data not shown). These results show digestion of the viral capsids by cathepsins is not needed for viral infection.

Loosening of JCV capsid increases infection but does not eliminate the requirement for low pH. Virus uncoating requires a conformational change in the virion. To simulate this, JCV was digested prior to infection with trypsin. This treatment causes changes in the virion particle, as seen by Western blot analysis. The major capsid protein, Vp1, appears as a doublet after trypsin treatment (Fig 5A). The minor capsid proteins also become accessible to digestion and appear as multiple bands (Fig. 5B). To find out whether this change allowed access to the viral genome, a DNase sensitivity assay was performed. After the virions were digested with trypsin, they were treated with DNase or mock treated with water. The capsids were removed with proteinase K (PK) and the viral genome was amplified with PCR. After trypsin digestion, the capsids became sensitive to DNase (Fig. 4C). However, when viral particles were examined by electron microscopy they did not appear to have gross conformational changes (Fig. 5D). These digested virions were also unable to cause hemolysis (data not shown). To evaluate whether this change in capsid structure affects ability of JCV to infect, the digested virions were used to infect SVG-A cells. Some SVG-A cells were pre-treated with NH_4Cl to see whether low pH would still be required. JCV infection was assayed 48 hours later using indirect immunofluorescence of large T. Surprisingly, the digested, virions became more infectious, but both digested and undigested viruses were still sensitive to elevated pH levels (Fig. 6A and B). These studies along with previous studies using bafilomycin A, indicate that low pH is required for continued JCV trafficking (1).

JCV requires ER calcium homeostasis. JCV has been shown to move from the endosome to the caveosome and finally to be deposited in the ER (33). The ER contains a high calcium environment. Polyomaviruses' structural stability can be modulated by calcium binding. SV40 and mouse polyoma structures have been determined and show two calcium binding sites at the base of the Vp1 pentamers (39). These binding sites are important to help adjacent pentamers interact and tie the capsid together. Additionally, JCV virus-like particles made of Vp1 alone can form in the presence of high calcium (8, 10). It is hypothesized this calcium coordination is critical for virus uncoating and genome delivery to the nucleus (18, 19). To determine whether ER calcium homeostasis is critical for JCV infection, SVG-A cells were treated with thapsigargin, an inhibitor of ER Ca^{2+}-ATPase. Thapsigargin causes calcium to be released from the ER and accumulate in the cytoplasm. SV40 infection is greatly reduced when CV-1 cells are treated with thapsigargin. JCV infection was scored 48 hours later using an indirect immunofluorescence assay of large T. When cells were pre-treated or treated 6 hours post-infection, JCV infectivity was greatly reduced. When treated 24 hours post-infection, however, there was little effect (Fig 7). This suggests ER calcium homeostasis is critical for JCV infection and that JCV requires the ER early during its infection.

DISCUSSION

Virus uncoating is a critical and rate-limiting step in the viral lifecycle. Viruses encounter cellular factors, which cause conformational changes in the structure that allow the release of the viral genome. Many enveloped viruses are able to fuse with the cell membrane and directly release their genome into the cytoplasm. However, non-enveloped viruses require cellular uptake to uncoat. Even upon cellular uptake, non-enveloped viruses must rely on low pH, proteases, or chaperones to assist in conformational changes and ultimately uncoating.

In this study, we described critical components in the early points of the JCV lifecycle. JCV requires a low pH environment to ensure it is properly trafficked throughout the cells. This low pH alone is unable to expose a lytic peptide in the virion, as determined by hemolysis assays (Fig. 3A) and limited proteolysis of the major protein Vp1 (Fig. 3B). Additionally, low pH-activated cathepsins are not required for JCV infection (Fig. 4). Although cathepsins can digest JCV *in vitro*, in the presence of reducing agents, they are not able to cause hemolysis to occur (data not shown). This suggests JCV enters the endosome and uses it solely as a docking area, where it can then be shuttled to the caveosome.

Virus capsid stability is a tightly controlled process. The capsid must provide protection from genome degradation, but not be so strong that it is unable to uncoat and release its genome at the proper time. The JCV virion contains multiple calcium binding sites (39). Low levels of calcium can cause capsids to disassemble (8). Therefore, changes in calcium levels can have great impact on virus stability and disassembly. It is hypothesized that SV40 partially uncoats in the ER, allowing it to simulate a misfolded

protein. This signals the ERAD pathway, which pulls the partially uncoated SV40 capsid from the ER into the cytoplasm. The proteasome and membrane proteins such as Derlin-1 further facilitate the retrotranslocation of SV40 (37). Once inside the cytoplasm, the low calcium levels cause further disassembly of the Vp1 outer capsid (18). This exposes the nuclear localization signal on the minor proteins, allowing nuclear import of the genome. JCV enters the ER and requires this high calcium environment, as shown by the decrease in infectivity upon thapsigargin treatment (Fig 7). This suggests that the timing of a low calcium environment is critical for efficient infectivity. When the calcium levels of the ER are too low the virus capsid may fall apart. This could cause the genome to be released in the ER where it is unable to be imported in the nucleus for gene expression.

JC virus infection can be stimulated by trypsin digestion. This digestion caused small changes in the capsid structure, as shown by Western Blot analysis and DNase sensitivity assays (Fig 5A-C). It is hypothesized the JCV is spread by the fecal-oral route. This would allow JCV to interact with intestinal proteases, including trypsin. Not only do these proteases not destroy the virus, they make it more infectious. Additionally, trypsin-like proteases can be expressed on the cell surface and could allow the virus to be digested during or after cell surface binding. This would be more beneficial to a virus, since it would decrease the likelihood of genome digestion. Papillomaviruses use this strategy. They require furin cleavage of their minor protein L2 to uncoat in the endosome of keratinocytes. Mature virions are unable to be cleaved and must have already undergone a conformational change (35). Papillomaviruses bind to cell surface heparan sulfate proteoglycans, causing the capsid to undergo a conformational change. Once inside the endosome, the destabilized papilloma capsid is further cleaved by furin. This

cleavage separates L2 and the genome from L1. L1 is maintained in the endosome, while L2 delivers the genome to the nucleus (14). It was also determined that if immature papillomaviruses were pre-digested with furin, they no longer required heparan sulfate binding (13). This shows a possible model for JCV entry where protease digestion in the gut or during cell attachment allows the virus to uncoat more efficiently later in its lifecycle.

ACKNOWLEDGEMENTS

We thank all the members of the Atwood Laboratory for critical discussion during the course of this work. Work in our laboratory is supported by grants from the National Cancer Institute (CA71878) and from the National Institute of Neurological Diseases and Stroke (NS43097) to WA. Megan L. Gasparovic was supported by a Ruth L. Kirschstein Predoctoral Fellowship #1F31NS053340-01.

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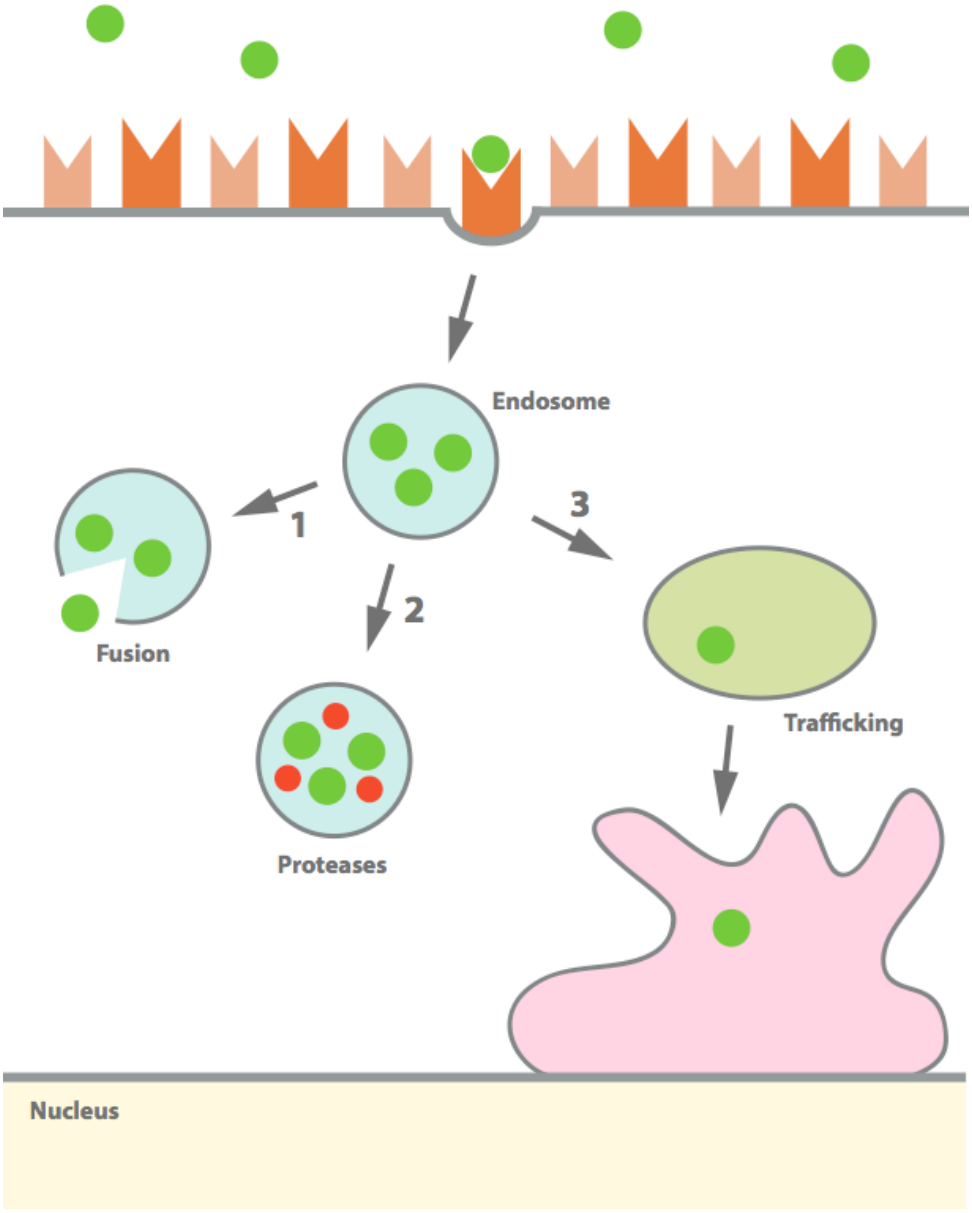

Figure 1: Roles of pH in virus entry. Viruses require low pH for entry for three reasons: membrane fusion, activation of proteases, or vesicle trafficking. Membrane fusion happens after the acidic environment allows conformation changes to occur within the virus. These conformational changes expose new protein components, which permit membrane insertion and genome release. Protease activation allows the capsid to be degraded by cellular proteases. This degradation often exposes buried proteins. Once exposed, these proteins are able to uncoat the virus through membrane fusion and viral genome release. Continued endomsomal sorting and trafficking also requires acidification of the endosomes. Without acidification, this sorting and fusing is halted.

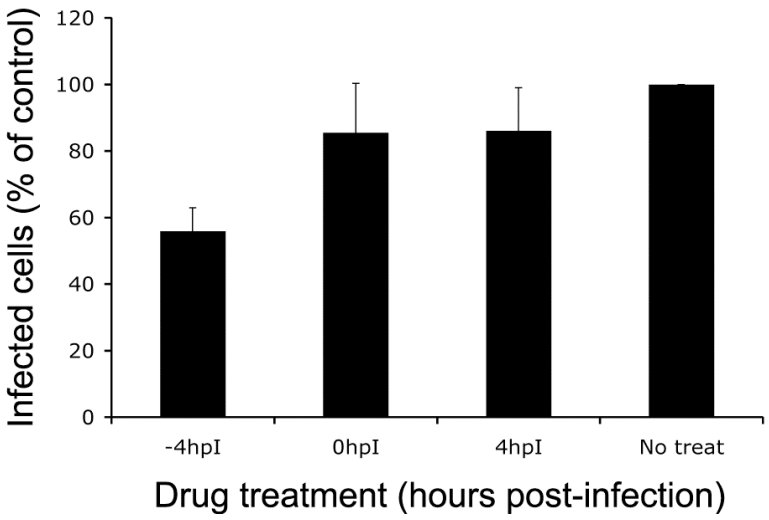

Figure 2: JCV requires low endosomal pH early during infection. SVG-A cells were treated with 25mM NH_4Cl at indicated time points. JCV protein expression was scored 48 hours post-infection with an indirect immunofluorescence assay. JCV requires acidification of endosome only during the first hours of its infection. All samples were performed in triplicate and an average of 10 visual fields were counted per experiment. Error bars indicate the standard deviation in the sample.

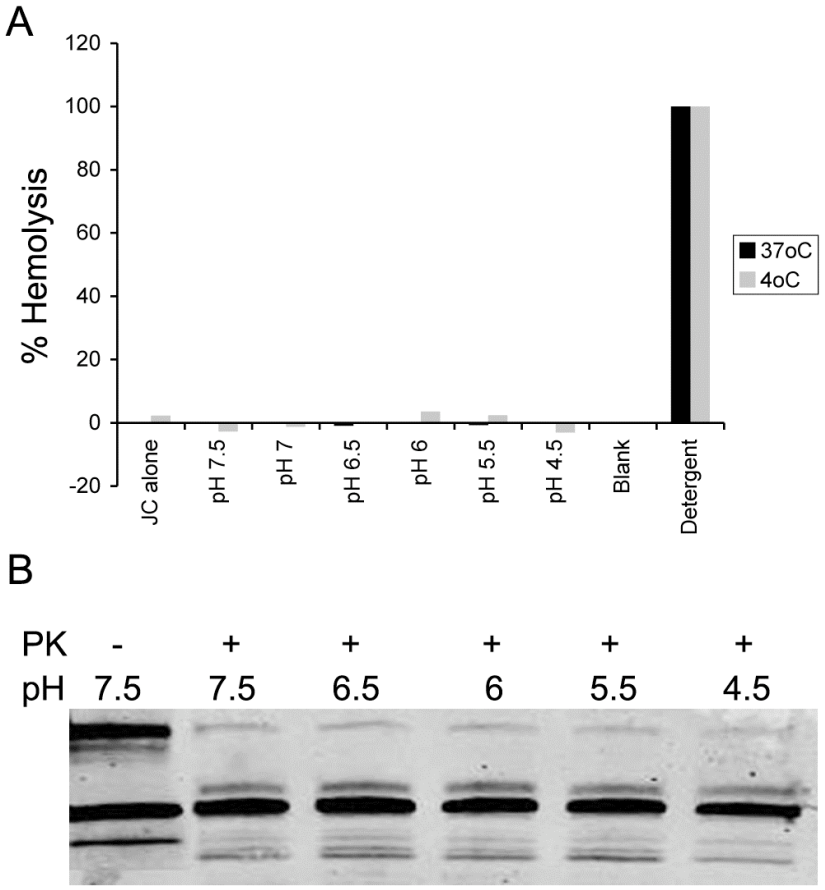

Figure 3: Low pH does not cause conformational change in JCV capsid. A. JCV was incubated with PBS pH 4.5-7.5 at either 37°C or 4°C. After incubation, virus was combined with red blood cells and the amount of hemoglobin released was read with a spectrophotometer. Upon exposure to low pH, JCV does not gain the ability to lyse red blood cells. B. JCV was incubated with PBS pH 4.5 – 7.5 for 1 hour at 37°C. JC virus was then subjected to limited proteolysis with proteinase K (PK) and the effect on Vp1 was analyzed by Western blot. JCV capsid is digested with PK, but lowering the pH does not cause the appearance of additional fragments.

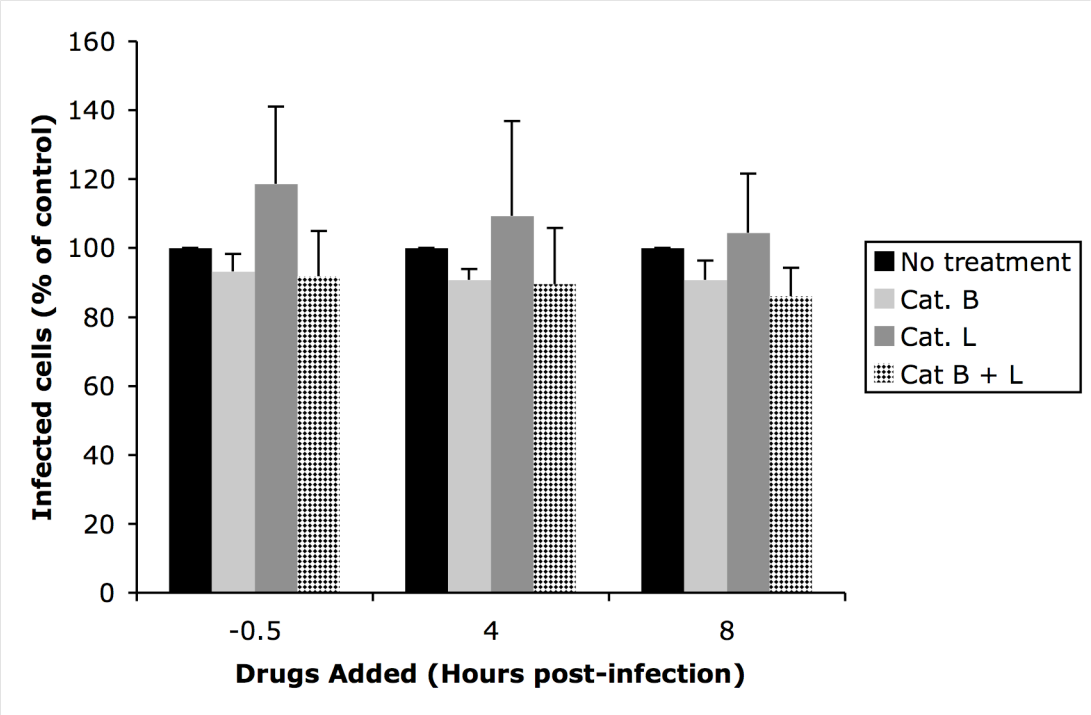

Figure 4: JCV does not require cathepsins. SVG-A cells were treated with inhibitors of cathepsins at indicated time points. Cathepsin B was inhibited with CA-074 at 20uM. Cathepsin L was inhibited with 1uM Z-FYdmk. Both cathepsin B and L were inhibited with 10uM Z-FYdmk. JCV protein expression was scored 48 hours post-infection with an indirect immunofluorescence assay. All samples were performed in triplicate and an average of 10 visual fields were counted per experiment. Error bars indicate the standard deviation in the sample.

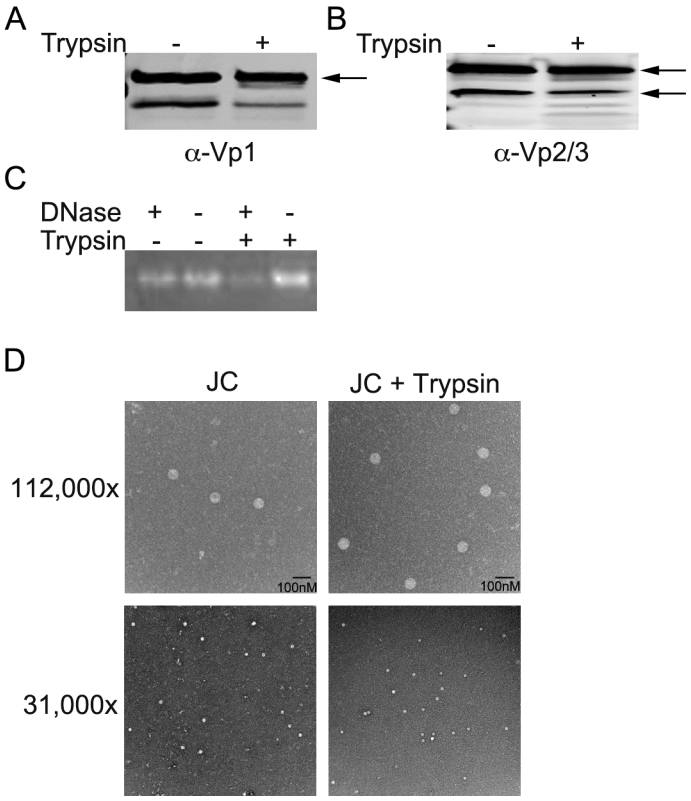

Figure 5: Trypsin digestion of JCV causes capsid loosening. CsCl-purified JCV was digested with trypsin. The reaction was stopped with 3x excess soybean trypsin inhibitor. A. Western blot analysis of JCV Vp1 with and without trypsin treatment. Arrow denotes location of Vp1. Upon trypsin digestion, Vp1 appears as a doublet. Experiment performed in triplicate and representative image is shown B. Western blot analysis of JCV Vp2/3 with and without trypsin treatment. Arrow denotes location of Vp2 and Vp3. Upon trypsin treatment Vp2/3 appear as multiple digest products. Experiment performed in triplicate and representative image is shown C. DNase protection assay of JCV. Virions were digested with trypsin, then digested with DNase or mock treated with water. The capsids were removed and the genome was amplified with PCR. After trypsin treatment, the JCV genome became sensitive to DNase. Experiment performed in triplicate and representative image is shown. D. Electron microscopy of negatively stained JCV virions with and without trypsin treatment. Virions do not appear different after trypsin treatment.

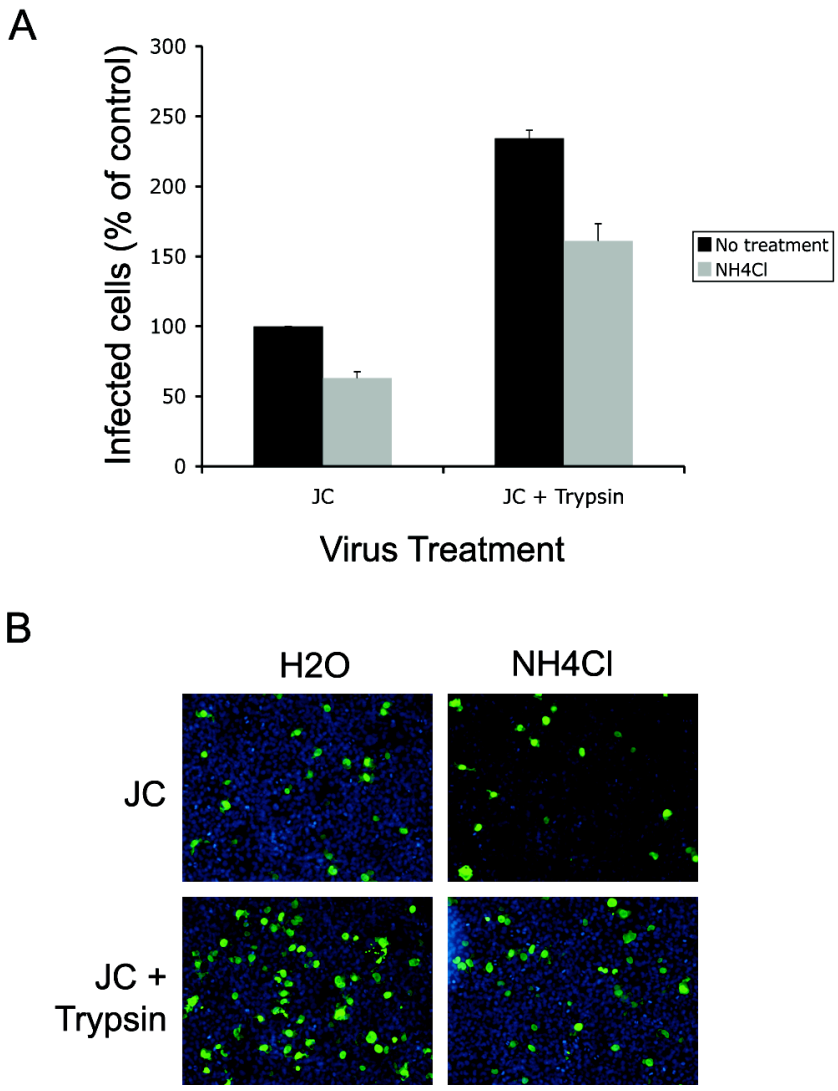

Figure 6: Trypsin digestion increases JCV infection. CsCl-purified JCV was digested with trypsin. This reaction was stopped with 3x excess soybean trypsin inhibitor. Treated JCV was used to infect untreated SVG-A cells and cells pre-treated with 25mM NH_4Cl. A. JCV infection was scored 48 hours later with an indirect immunofluorescence assay. Positive cells were counted; an average of three experiments is shown. Error bars indicate the standard deviation in the samples. JCV infection is increased with trypsin digestion, but it still requires acidification of the endosomes. B. Representative immunofluorescence images. Cells were mounted on slides with DAPI medium.

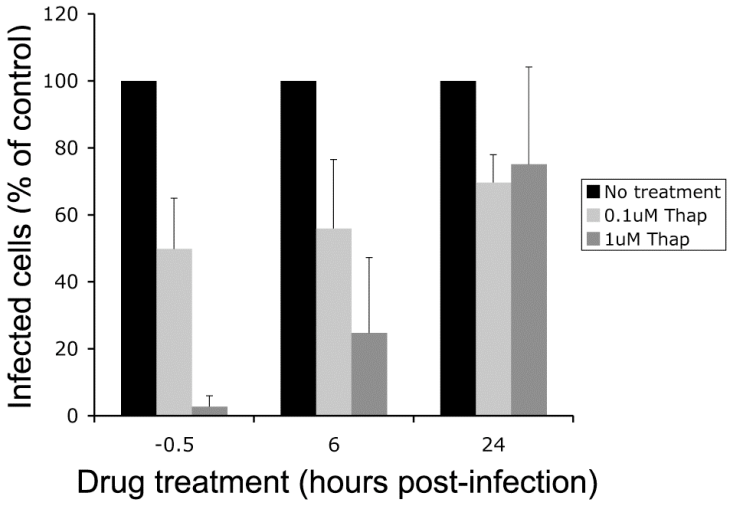

Figure 7: JCV requires high ER calcium. SVG-A cells were treated at indicated time points with thapsigargin, which depletes ER calcium stores. SV40 infection was inhibited with 1uM thapsigargin. JCV protein expression was scored 48 hours post-infection with an indirect immunofluorescence assay. All samples were performed in triplicate and an average of 10 visual fields were counted per experiment. Error bars indicate the standard deviation in the sample.

CHAPTER 3

MODULATION OF PML PROTEIN EXPRESSION BOTH NEGATIVELY AND POSITIVELY REGULATES JCV INFECTION

Modulation of PML protein expression both negatively and positively regulates JCV infection

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ABSTRACT

JC virus (JCV) is a human polyomavirus for which 70% of the human population is seropositive. It is responsible for the fatal demyelinating disease Progressive Multifocal Leukoencephalopathy. JCV binds to cells using the serotonin receptor 5-HT_{2A}R and α(2-6)- or α(2-3)-linked sialic acid. It enters cells using clathrin-dependent endocytosis, after which it traffics to the early endosome and the endoplasmic reticulum. The genome is delivered to the nucleus where it is transcribed. We found that the early regulatory protein large T accumulates in microdomains in the nucleus adjacent to ND-10 or PML domains. This prompted an analysis of whether localization to these domains regulated JCV infection. We modulated PML, the backbone of ND-10, and determined that decreased nuclear expression of PML protein enhanced viral infection. JCV infection itself does not modulate PML protein expression or nuclear localization indicating that JCV does not rely on PML, but changes in PML expression have drastic affects on JCV infection.

INTRODUCTION

JC virus (JCV) is a human polyomavirus for which 70% of the human population is seropositive (31). JCV causes the fatal demyelinating disease Progressive Multifocal Leukoencephalopathy, which occurs due to reactivation of latent JC virus (32). Reactivation is triggered by immunosupression that allows the virus to migrate from peripheral sites into the central nervous system (CNS). In the CNS, JCV infects and lytically destroys the myelin-producing oligodendrocytes (1, 21, 36).

JCV is a small, non-enveloped, double stranded DNA virus with transcriptional units organized into early and late regions (27). These regions are divided by the non-coding control region, containing a bidirectional promoter and the viral origin of replication (4, 10). The early region contains small t, large T, T'_{135}, T'_{136} and T'_{165}. All of these products are produced from alternative splicing of the viral early mRNA (46). Large T (LT) antigen is a regulatory protein involved in deregulation of the cell cycle and DNA replication. LT is composed of several domains that interact with cellular factors to promote these actions (15, 44). After replication, JCV LT interacts with the late promoter and drives the expression of the viral capsid proteins Vp1, 2, and 3 (43).

JCV attaches to host cells by binding to either α(2-3)- or α(2-6)-linked sialic acid and the serotonin receptor 5-HT_{2A}R (5, 6, 19). After binding, JCV is internalized using clathrin-dependent endocytosis and traffics to the early endosome (33, 34). The virus then traffics to caveosomes in a pH dependent step and is then delivered to the endoplasmic reticulum (ER) (35). Initial virus uncoating likely takes place in the ER by analogy with the mouse polyomavirus and SV40 but the mechanism and site of uncoating have not

been formally demonstrated for JCV (20, 38). Ultimately the virus genome is delivered to the nucleus where transcription, DNA replication, and virus assembly take place.

Upon entering the nucleus, DNA viruses can localize to distinct regions in the nuclear space where they regulate genome production and orchestrate efficient packaging of progeny virions. Nuclear domains are heterogeneous groups of proteins located within the nuclear matrix. They are connected to other nuclear components through a scaffolding of microtubules and microfilaments that allows them to dynamically change their localizations, protein contents, and responses to stimuli. These domains can be distinctly identified through their morphology and antigenic responses. Examples of the domains include the cajal bodies, the nucleolus, and promyelocytic leukemia domain (PML, also known as nuclear domain 10 or ND-10). Each domain performs a unique function, such as ribosome biogenesis, storage, and responses to cellular stresses (7, 8, 50).

ND-10 or PML domains appear in a speckled pattern in the nucleus with immunofluorescent staining. However, the size and number of the domains varies by cell type. PML domains are mobile structures whose movements are ATP- and myosin-dependent. There are many proteins associated with this domain including Sp100, Daxx, and CREB binding protein. For these proteins PML seems to provide the scaffolding and allow for recruitment of other proteins. Daxx is a pro-apoptotic Fas-interacting protein, recruited to this domain through its C' terminal region in association with SUMOylated PML. Daxx undergoes transient interactions with PML domains and can be recruited to heterochromatic domains by ATRX (13). Sp100 has a DNA binding domain and has been implicated in transcriptional modulation (29). Many transcription factors, such as CREB

binding protein (CBP), are also localized to ND-10. Cells can survive without the presence of PML, suggesting possible redundancies in its role (50). The loss of PML is not without consequences: acute promyelocytic leukemia (APL) has been linked to a chromosomal translocation that causes a fusion between PML and the retinoic acid receptor, RARα. This fusion, in turn, causes PML to be re-localized to the cytoplasm, where it is unavailable to respond to cell stress (50). Currently, treatment of APL patients with arsenite that causes the relocalization and degradation of the PML-RAR fusion, leads to clinical remission of the disease (47). PML protein can be modulated not only by arsenic but also by other heavy metals and stress. This modulation leads to a changes PML protein localization and levels which will then influence the behavior of other domain components (24).

Many viruses have antagonistic relationships with the PML domain. However, the PML domain is a common site of genome deposition by DNA viruses (12). Adenovirus type 5 immediate protein pIX has been shown to form inclusion bodies in the nucleus. These inclusion bodies consist of PML protein surrounded by pIX as a way to modulate PML function and allow virus infection to proceed (37). Herpesviruses also target the PML protein early in infection. Herpes simplex virus (HSV-1) immediate early protein ICP0 disrupts the ND-10 domain and causes PML to be degraded (9, 23). Papillomaviruses, which are similar to polyomaviruses, have been shown to localize to ND-10 domains. This localization is dependent on the minor protein L2. L2 early during infection deposits the genome in these domains and also accumulates there during viral assembly. However, this localization does not require the presence of the PML protein (3, 11, 28). Other polyomavirus family members, simian virus 40 (SV40) and BKV, have

been shown to replicate adjacent to PML nuclear bodies (14). This replication is dependent on having a complete viral origin with LT binding sites and the expression of the LT protein itself (45). JC virus minor proteins have been shown to accumulate in PML nuclear bodies. Assembled JC virus has been shown to reside in PML structures in post-mortem brains of patients with Progressive Multifocal Leukoencephalopathy (39, 40).

In our study, we evaluated the relationship between JCV early regulatory protein large T and PML domains. We first observed that LT protein would accumulate adjacent to PML domains. Next we determined that JCV was able to infect cells where PML had been knocked down with shRNA. In order to determine a larger global effect of PML, arsenite was used to eliminate PML in all cells. Surprisingly, elimination of PML protein enhanced JCV infection and the enhancement was manifest at the transcriptional level. Conversely, treatment of cells with IFN-β increased PML protein expression in the nucleus and led to a drastic inhibition of virus infection and early gene expression. Interferon was no longer able to decrease viral infection if PML protein had previously been eliminated with arsenite. The viral microdomains were not disrupted when PML was eliminated by arsenite. However, the domains are more closely associated with LT when PML is upregulated in INF-β samples. PML protein was not disrupted by JCV over long-term infections, indicating that the virus is not degrading or relocalizing PML during infection. Taken together, these findings indicate that JCV infection is regulated by nuclear PML domains and their components.

MATERIALS AND METHODS

Cells, viruses, and antibodies. SVG-A cells are a subclone of the original SVG human glial cell line established by transformation of human fetal glial cells by an origin-defective SV40 mutant (22). SVG-A cells were maintained in a humidified 37°C CO_2 incubator in Eagle's minimal essential medium (EMEM) (Mediatech Inc., Herndon, Va.), supplemented with 10% heat-inactivated fetal bovine serum (Mediatech Inc.). The PAB597 hybridoma produces a monoclonal antibody against the SV40 major capsid protein VP1 and was a generous gift from Ed Harlow. This antibody has previously been shown to cross-react with JCV VP1 (2). The PAB962 hybridoma produces a monoclonal antibody against the JCV large T antigen, which was a generous gift from the Tevethia lab. The Mad-1/SVEΔ strain of JCV was used in these experiments as it has enhanced growth kinetics over wild type strains of the virus. The coding sequences of the Mad-1/SVEΔ strain is entirely from the Mad-1 strain. The regulatory region has elements of both Mad-1 and SV40 (17, 18). Rabbit serum containing anti-JCV neutralizing antibody was used to neutralize infection. Other antibodies used were directed against PML H-238 and PGM3 (these detect all isoforms of PML) (Santa Cruz Biotech, Santa Cruz, CA), Daxx (Abcam, Cambridge, MA), Sp100 H-60 (Santa Cruz Biotech, Santa Cruz, CA), and tubulin (Santa Cruz Biotech, Santa Cruz, CA).

Indirect immunofluorescence. SVG-A cells were grown to 50% confluence on coverslips. Cells were either treated with 1uM arsenite, 3uM arsenite, or 500IU/mL interferon beta 24h prior to infection. Drugs were removed during infection and the cells

were incubated with JCV at 37°C for 1.5 hrs. At the end of the incubation, cells were rinsed in EMEM then fed with EMEM (drugs were replaced and maintained for the duration of the experiment). For 24h post-infection drug treatment, medium was removed and replaced with medium containing drug concentrations described above. JCV infection was assayed 48 hrs post-infection for LT or 72 hrs for Vp1. Cells were fixed in 2% paraformaldehyde (PFA) for 20 mins and washed several times in phosphate-buffered saline (PBS) (137 mM NaCl, 2.682 mM KCl, 8.1 mM Na2HPO4, 1.47 mM KH2PO4, pH 7.2). Cells were permeabilized in 0.5% Triton-X 100 at room temperature for 15 mins, then incubated with a 1:10 dilution of the PAB597 or PAB962 monoclonal antibody in PBS at 37°C for 1h. Cells were washed three times in PBS, incubated with a 1:500 dilution of goat anti-mouse Alexa Fluor 488 (Molecular Probes, Carlsbad, CA) at 37°C for 45 min, and rinsed in PBS. Coverslips were mounted onto slides with mounting medium containing DAPI (Vector Labs, Burlingame,Calif.). JCV-positive cells were visualized on a Nikon epifluorescence microscope Eclipse E800 (Nikon Inc., Melville, N.Y.) and scored by counting. At least ten visual fields were counted for each sample.

Confocal microscopy. SVG-A cells were grown to 50% confluence on coverslips. Cells were treated either with 1uM arsenite, 3uM arsenite, or 500IU/mL interferon beta 24 hrs prior to infection. Drugs were removed during infection and the cells were incubated with JCV at 37°C for 1.5 hrs. At the end of the incubation, cells were rinsed in EMEM and fed with EMEM (drugs were replaced and maintained for the duration of the experiment). At 48 hrs post-infection, cells were fixed in 2% PFA for 20 mins, washed several times in PBS, permeabilized in 1% Triton-X 100 at RT for five mins, blocked in 5% bovine serum

albumin (BSA) at RT for 1h, and incubated with PAB962 (1:10) and either PML, Sp100, or Daxx (all 1:50) at RT for 1h. Cells were then washed four times in PBS with 0.5% BSA and 0.05% Tween-20 (PBS-BT) and incubated with a 1:1,000 dilution of goat anti-mouse Alexa Fluor 488 (Molecular Probes, Carlsbad, CA) and goat anti-rabbit Alexa Fluor 594 (Molecular Probes, Carlsbad, CA) at 37°C for 45 min. Cells were washed four times in PBS-BT, one time in PBS, and one time in diH2O. Coverslips were mounted onto slides with mounting medium containing DAPI (Vector Labs, Burlingame, Calif.). Cells were visualized on a Zeiss LSM 510 meta laser-scanning confocal microscope (Carl Zeiss, New York, NY) using a 63X objective.

shRNA knockdown. shRNA constructs were generated using siSTRIKE plasmid (Promega, Madison, WI) and primers:

[(PML):

5'accgAGATGCAGCTGTATCCAAGTTCAAGAGACTTGGATACAGCTGCATCTTTTTTTc3';

(PML2):

5'tgcagAAAAAAAGATGCAGCTGTATCCAAGTCTCTTGAACTTGGATACAGCTGCATCT-3'; (Luc):

5’accgGTGCGTTGCTAGTACCAACTTCAAGA GAGTTGGTACTAGCAACGCACTTTTTTc; (Luc2):

5’tgcagAAAAAAGTGCGTTGCTAGTACCAACTCTCTTGAAGTTGGTACTAGCAACGCAC]. SVG-A cells at 50% confluence were transfected with 1ug of either PML plasmids or control luciferase plasmid using FuGene per manufacturers directions. Knockdown was monitored using indirect immunofluorescence of PML at 24-hr intervals as described. Cells were fixed in 2% PFA for 20 mins, washed several times in PBS, and permeabilized in 0.5% Triton-X 100 at RT for 15 mins. Cells were then incubated with PML antibody (1:50) at 37°C for 1 hr, washed three times in PBS, incubated with goat anti-rabbit Alexa

Fluor 594 (1:500) (Molecular Probes) at 37°C for 45 min, and rinsed in PBS. Coverslips were mounted onto slides with 100% glycerol. Eight days post-transfection, transfected cells were infected with JCV at 37°C for 1.5 hrs. At the end of the incubation, cells were rinsed in EMEM then fed with EMEM. JCV infection was assayed 72 hrs for Vp1. Cells were fixed in 2% PFA for 20 mins and washed several times in PBS. Cells were permeabilized in 0.5% Triton-X 100 a RT for 15 mins, then incubated with a 1:10 dilution of the PAB597 monoclonal antibody in PBS at 37°C for 1h. Cells were washed three times in PBS, incubated with a 1:500 dilution of goat anti-mouse Alexa Fluor 594 (Molecular Probes, Carlsbad, CA) at 37°C for 45 min, and rinsed in PBS. Coverslips were mounted on slides with 100% glycerol. Cells were visualized on a Nikon epifluorescence microscope (Eclipse E800; Nikon Inc., Melville, N.Y.).

Western blot analysis. SVG-A cells were grown to 70% confluence in 75-cm^2 flasks and treated with 3uM arsenite or 500IU/mL interferon beta in EMEM. Cells were then harvested 24 or 48 hrs post-treatment by washing twice in cold PBS and lysing in ice cold radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris HCl, pH 7.4, 0150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor cocktail [Sigma-Aldrich], 1 mM sodium orthovanadate) for 30 mins on ice. Samples were collected by pelleting membranes by centrifugation at 13,000rpm for 10 mins and then removing the protein-containing supernatant. Protein samples were loaded onto a 4-15% Tris-HCl polyacrylamide gel (Bio-Rad, Hercules, CA), run at 30mA, transferred to nitrocellulose membranes using a mini-*trans* blot apparatus (Bio-Rad), and blocked with 5% milk in PBS containing 0.05%

Tween 20 (PBS-T). Blots were probed with the respective antibodies all diluted in 5% milk in PBS-T, washed in PBS-T, and then incubated with goat anti-rabbit Alexa Fluor 680 (Molecular Probes) antibody diluted 1:5,000 in 5% milk in PBS-T. This was followed by further washes with PBS-Tween 20 and one in PBS. Blots were viewed using an infrared scanner (LI-COR, Lincoln, NE) and analyzed using Odyssey software (LI-COR).

Nuclear and cytoplasmic extracts. SVG-A cells were grown to 70% confluence in 150-cm^2 flasks and treated with 3uM arsenite or 500IU/mL interferon beta in EMEM. Cells were then harvested 1, 4, and 24 hrs post-treatment by scraping. Cell pellets were washed two times in cold PBS and cells were pelleted at 2,000rpm at 4^oC. Pellets were resuspended in five times cell pellet volume of pre-lysis buffer (100mM HEPES pH 7.9; 15mM $MgCl_2$; 100mM KCl, 0.01M DTT and protease inhibitors) on ice for 15 mins. Cells were pelleted at 2,000rpm for five minutes and supernatant was removed. Pellet was resuspended in twice the pellet volume of lysis buffer and a syringe was used to disrupt the membrane. The samples were centrifuged at 10,500rpm for 20 mins and the cytosolic containing supernatant was frozen at -20°C. The remaining pellet was resuspended in 2/3 cell pellet volume of extraction buffer (20mM HEPES H 7.9; 1.5mM $MgCl_2$; 0.42M NaCl; 0.2mM EDTA, 25% (v/v) glycerol, 0.01M DTT and protease inhibitors) and a syringe was used to disrupt the nuclear membrane. The samples were then incubated on a rocking platform at 4^oC for 30 mins. Samples were collected by centrifugation at 10,000rpm for 15 mins and the nuclear-containing supernatant was

frozen at -20°C. Samples were then subjected to western blot analysis as previously described.

Neutralization of JCV. 50% confluent SVG-A cells were pre-treated with EMEM or EMEM containing 3uM arsenite for 24 hrs. SVG-A cells were then infected with JCV for the indicated time at 37^{o}C. Cells were washed in EMEM and media containing anti-JCV antiserum was added to the cells. Viral infection was assayed 72 hrs later for Vp1 as previously described.

qPCR of JCV transcripts. SVG-A cells were grown to 50% confluence on 6-well plates in triplicate. Cells were either treated with 1uM arsenite, 3uM arsenite, or 500IU/mL interferon beta 24 hrs prior to infection. Drugs were removed during infection and the cells were incubated with JCV at 37°C for 1.5 hrs. At the end of the incubation, cells were rinsed in EMEM and fed in EMEM (drugs were replaced and maintained for the duration of the experiment). For 24h post-infection drug treatment, medium was removed and replaced with medium containing above described drug concentrations. 48h post-infection cells were washed with cold PBS and detached with trypsin. Cells were pelleted at 2,000rpm for five mins and washed two times with cold PBS. RNA was extracted using RNeasy Kit (Qiagen, Germantown, MD). Sample concentrations were determined and 1ug of RNA was used for one-step PCR with probes (BioRad). Primers used (LT): ttcttcatggcaaaacaggtctt and ttccaccaggattcccattc; Probe used: ccacttctcattaaatg as previously described (Applied Biosystems, Foster City, CA) (25). GAPDH was used as

an internal control. GAPDH primers and probes were obtained from Applied Biosystems. All samples were performed in triplicate.

RESULTS

JC virus is capable of infecting cells in the absence of PML protein. When assaying for JCV infection using LT protein we noticed that it accumulated in a distinct punctate pattern in the nucleus. As other polyomaviruses are known to replicate adjacent to PML domains, we assayed whether the LT accumulations were associated with PML protein. We performed indirect immunofluorescence analysis of LT and PML protein 48 hours post-infection on the human glial cell line, SVG-A. Both proteins exhibited a punctate, speckled pattern in the nucleus, and LT associated adjacent to many of the PML domains (Fig. 1A). The SVG-A cell line expresses SV40 LT antigen. However, SV40 large T is diffuse throughout the nucleus and does not accumulate in a punctate pattern in these cells (data not shown).

To determine whether JC virus requires PML protein for infection, permissive SVG-A cells were transfected with shRNA constructs against PML protein or luciferase as a negative control. The ability of these constructs to eliminate PML was monitored with indirect immunofluorescence of the PML protein. The constructs expressing the shRNA also express GFP allowing us to identify shRNA expressing cells. After monitoring the levels of PML protein in GFP positive cells, it was determined there was complete knockdown of PML eight days post-transfection (Fig. 1B). After eight days, transfected cells were infected with JCV. JCV infectivity was monitored 72 hours post-infection. At that time, PML protein was still knocked down in the PML samples, but was unaffected in the samples that received the luciferase construct (Fig. 1B). JCV was able to infect both untreated and treated samples indicating that it does not require PML protein for a productive infection.

JCV LT accumulated in microdomains in the nucleus that are independent of PML. While shRNA indicated JCV is able to infect in the absence of PML, the percentage of cells receiving the knockdown was not sufficient to determine a global effect on infection. To see this global effect, ND-10 components were modulated with arsenite or interferon beta (INF-β) treatment. Arsenite is known to disrupt and degrade PML protein and INF-β is known to increase PML protein and transcript (26). The concentration of arsenite used is in the therapeutic range used to treat APL and did not cause toxicity in our cell line (data not shown) (47). Previous work in our lab determined that INF-β treatment was inhibitory to JCV infection (30). Permissive SVG-A cells were treated with 1uM arsenite, 3uM arsenite, or 500IU/mL INF-β. After 48 hours of treatment, samples were stained by indirect immunofluorescence for PML, Sp100, and Daxx, and their cellular localization was evaluated using confocal microscopy. In the untreated samples, PML, Sp100, and Daxx all exhibit a pattern of expression with punctate domains speckled throughout the nucleus, which is the characteristic distribution of proteins in ND-10. However, upon arsenite treatment PML protein is eliminated and can be seen in the cytoplasm of some samples. Arsenite has no effect on the punctate, speckled pattern of Sp100, while Daxx expression was not only punctate but also diffuse throughout the nucleus. In the presence of interferon treatment, indirect immunofluorescence for PML and Sp100 samples revealed an increase number and size of the domains; yet Daxx expression is unaffected (Fig. 2A). To determine whether the overall protein levels of PML and Daxx were affected by arsenite and INF-β, SDS-PAGE and Western blot analysis were performed. After 24 or 48 hours of treatment, the PML

protein is eliminated from arsenite treated cells and increased with INF-β treatment. Daxx protein levels remain constant over the time course (Fig. 2B). These results indicate PML protein levels can be modulated using arsenite or interferon beta.

To determine whether viral proteins directly interact with ND-10 components, the localization of JCV LT protein and ND-10 proteins were evaluated 48 hours post infection. JCV LT is seen throughout the nucleus, but accumulates in larger speckled regions, or viral microdomains. These accumulations are adjacent to PML, Sp100, and Daxx. There does not seem to be complete overlap of the ND-10 components and LT (Fig. 3). Given that JCV LT accumulates in microdomains we wanted to determine whether the domains were dependent on the presence of PML. Upon arsenite treatment, when PML is removed from the nucleus, the LT microdomains are unaffected. This indicates the virus does not use PML as a scaffold for its microdomains. LT itself could contain an element that allows it to create these domains. It has its own nuclear localization signal and could also contain additional signals for localization. When the samples are treated with INF-β and PML protein is increased we see a large percentage of LT microdomains associating with ND-10 components. This increase in ND-10 association may partially explain the inhibitory effects seen with IFN-β treatment.

Arsenite treatment eliminates PML protein and enhances JCV infection. We then evaluated whether the modulation of PML proteins levels would affect JCV infection. SVG-A cells were either treated 24 hours before infection, at the time of infection, or 24 hours post-infection. Cells infected with JCV were scored 48 hours post-infection using an indirect immunofluorescence assay for LT protein (Fig. 4B). The amount of JCV-

positive cells were quantified and compared to those receiving no treatment. Cells pre-treated or treated at the time of infection with arsenite showed a 2-fold increase in infection. However, this increase was not seen when arsenite was added 24 hours post infection. Interferon beta treated cells showed the opposite trend: cells treated 24 hours prior to infection or at the time of infection show a 95-55% decrease in JCV LT positive cells (Fig. 4A). Similarly, arsenite treatment led to an increase in viral protein expression and INF-β led to a decrease in viral protein expression when the late viral protein Vp1 was monitored (data not shown).

Interferon-β can upregulate many proteins in the cell. To determine whether the effect of interferon on JCV was mediate through PML, SVG-A cells were pre-treated 24 hours prior to JCV infection with arsenite to eliminate PML. The SVG-A cells were infected and INF-β was added at the time of infection. Infection was evaluated 48 hours later with an indirect immunofluorescence assay of LT (Fig 5B). JCV infection was no longer inhibited by INF-β when PML was removed with arsenite (Fig 5A). These results indicate changes in PML protein level have dramatic effects on viral gene expression and infection. Consistent with our previous shRNA findings, JCV is not only able to infect cells in the absence of PML, its infectivity is drastically increased when PML is removed. When PML protein is upregulated with interferon, JCV infection is drastically decreased. Additionally, INF-β decreases JCV infection in a PML dependent manner.

Arsenite has no effect on JCV entry but does increase viral transcripts. Upon arsenite treatment, there are more cells infected with JCV. This indicates that arsenite induces a global change in the cells that makes them more permissive to viral infection.

While arsenite decreases PML levels it does this through a series of signaling cascades. To evaluate which step of the viral lifecycle was enhanced by arsenite, we began by analyzing viral entry. Effects on entry were evaluated by infecting cells that were either pre-treated with arsenite or treated with media alone. JCV infection was neutralized at regular intervals post-infection using neutralizing anti-JCV rabbit serum. Infection was scored 48 hours later using an indirect immunofluorescence assay of LT (Fig. 6A). Both untreated and arsenite treated cells exhibited the same rate of neutralization, suggesting that the arsenite dependent enhancement in JCV infection is not due to an increase in viral entry.

ND-10 domains contain a variety of transcriptional regulators whose localization and activity are modulated by other domain components. To determine if viral transcripts were increased during infection, SVG-A cells were treated with arsenite and INF-β. Cells were either pre-treated, treated at the time of infection or 24 hours post-infection. Cells were harvested 48 hours post-infection and viral transcript levels were assayed using qPCR with viral specific primers and probes to LT. Viral transcript levels mirror viral protein levels which had received similar treatments (Fig 6B). These data demonstrate that upon arsenite treatment, there is an increase in viral transcript levels. Taken together, this suggests that removing PML from ND-10 releases a repression on viral transcription.

JCV does not eliminate PML during infection. Many viruses interact with PML and modulate its expression during infection. To evaluate PML protein during JCV infection, permissive SVG-A cells were infected with JC virus. PML and Vp1 protein levels were monitored over a long time course of infection. Whole cell extracts were collected 7, 14,

and 21 days post-infection and their PML and viral protein levels were monitored by Western blot analysis (Fig. 7A). Tubulin levels were also evaluated as a control. JCV protein levels increase over time, but the presence of PML protein remains throughout the infection. The decrease in PML at 21 days post-infection is due to cytopathic effects (CPE) of the virus. When quantified by infrared scanning of proteins, PML is expressed at the same level relative to tubulin as the no infection control (data not shown). To determine whether PML is re-localized during infection, cytoplasmic and nuclear extracts were collected at 5, 10, and 15 days post-infection, all days prior to the development of CPE (Fig. 7B). Cells collected at these time points were also stained for the presence of JCV Vp1 and PML and evaluated by confocal microscopy (Fig. 7C). Over these time points, JCV infection spreads throughout the culture. However, there is no effect on the nuclear localization of the PML protein, since it is still observed in all samples. PML also maintains its characteristic nuclear speckled pattern over the time of infection. This is also confirmed through immunoblot analysis of the cytoplasmic and nuclear extracts (Fig. 7B). PML has many isoforms and post-translational modifications, which can be modulated by the cell cycle or other cellular proteins. We see that PML has more bands in the cytoplasmic fractions as the time in culture increases. This is probably due to increased numbers of cells in different phases of the cell cycle. Although other viruses are able to eliminate PML or sequester it during infection, we demonstrate JCV is unable to remove or modulate PML during infection.

DISCUSSION

DNA viruses deliver their genomes to the nucleus of the host cell. Within the nucleus they are able to modulate host cell proteins and activities in order to transcribe and replicate their genomes. This can occur through a variety of mechanisms. Polyomaviruses use their early regulatory protein LT to interact with p53 and Rb, thereby regulating the cell cycle and allowing for viral replication (41, 42, 48). Other DNA viruses interact with nuclear domain components such as PML. Herpesviruses cause PML to be degraded through a proteasome-dependent pathway to efficiently complete their lifecycle (9, 23), while adenovirus sequesters PML to remove PML's repressive effects on its lifecycle (37). We were interested in exploring the relationship between JC virus regulatory proteins and nuclear domain components. Previous studies have shown viral capsid protein Vp2 and Vp3 are able to localize adjacent to PML domains. It is hypothesized that this localization is necessary for packaging and assembly of JCV virions (39, 40). Using shRNA, we were able to show that PML protein is not required for JC virus to produce its early and late proteins as determined by indirect immunofluorescence.

PML domains are sites where many proteins, including a variety of transcription factors, are localized depending on their post-translational modification status. Treatment of cells with arsenite has been shown to cause changes in the SUMOylation and phosphorylation status of PML (16, 49). These changes affect the activities of transcriptional regulators within the domain (8). Arsenite has been used in the treatment of acute promyelocytic leukemia (APL) since the 1990's, the concentrations that were used in our experiments are in the same therapeutic range as used to treat APL. Arsenite

was found to be more effective at causing remission than the *all-trans* retinoic acid treatment used previously. It is also used to treat APL that is resistant to retinoic acid treatment (47).

In our experiments, altering PML protein had drastic effects on the capacity of JCV to infect SVG-A cells. Upon arsenite treatment, PML is eliminated and infection is enhanced. The opposite response is seen when PML is upregulated with interferon-β, thereby inhibiting infection. This inhibition by interferon-β was dependent on the presence of PML since it is no longer able to be repressive in arsenite treated cells were PML is eliminated. We did not observe any specific viral transcription factors activated from the cytoplasm in response to changes in PML (data not shown). However, there are many other regulators within the ND-10 domains, which can be repressed by PML or other domain components. This is supported by the increase in viral transcript levels seen with arsenite treatment.

To understand whether any JCV proteins interact with the ND-10 domain or regulate its localization during infection, we evaluated LT microdomains in association with ND-10 components, as well as levels and localization of PML itself during a long-term infection. When the early viral protein large T was evaluated, we noted it accumulated in microdomains in the nucleus. These viral domains were localized adjacent to ND-10 components PML, Sp100, and Daxx. When PML was eliminated with arsenite treatment, these viral domains were unaffected, suggesting they do not require PML as a scaffold protein. Upon interferon treatment, however, the viral domains became smaller and more closely associated with ND-10 components. This suggests the antiviral nature of PML domains could be through sequestering viral proteins or

transcription factors within the PML domains. JCV did not alter the expression of PML during infection: PML was seen at constant levels throughout JCV infection. PML was also not re-localized during infection suggesting PML removal is not necessary to achieve infection by JCV.

It is beneficial for viruses to localize their processes to distinct regions, since it makes the process of viral replication and packaging most efficient. Why would polyomaviruses choose to localize close to domains known to have antiviral properties? PML domains are also sites of many regulators, including cell cycle regulators and transcription factors. Polyomaviruses are small viruses that require many cellular factors to efficiently complete their lifecycles. Cell cycle regulators such as p53 are associated with PML domains and are also critical for LT induction of S-phase in order to allow for viral replication. SV40 and BKV require localization close to PML domains for their replication. Overall these domains are very complex, tightly localized, and have many properties that would be valuable for viruses to exploit.

ACKNOWLEDGEMENTS

We thank all the members of the Atwood Laboratory for critical discussion during the course of this work. Work in our laboratory is supported by grants from the National Cancer Institute (CA71878) and from the National Institute of Neurological Diseases and Stroke (NS43097) to WA. Megan L. Gasparovic was supported by a Ruth L. Kirschstein Predoctoral Fellowship #1F31NS053340-01.

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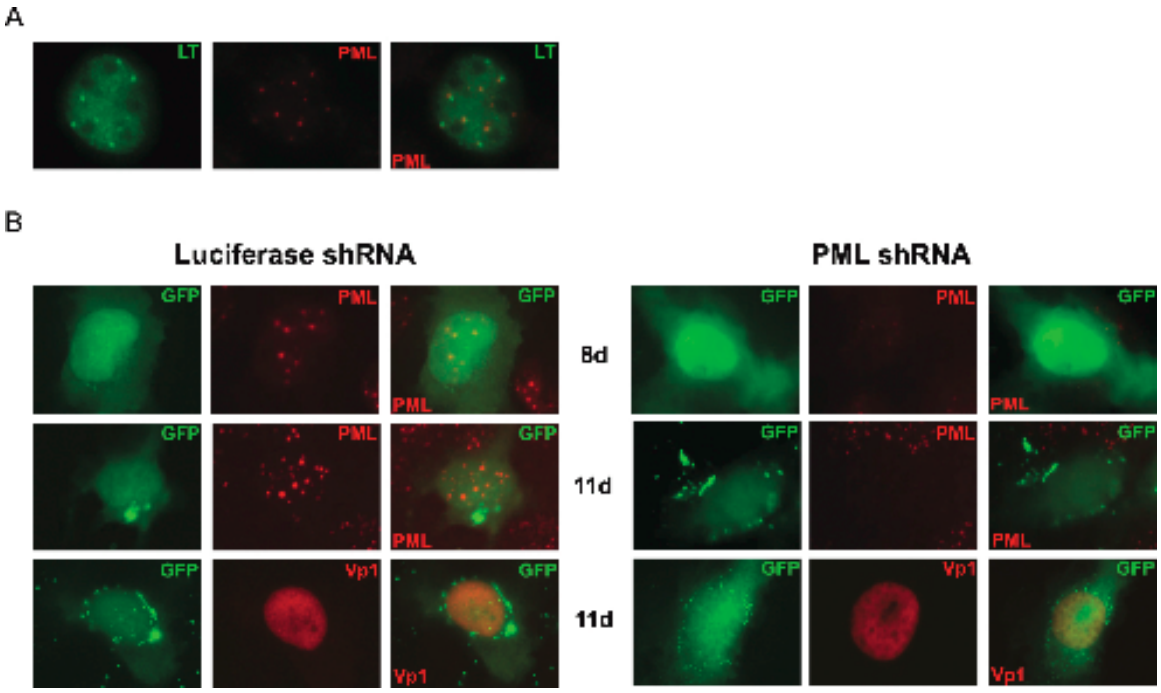

Figure 1: shRNA knockdown of PML has no effect on JCV infection. (A) Untreated SVG-A cells were infected with JCV and assayed 48h post-infection by indirect immunofluorescence analysis of JCV large T (LT) and PML protein expression. Samples were analyzed with epifluorescence using a magnification of 400. JCV LT is seen to accumulate in microdomains, which are adjacent to PML domains. (B) SVG-A cells were transfected with shRNA constructs directed at either a luciferase control or the PML protein. The plasmids containing the hairpins also express GFP. After 8 days PML was knocked down in all GFP expressing samples and these transfected cells were infected with JCV. JCV infection was monitored 72 hrs later for the presence of JCV Vp1 protein or PML protein by indirect immunofluorescence in GFP-positive cells. Samples were analyzed with epifluorescence using a magnification of 400. It was determined that JCV was able to infect cells containing the control luciferase constructs equally well as those cells where PML protein was no longer expressed (data not shown).

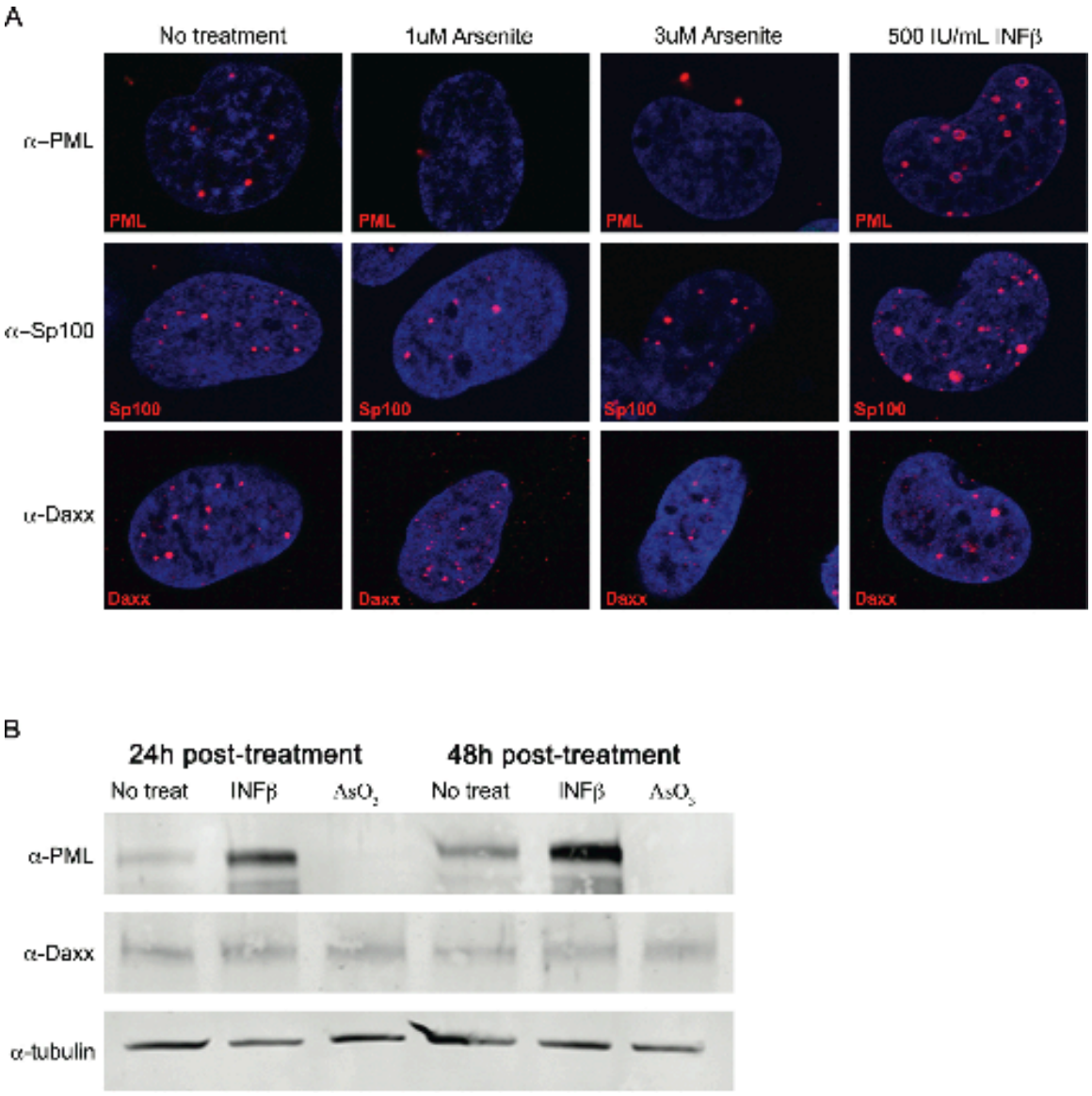

Figure 2: Arsenite treatment eliminates PML protein. SVG-A cells were treated with media (no treatment), 1uM arsenite, 3uM arsenite, or 500IU/mL INF-β for 48 hrs. (A) Indirect immunofluorescence of SVG-A cells and ND-10 components. Samples were mounted on slides with DAPI medium and analyzed using confocal microscopy using a 63X objective. Arsenite-treated samples showed PML re-localized from the nucleus to the cytoplasm, but Sp100 and Daxx samples were unaffected. Interferon beta treated samples of PML and Sp100 showed increases in domain size and number, but showed no effect on Daxx. (B) Whole cell lysates of SVG-A cells 24 and 48 hours post-treatment. PML protein was eliminated with arsenite treatment and increased with interferon beta treatment. Daxx protein levels remained unaffected. Tubulin was used as a loading control.

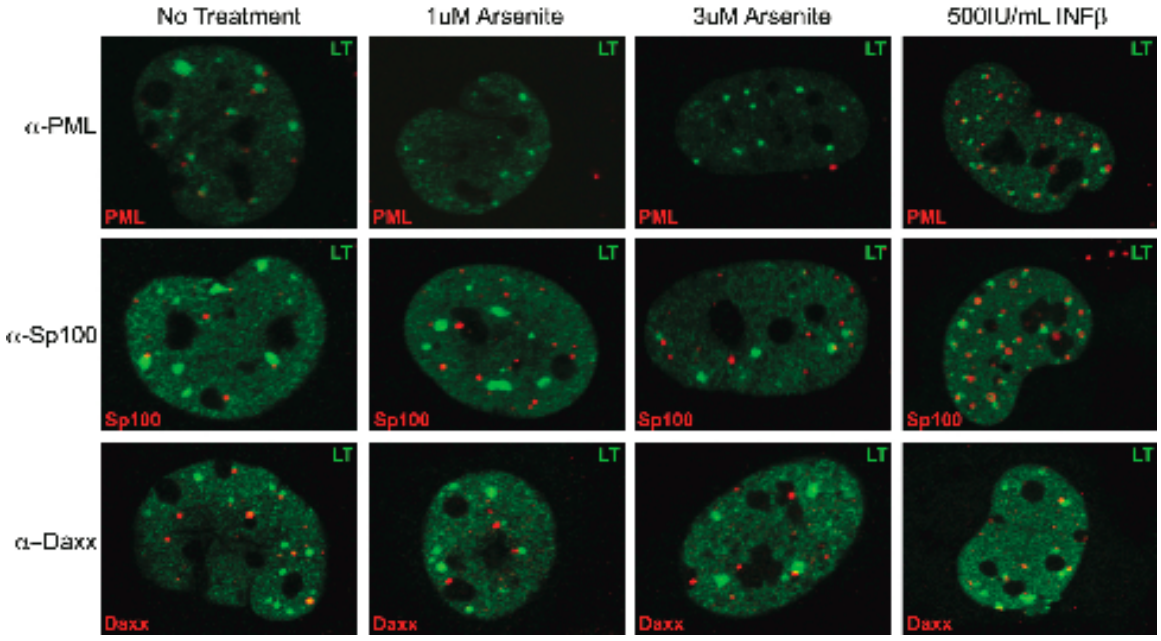

Figure 3: Large T accumulates in microdomains in the nucleus, which are independent of PML protein. SVG-A cells were treated with media (no treatment), 1uM arsenite, 3uM arsenite, or 500IU/mL INF-β. Treated cells were infected with JCV and 48h post-infection samples were analyzed by indirect immunofluorescence and visualized using confocal microscopy with a 63X objective. LT was observed in the nucleus in accumulated microdomains. These accumulations were adjacent to the ND-10 components. When PML was removed using arsenite treatment, the microdomains were unaffected. When ND-10 domains components were upregulated with INF-β treatment, there was greater overlap of LT and the ND-10 proteins.

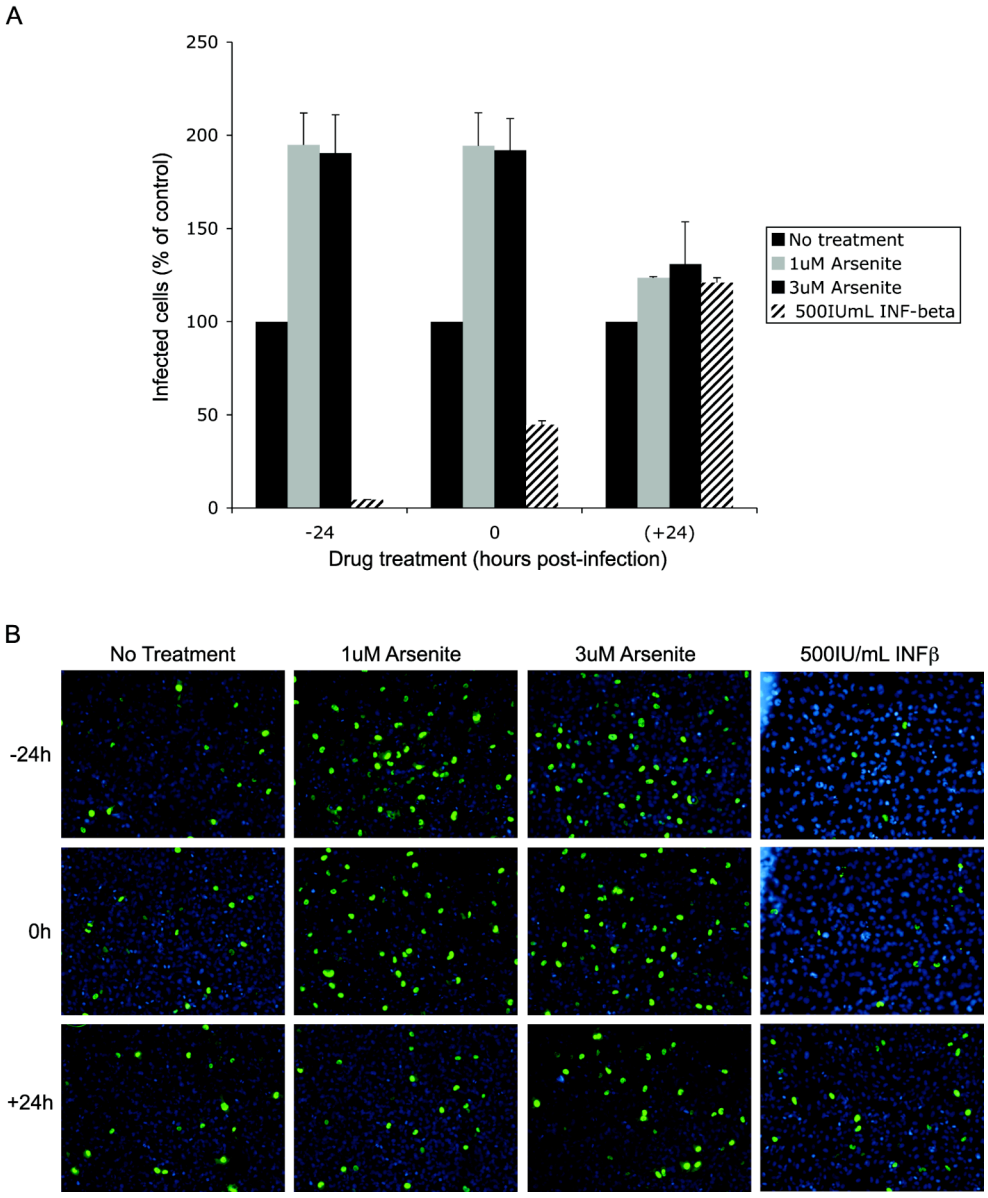

Figure 4: Removal of PML protein enhances JCV infection. SVG-A cells were treated with media (no treatment), 1uM arsenite, 3uM arsenite, or 500IU/mL INF-β at indicated time points. Treated cells were infected with JCV and infection was scored 48 hrs later using indirect immunofluorescence of JCV LT. (A) Positive cells were quantified and the data represent an average of three infections compared to no treatment (set at 100%). There is a drastic increase in infection when samples are pre-treated with arsenite. Conversely, there is a decrease in infection when samples are treated with INF-β. There is no effect when samples are treated 24 hrs post-infection. Error bars represent the standard deviation of the samples. (B) Representative indirect immunofluorescence images of JCV LT 48 hrs post-infection. Samples were mounted on slides with DAPI medium.

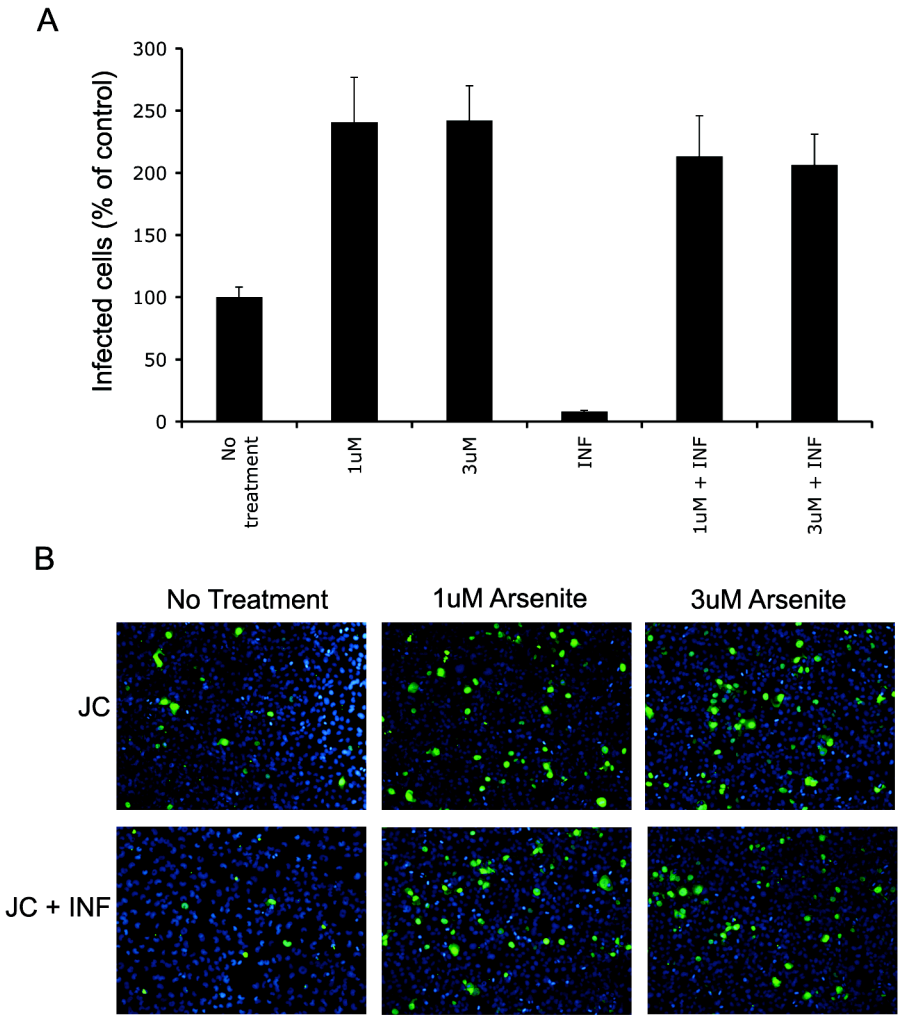

Figure 5: Interferon-β requires PML to inhibit JCV infection. SVG-A cells were pre-treated 24 hours prior to infection with media (no treatment), 1uM arsenite, or 3uM arsenite. Cells were infected with JCV and interferon-β was added at the time of infection. Infection was scored 48 hrs later using indirect immunofluorescence of JCV LT. (A) Positive cells were quantified and the data represent an average of three infections compared to no treatment (set at 100%). There is a drastic increase in infection when samples are pre-treated with arsenite. Conversely, there is a decrease in infection when samples are treated with INF-β. However, interferon-β is no longer able to inhibit infection in samples were PML was eliminated with arsenite. Error bars represent the standard deviation of the samples. (B) Representative indirect immunofluorescence images of JCV LT 48 hrs post-infection. Samples were mounted on slides with DAPI medium.

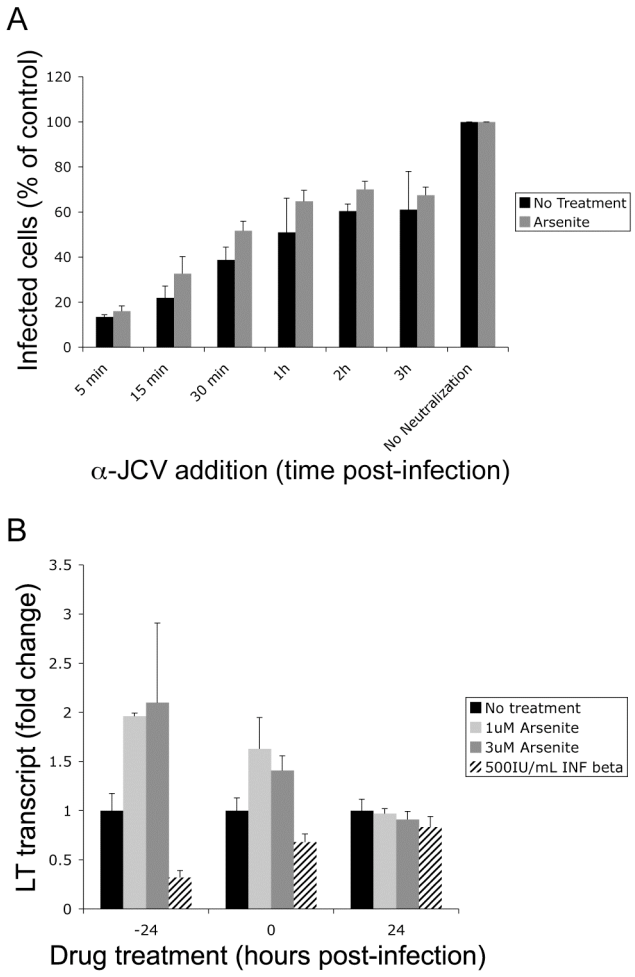

Figure 6: Arsenite does not enhance JCV entry but increases viral transcripts. (A) SVG-A cells untreated or pre-treated with arsenite were infected with JCV. The infection was neutralized at indicated times post-infection with an anti-JCV rabbit serum. JCV positive cells were scored 48 hrs post-infection using indirect immunofluorescence of JCV LT. The average of three trials is plotted compared to no neutralization controls set as 100%. Error bars represent the standard deviation of the samples. (B) Cellular RNA was harvested from SVG-A cells treated with media (no treat), 1uM arsenite, 3uM arsenite, or 500IU/mL interferon beta at indicated times. Viral transcripts were detected using qPCR with JCV specific LT primers and probes. All samples were performed in triplicate. An average of three experiments is represented. Error bars indicate the standard deviation of the samples. Similar to infection results, arsenite increased viral transcripts while INF-β decreased viral transcripts. There was no effect when treatment was performed 24 hrs post-infection.

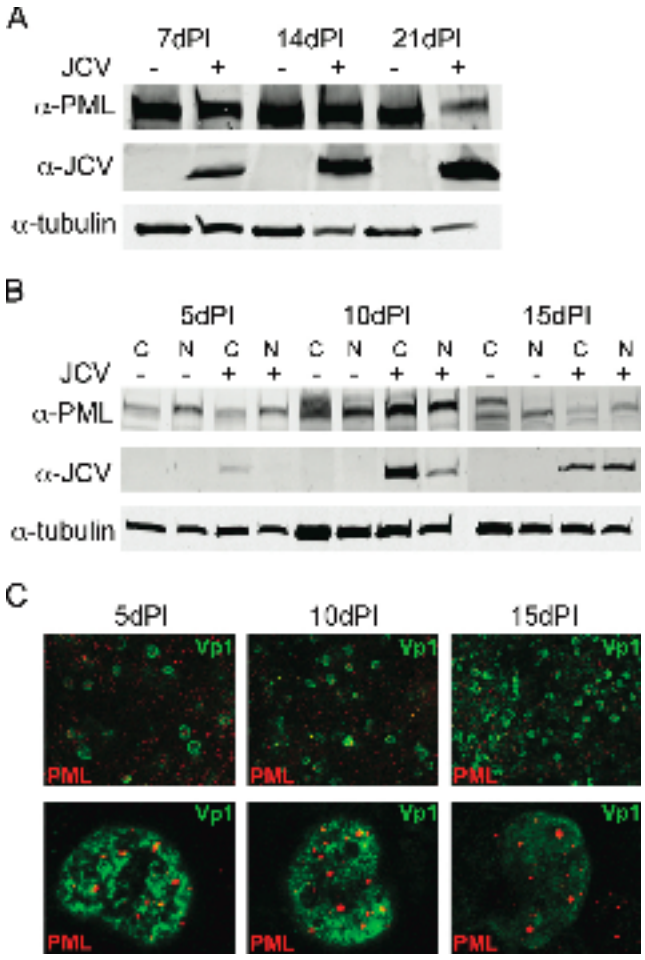

Figure 7: JCV does not eliminate PML protein during infection. (A) Whole cell lysates of uninfected and JCV infected SVG-A cells 7, 14, and 21 days post-infection (dPI). JCV infection spreads over time, but PML protein levels remained throughout the infection. (B) Cytoplasmic (C) and nuclear (N) extracts from uninfected and JCV infected cells at 5, 10, and 15 days post-infection (dPI). There was no change in the cellular localization of PML during the course of JCV infection. (C) Representative indirect immunofluorescence images of JCV VP1 and PML 5, 10, and 15 days post-infection (dPI). Top panels represent 20X field of JCV infection, which spreads throughout the culture over the course of infection. Bottom panels represent 63X images of Vp1 positive nuclei showing no change in the appearance of PML domains during the infection.

CHAPTER 4

THE JC VIRUS (JCV) MINOR CAPSID PROTEINS VP2 AND VP3 ARE ESSENTIAL FOR VIRUS PROPAGATION

The JC Virus (JCV) Minor Capsid Proteins Vp2 and Vp3 are Essential for Virus Propagation

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Running title: VP2 and VP3 are Essential for JCV Growth

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ABSTRACT

Virus encoded capsid proteins play a major role in the life cycle of all viruses. The JC virus (JCV) capsid is composed of 72 pentamers of the major capsid protein Vp1 with one of the minor coat proteins Vp2 or Vp3 in the center of each pentamer. Vp3 is identical to two thirds of Vp2 and these proteins share a DNA binding domain, a nuclear localization signal and a Vp1 interacting domain. We demonstrate here that both the minor proteins and the myristylation site on Vp2 are essential for the viral lifecycle, including the proper packaging of its genome.

INTRODUCTION

JC virus (JCV) is the causative agent of the fatal demyelinating disease progressive multifocal leukoencephalopathy (PML) (22). PML develops from a lytic infection of the myelin-producing oligodendrocytes in the central nervous system. JCV is widespread in the human population, where it is estimated that 70% of people are seropositive for the virus (21). JCV is generally latent but can traffic to the CNS upon immunosupression, lytically infecting oligodendrocytes and causing PML (1, 15, 24).

JCV contains a small, non-enveloped double-stranded DNA genome that is organized into early and late coding regions. These regions are divided by the regulatory region containing a bidirectional promoter and the viral origin of DNA replication. The late region contains agno, Vp1, Vp2, and Vp3. The V antigens (VAg) make up the virus capsid, consisting of 360 molecules of the major coat protein Vp1. These are arranged in 72 pentamers, creating the icosahedral shape. One of the minor coat proteins, Vp2 or Vp3, lies in the center of each pentamer. Vp3 is identical to the C' terminal two-thirds of Vp2; this shared domain is comprised of the nuclear localization signal (NLS), the DNA binding domain and Vp1 interacting domain (2, 4, 5, 8) (Fig. 1B). Vp2 is also modified by a myristylation moiety on its N' terminus. Myristylation is a fatty acid that is added co-translationally. Myristoyl proteins may be either cytoplasmic or membrane-associated. The myristoyl group is transferred by the enzyme N-myristoyl transferase (NMT) after the methionine is removed and an N' terminal glycine residue is recognized (23).

The viral DNA is packaged with histones H2A, H2B, H3 and H4 and creates a mini-chromosome structure that is almost indistinguishable from the hosts chromatin

(20). The host cell type specificity can be controlled through transcriptional blocks to infection as well as virus-receptor interactions (3, 7, 19).

Previous work evaluating the role of minor coat proteins in the related polyomavirus, SV40, found that Vp2 was dispensable but Vp3 was necessary for infection. They also suggested that the importance of Vp3 could lie in its activation of poly-(ADP-Ribose) polymerase (PARP) (9). This over-activation of PARP is thought to deplete intracellular ATP and cause necrosis, thereby releasing the virus (9, 10). The SV40 minor proteins have also been shown to contain lytic properties in bacteria that could aid in virus release from the cell (6).

Similar work has also been performed for mouse polyoma (Py) where Vp2, Vp3 and Vp2 myristylation mutants have been produced by two different labs. Both labs determined that Vp2 and Vp3 are essential for virus production and infection. The myristylation site is also necessary and has been postulated as important for both exit from the cell and re-infection (16, 25).

METHODS, RESULTS, AND DISCUSSION

To determine the role of the minor proteins in the human polyomavirus, JCV, we used site-directed mutagenesis to eliminate Vp2 and Vp3 and the myristylation site on Vp2. The Mad1-SVEΔ strain of JCV was linearized at the BamHI sites and subcloned into pUC19 for viral DNA propagation in bacteria. The start sites of Vp2 and Vp3 were replaced by alanine residues by site-directed mutagenesis using GeneEditor™ (Promega). The results of the mutagenesis were confirmed by sequencing (Primers used for mutagenesis (5'-3') 1. gtgttttcaggttcGCgggtgccgcacttg (Vp2) 2. cagcagccagctGCggctttacaatta (Vp3) Mismatched nucleotides are shown in uppercase). The double mutant was also created using both primers to eliminate both Vp2 and Vp3. Additional mutants were created by the removal of the myristylation site at position two of Vp2 by changing the glycine to either an alanine, glutamate, glutamine or histidine (Primers used for mutagenesis (5'-3') 3 ggttcatcgCtgccgcac (G2A) 4. gttttcaggttcatgGAAgccgcacttgcac (G2E); 5. gttttcaggttcatgCATgccgcacttgcac (G2H); 6. gttttcaggttcatgCAAgccgcacttgcac (G2Q) Mismatched nucleotides are shown in uppercase.) (Fig. 1A).

Mutant and wild type genomes were linearized and equal amounts of viral DNA were transfected into permissive SVG-A cells using lipofectAMINE™ (Invitrogen) and Plus™ (Invitrogen) reagents. They were scored 84 hours post-transfection for viral expression by indirect immunofluorescence assay of Vp1. At 84 hours post-transfection Vp1 was expressed in approximately 5% of the cells, which is consistent with the transfection efficiency of these cells. Vp1 has a weak monopartite NLS in contrast to the

strong bipartite NLS of its family members SV40 and BK virus (11, 26). JCV therefore relies on the presence of the minor proteins for efficient nuclear localization. All samples containing one minor protein correctly localized Vp1 to the nucleus, indicating the mutant DNA is able to be expressed and that only one minor protein is needed for Vp1 import (Fig. 2A). The double mutant ($Vp2^-/Vp3^-$) did not localize Vp1 to the nucleus as efficiently as either of the single mutants, which is consistent with the weak NLS in JCV Vp1. (Fig. 2A). After following the infection for 19 days, the mutants were unable to efficiently propagate the infection. In contrast, wild type virus spread to 85% of the cells (Figure 2B, 2C). This suggests that JCV requires Vp2, the myristylation site on Vp2, and Vp3 for completion of its lifecycle. This also shows that Vp2 and Vp3 have distinct functions in the viral lifecycle as neither can be complemented by the other protein.

The lack of the minor proteins could effect many aspects of the viral lifecycle. These proteins have been shown to localize to ND10 domains in the nucleus so their absence could cause mislocalization and therefore assembly defects (27). To address an assembly defect in the life cycle, a DNase protection assay was performed. Virus was harvested from transfected cells at 4, 13 and 22 days post-transfection and genome protection by the capsid was tested with DNase treatment. The virus was harvested by freeze-thaw, sonication, membrane lysis with 2.5% deoxycholate and centrifugation to remove cell debris. Equal amounts of virus harvested from the cells were treated with 20-fold excess DNaseI or mock treated with water. The capsids were removed using Proteinase K and the genomes were purified and amplified by PCR (Primers (5'-3') $JCV_{for}401$: GTG AAG ACA GTG TAG ACG G and $JCV_{rev}1070$: GAA TTT CCT GAG AGG TTA AGC). Cells that received no viral DNA were used as a control to show that

cellular DNA was not being amplified. At all of the time points wild type (WT) virus showed protection of its DNA, whereas the mutants were unable to protect their DNA (Fig 3: compare 3,5,8 and 10). This lack of amplification in the mutant samples is not due to lack of viral DNA as shown by the mock treated lanes. (Fig 3: compare 4,6,9, and 11). At the four day time point the wild type and mutants were expressed equally, suggesting the result is not due to excess wild type virus.

From these results we concluded that both of the minor proteins and the myristylation of JCV Vp2 are necessary for efficient propagation of the virus. Neither minor protein is able to functionally substitute for the other. Both minor proteins and the myristylation site are needed for the correct packaging of the virus. Current studies in the lab are underway to further understand the assembly defects. These studies will continue to evaluate whether there is a difference in the localization of the capsid proteins or the genome in the mutant viruses.

Our results are also consistent with current findings in mouse polyoma. When the myristylation moiety of Py is mutated to histidine, glutamine and glutamate, the viruses have lower viral burst and therefore have fewer infected cells over time (16). Also, when the myristylation site is mutated to a glutamate, electron microscopy studies show an altered morphology. These mutants were able to create capsid-like structures but were less regular and less compact (14).

Myristoylated proteins have been known to play a key role in assembly for other non-enveloped viruses such as polio (17, 18). It is possible that the myristoyl moiety makes key contacts within the virion that are necessary for the structural integrity of the

virus. Additionally, the assembly defects could be because of unique protein interactions between the minor proteins and cellular chaperones.

The inability of the virus to protect DNA when it is lacking the minor proteins is also consistent with recent virus-like particle (VLP) studies. These studies showed JCV VLPs, consisting of Vp1 alone, are unable to protect genome size DNA from DNase degradation (28). They also showed that under physiological conditions Vp2/3 is required for SV40 Vp1 to form VLPs (12, 13).

ACKNOWLEDEGMENTS

We thank all the members of the Atwood Laboratory for critical discussion during the course of this work. Work in our laboratory was supported by a grant from the National Cancer Institute, R01 CA71878, and by a grant from the National Institute of Neurological Disorders and Stroke, R01 NS43097 to WA. Megan L. Gasparovic is supported by a Ruth L. Kirschstein Predoctoral Fellowship #1F31NS053340-01.

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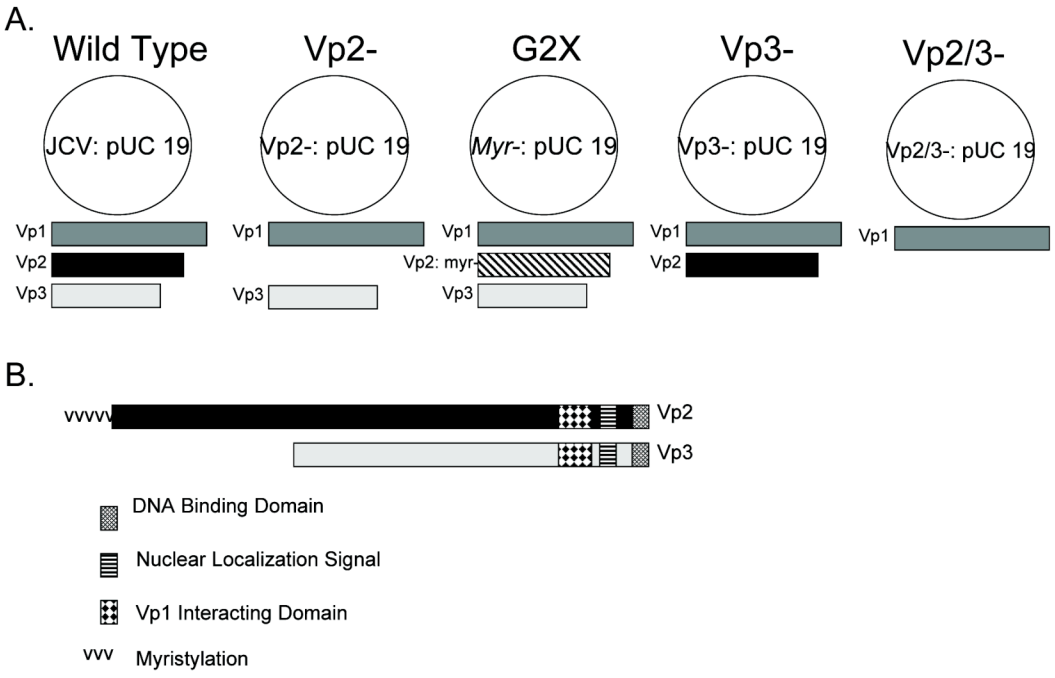
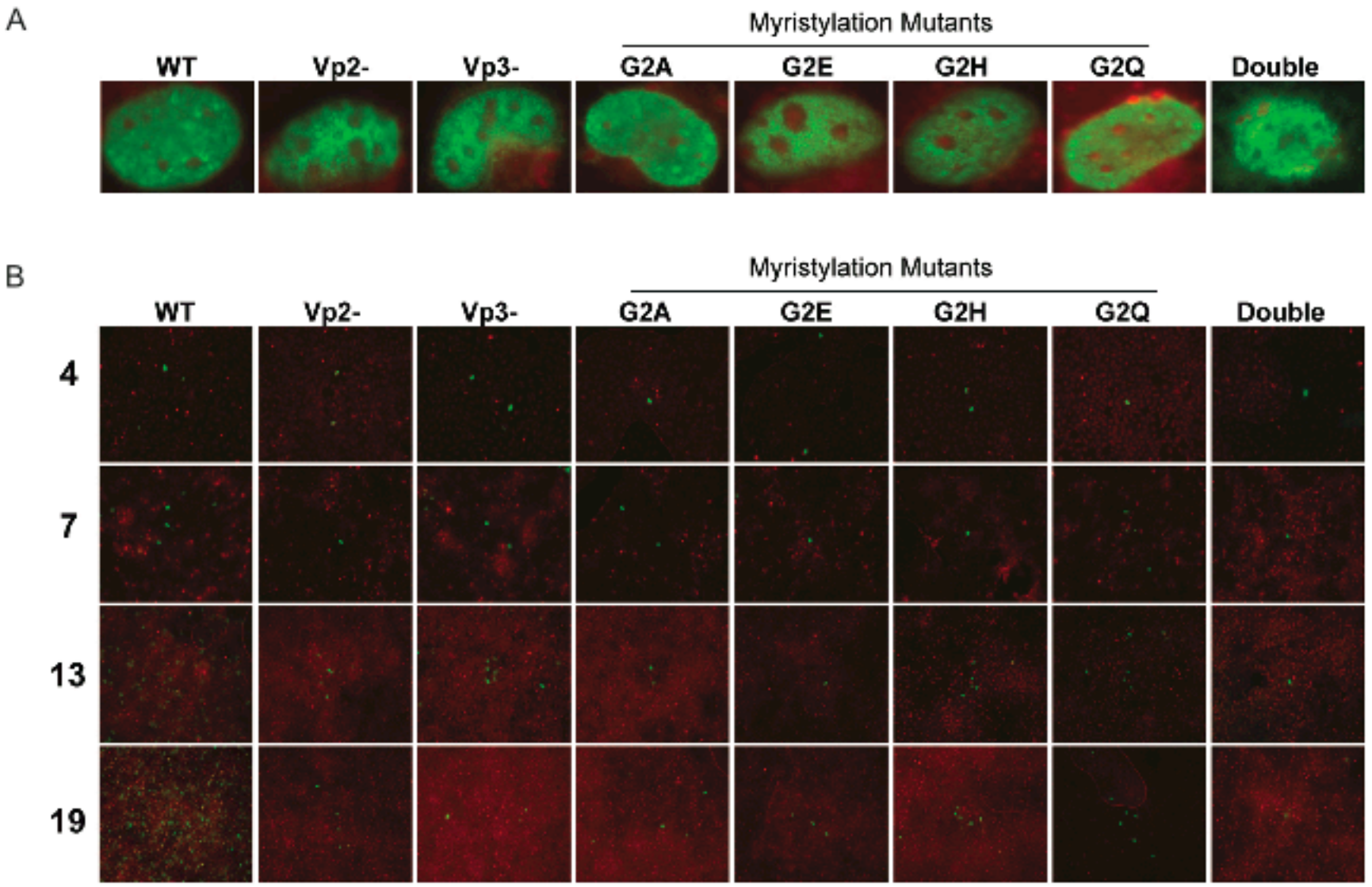


Figure 1: Diagram of minor proteins functional domains and the mutants created by site-directed mutagenesis (A) Diagram of capsid proteins that will be remaining after site-directed mutagenesis. (B) Diagram of Vp2 and Vp3 and their functional domains.


A
Myristylation Mutants
WT
Vp2-
Vp3-
G2A
G2E
G2H
G2Q
Double
B
Myristylation Mutants
WT
Vp2-
Vp3-
G2A
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G2Q
Double
4
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13
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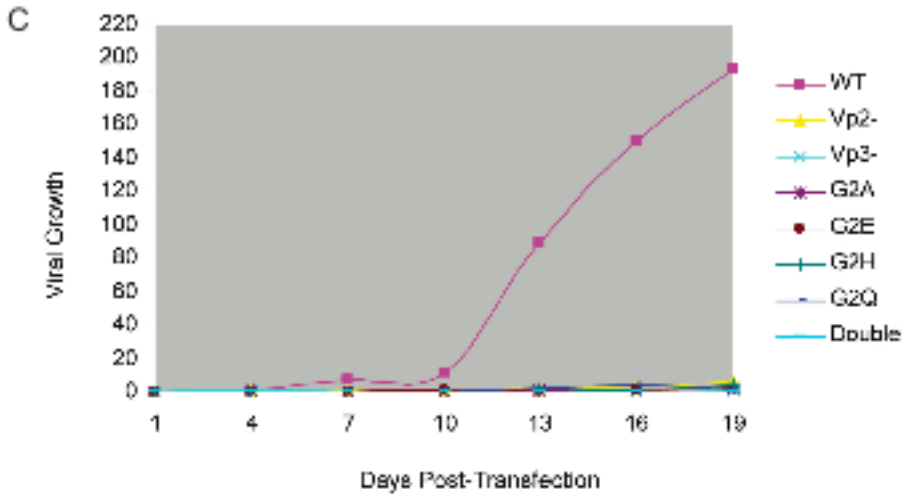

Figure 2: Viral propagation requires the minor proteins. (A) Nuclear localization was monitored by indirect immunofluorescence of Vp1. All mutants except the double mutant properly localize Vp1. (B) Viral growth was monitored by indirect immunofluorescence of Vp1. All mutants are expressed at 4 days post-transfection but fail to grow over time. (C) A growth curve was created for Wild-type (WT) and mutant viruses by determining the average number of Vp1 positive cells/250 cells counted

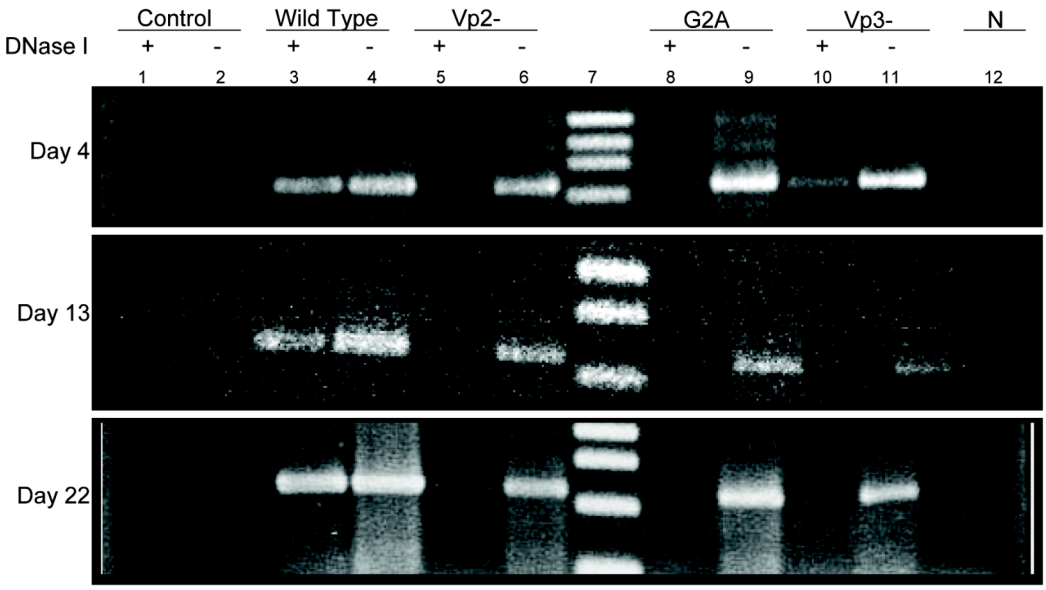

Figure 3: DNase sensitivity assay. An equal amount of virus was obtained from each time point. The virus samples were subjected to 20 fold excess DNase I treatment or mock treated with water. The capsids were then removed with Proteinase K treatment and the genomes were amplified by PCR. Control= cells that received no viral DNA.N= PCR control that received no input DNA.

CHAPTER 5

DISCUSSION

The main focus of this thesis is to understand which cellular and viral factors contribute to the entry and gene expression of JC virus. Polyomaviruses are small, non-enveloped viruses that produce a small number of viral proteins. Therefore, they rely upon cellular factors for aid in their lifecycle. After cell surface binding, JCV enters cells and is trafficked to the early endosome (26). From there, the virus moves to the caveosome and the endoplasmic reticulum (ER) (27). Current work with SV40 and mouse polyoma shows they uncoat in the endoplasmic reticulum (21, 28, 32). We know that JCV, unlike SV40, requires low pH during its lifecycle (1). We were interested in the role that low pH played in JCV. We studied the role of low pH using inhibitors to cellular proteases and monitored conformational changes and uncoating. Additionally, we used modulated ER calcium levels to determine their effect on viral gene expression. After uncoating, the viral DNA is delivered to the nucleus. SV40 and BKV require the ND-10 or PML domain for viral DNA replication (20, 42). We characterized the role the PML domain plays in JCV infection and gene expression using shRNA to remove PML protein or through chemical modulation of the domain. Additionally, we hypothesized that all viral gene components were necessary for infection. We investigated the role of the minor capsid proteins using site-directed mutagenesis on Vp2, Vp3, and the myristoylation site on Vp2.

Virus Entry and Trafficking

We began investigating the factors responsible for genome delivery. After initial receptor binding, JCV is internalized using clathrin-dependent endocytosis (23). JCV is delivered to the early endosome and requires this low pH environment for its infection (1,

27). When permissive SVG-A cells were treated with the weak base ammonium chloride (NH_4Cl), JCV infection was reduced. We evaluated the time in which low pH was required by treating SVG-A cells with NH_4Cl in four hour intervals prior to infection and throughout the first eight hours of its infection. We used indirect immunofluorescence of large T to assay virus infection and determined low pH was only necessary during the first two to three hours of virus internalization. Because of this, we decided to characterize the role of the low pH environment. Viruses require low endosomal pH for conformational change leading to membrane fusion, digestion by proteases, or continued vesicular trafficking. To evaluate whether low pH causes a conformational change in the JCV capsid, limited proteolysis and Western blot analysis were employed. Purified JCV virions were incubated with low pH conditions and then digested with proteinase K. No conformational change was seen in the assay. Proteinase K is a nonspecific protease capable of digesting JCV thereby possibly eliminating digest products. However, a conformation change was not detected when purified JCV was incubated in low pH conditions then probed with bis-ANS binding to detect hydrophobic residues. Additionally, a hemolysis assay confirmed JCV did not become capable of lysing red blood cells after its incubation in low pH conditions.

An additional way viruses can undergo conformation change is by protease digestion. Cathepsins are cysteine proteases activated by low pH environments. They are found in the endosomal/lysomsomal pathway (17). Many viruses that require low pH and traffic to the endosome use cathepsin for conformation change. One of the most well documented cases is reoviruses. Reovirus capsids are composed of two protein layers. The outer layer must be digested to expose the interior protein μ1. μ1 has a

myristoylation site and is capable of penetrating the endosomal membrane after its exposure. The digested form of a reovirus is termed the "infectious subvirion particle" and can also be created by protease digestion within the intestinal tract (2, 13). To determine whether cathepsins were necessary for JCV infection, SVG-A cells were treated with inhibitors of both cathepsin B and L. These inhibitors were used at concentrations known to inhibit virus infection and inhibit cathepsin activity *in vitro*. Treated SVG-A cells were infected with JCV and infection was monitored with indirect immunofluorescence of Vp1. Cathepsin inhibitors did not block JCV infection, suggesting they are not required for genome release. Additionally, cysteine proteases and calcium-activated proteases were blocked by treating SVG-A cells during JCV infection. These again had no effect on viral gene expression.

In contrast to the inhibitor studies, when JCV was digested with trypsin prior to infection, JCV gene expression was enhanced. Trypsin digestion is capable of causing changes in the viral capsid proteins. SDS-PAGE and Western blot analysis showed Vp1 was digested and becomes a doublet. This is likely due to a cleavage in the C' terminal tail of the Vp1 molecule, which will loosen the contacts between pentamers. Trypsin digestion is used during crystallization of the pentamers, when it cleaves the C' terminus and keeps the pentamers from self-assembling (6, 39). Additionally, Vp2/3 were digested by trypsin treatment and appear as multiple bands, suggesting trypsin is able to access the interior parts of the virion. A DNase protection assay also showed the JCV virion becoming sensitive to DNase after trypsin digestion. However, the capsids did not fall apart and retained their characteristic icosahedral shape, as determined by electron microscopy. The changes in the pentamer created by trypsin digestion are not capable of

relieving the requirement of an acidic endosome, as digested JCV is still inhibited by ammonium chloride treatment. The loosening of pentamer contacts could be helping the virus uncoat faster, which would lead to an increase in infection. The outside of oligodendrocytes contains trypsin-like proteases. These proteases could be capable of digesting JCV during its cell surface binding. It would be interesting to inhibit trypsin and trypsin-like proteases within the cell to determine whether JCV infection is decreased in their absence.

These experiments show that JCV does not lyse membranes in low pH conditions, does not require digestion by cathepsin, and still requires low pH after trypsin digestion. Suggesting JCV traffics to the early endosome and requires low pH for continued movement throughout the cell. bafilomycin A which raises endosomal pH but also blocks vesicular trafficking completely inhibits JCV infection (1). Additionally, the monitoring labeled JCV particles showed JCV moves from the endosome to the caveosome. The virus becomes unable to associate with docked vesicles when the pH of the endosome is raised. From the caveosome, JCV traffics to the ER (27).

Mouse polyoma is able to insert into membranes only after exposure to chaperones in the endoplasmic reticulum (28). The ER protein ERp29 is able to cause conformational changes in mouse polyoma capsid, as determined by limited proteolysis (21). This allows the minor capsid proteins to be exposed, which penetrate or insert into membranes (28). SV40 minor proteins can also penetrate membranes. SV40 Vp2 is predicted to have five transmembrane domains. Vp3 shares four of these domains. Both SV40 minor proteins are capable of inserting into the ER membrane in a Vp1-dependent manner (9). Additionally, the overexpression of SV40 Vp2 and Vp3 are able to lyse

bacteria (9, 11). These data suggest polyomavirus penetration and genome release require exposure of the minor proteins, most likely because of the myristoylation site on Vp2. SV40 also requires parts of the ERAD pathway to release its genome into the cytoplasm. SV40 infection is inhibited when Derlin-1, a membrane protein used to retrotranslocate ERAD proteins, is removed. Additionally, SV40 requires the proteasome during its first eight hours of infection. The proteasome interacts with the ERAD pathway and can assist in protein retrotranslocation. When SVG-A cells were treated with two proteasome inhibitors, lactacystin and MG-132, there was no effect on JCV infection. Currently JCV has not been shown to insert into membranes or cause cell lysis. However, we were unable to overexpress JCV Vp2 and Vp3 alone, suggesting they too possess lytic properties. It would be useful to confirm whether JCV can insert into membranes. This could be answered by incubating purified JCV with ER extracts and then performing a hemolysis assay to determine whether ER conditions uncoat JCV and lead to membrane fusion. Additionally, conformational changes within the capsid could be monitored by binding of bis-ANS after incubation with ER extracts.

The ER not only provides chaperones to assist in viral uncoating, it is also a site of high calcium concentration. Polyomavirus particles have calcium binding sites at the base of each Vp1 pentamer (38). These ions make important structural contacts, which hold the capsid together (5). It is hypothesized that once the genomes are partially uncoated in the ER, they are transported to the cytoplasm. The low calcium conditions within the cytoplasm would lead to full disassemble and exposure of the nuclear localization signal, leading to nuclear import. Both JCV and SV40 infections are inhibited when calcium is depleted from the ER using thapsigargin (32). This inhibition is

most likely due to dissociation of the genome from the capsid proteins. The genome would then be unable to be imported to the nucleus. It would be interesting to determine whether a labeled JCV genome would become trapped within the ER when calcium levels are depleted.

Nuclear Entry and Nuclear Domains

After polyomaviruses uncoat and have their genome delivered to the cytoplasm, they must be imported to the nucleus for viral gene expression. Virions are 40-45nm, making them too large to fit through nuclear pore complexes in a fully assembled state. This supports the hypothesis that partial uncoating must occur prior to the JCV virion encountering the nuclear pore complex. Additionally, the nuclear localization signal (NLS) on JCV Vp1 is weak and requires Vp2 or Vp3 for efficient import (7, 33). For the minor proteins to assist with import, the virus must have undergone a conformational change to expose these interior proteins. JCV Vp1 has been shown to use the traditional nuclear import pathway for its delivery to the nucleus (Figure 1) (25). These studies used virus-like particles (VLPs), which consist of Vp1 alone. The nuclear entry of Vp1 requires an intact nuclear pore complex and both importin α and β. Nuclear entry of JCV was inhibited when the nuclear pore complex was blocked with wheat germ agglutinin or antibodies to the nuclear pore proteins. Additionally, if the cytoplasm was depleted by digitonin membrane permeabilization, JCV was unable to import into the nucleus, suggesting it requires interactions with cytoplasmic import factors. SV40 also uses this traditional pathway for it nuclear import. However, SV40 nuclear import has been described as the interaction of minor protein Vp3 with the nuclear pore complex and

importin α and β (8). JCV VLPs were unable to be created with the minor proteins, but they are likely to use this same pathway for nuclear import.

Once inside the nucleus, the viral genome can be transcribed and replicated. The nucleus is organized into a number of domains. These domains are not membrane bound but are accumulations of proteins that interact with one another to perform a unique function. Examples of domains include the nucleolus, the cajal bodies, and the ND-10 domain. These domain and their associated proteins are involved in ribosome biogenesis, stress response, DNA repair, or protein storage (44).

The ND-10 or PML domain has a unique relationship with viruses. The backbone of this domain is the promyelocytic leukemia protein (PML). Its localization to the domains is based upon its SUMOylation status. When PML is modified by SUMO, it is associated with the domain. When the SUMO modification is removed, PML is degraded. PML can also have additional post-translational modifications. These modifications are modulated by the cell cycle or as responses to stress (4). When PML undergoes a modification, it changes which other factors are also associated with the domain. Sp100, another domain resident, also requires SUMO modifications for its domain localization. Additionally, there are many transcription factors and cell cycle regulators in the domain (40, 43).

PML and Sp100 are interferon responsive. This has caused the ND-10 domain to be characterized as anti-viral. Many viruses also see this domain as inhibitory and disrupt them early during infection. Herpes simplex virus (HSV-1) immediate early protein ICP0 targets the PML protein directly. ICP0 causes de-SUMOylation and proteasome-dependent degradation of PML. HSV-1 lytic infection cannot proceed unless PML has

been degraded (14). Adenoviruses also require PML protein removal. However, PML is not degraded in adenovirus infections. Instead, adenovirus protein pIX accumulates around PML and sequesters it, removing its repressive effect (30). Like HSV-1, adenovirus infection is not productive unless PML is removed.

Polyomaviruses and former family member papillomaviruses have a different relationship with PML domains. These DNA viruses deposit their genomes adjacent to the PML domains. SV40 and BKV have been shown to replicate adjacent to the PML domains. To localize to this domain, SV40 and BKV require the production of large T and an intact origin (20, 42). Papillomaviruses' minor protein L2 contains a localization signal. It is responsible for depositing the papilloma genome at the PML domain (12). L2 is also responsible for recruiting the minor protein L1 and the regulatory protein E2 to the PML domain (3). JCV minor proteins have been shown to localize to the PML domain; it is hypothesized to be a site of virion assembly (35). Virions can be found adjacent to the PML domain in post-mortem brain tissue of patients with Progressive Multifocal Leukoencephalopathy (34).

While evaluating viral protein expression with indirect immunofluorescence, we noticed large T accumulated in a speckled pattern within the nucleus. When PML protein expression was analyzed in large T positive cells, we found they were adjacent to one another. To evaluate whether PML was critical for JCV infection, SVG-A cells were transfected with shRNA constructs against PML or luciferase. The PML shRNA construct knocked down PML expression eight days post-transfection; the luciferase control had no effect on PML. After eight days, transfected cells were infected with JCV and evaluated 72 hours later. Both samples were infected with equivalent efficiency,

indicating PML protein was not required for JCV protein expression. The transfection efficiency in these experiments was low. To determine the effect of PML on a global level, SVG-A cells were treated with arsenite or interferon beta (INF-β). Arsenite can cause PML degradation, while INF-β can increase PML protein. Other stresses such as heat shock and cadmium were unable to causes changes in PML protein levels. This modulation of PML protein was confirmed in SVG-A cells using indirect immunofluorescence and confocal microscopy or SDS-PAGE and Western Blot analysis. We also evaluated how Sp100 and Daxx, additional PML domain components, responded to these treatments. Arsenite treatment had no effect on Sp100, but similar to PML, INF-β increased Sp100 protein expression. Daxx was slightly dispersed upon arsenite treatment but was unaffected by INF-β treatment. The change in PML protein had a drastic effect on JCV infection. When cells were treated with arsenite and PML was removed, JCV protein expression was significantly increased. Conversely, when cells were treated with INF-β, JCV infection was decreased. However, INF-β was no longer inhibitory to JCV when PML was eliminated with arsenite.

When large T was examined after arsenite and INF-β treatment with indirect immunofluorescence and confocal microscopy, we saw the viral accumulations were always present, regardless of PML protein status. This suggests PML does not provide a scaffold for these viral microdomains. Removal of PML with arsenite did not affect viral entry. Cells pre-treated with arsenite had the same rate of viral neutralization as untreated cells. However, loss of PML did affect viral transcripts. RT-PCR of viral transcripts harvested from untreated cells, arsenite, or INF-β treated cells showed increase in transcripts when PML was removed. This suggests a model where PML is able to

actively recruit or modify transcriptional regulators. ND10 domains are not merely storage compartments, but are highly regulated areas. Transcriptional regulators are moved into and out of the domain depending on the cell cycle and stress. Increases in PML due to interferon treatment could bring transcriptional repressors to the domain or silence necessary viral transcription factors. Additionally, histone deacetylases (HDAC) are present in the ND10 domain. PML could cause silencing of viral gene expression through activation of HDAC complexes. It would be interesting to evaluate the effects of HDAC repressors on JCV infection and whether they would have a different effect in presence and absence of PML.

We also evaluated whether JCV actively removed PML protein during its infection. SVG-A cells were infected and the level of PML protein was evaluated over the course of JCV infection using indirect immunofluorescence and Western blot analysis. Whole cell extracts were collected 7, 14, and 21 days post-infection. PML protein was not decreased in these samples relative to tubulin. JCV protein levels increased over this time course. Nuclear and cytoplasmic extracts were also collected throughout JCV infection. At 5, 10, and 15 days post-infection, PML protein was found to have the same cytoplasmic and nuclear localization as non-infected cells. Using indirect immunofluorescence of PML and JCV, we found PML maintained its speckled nuclear appearance even as JCV infection spread throughout the culture. These data show JCV does not remove PML protein during its infection. We know JCV large T accumulates adjacent to the ND10 domain. SV40 and BKV replicate here as well. JCV replication could be monitored with *in situ* hybridization. It is interesting that JCV is able to be close to the PML bodies without eliminating them. This indicates that there are pro-

viral factors active within the domain that assist in viral gene expression, even when PML is present.

JCV Minor Proteins

Once the JCV genome has been deposited in the nucleus, the early viral genes are produced. These early genes are the five T antigen proteins: small T, T'_{135}, T'_{136}, T'_{165}, and large T (24). Each of these proteins is an alternatively spliced product of the same mRNA transcript. They are all regulatory proteins. The role large T plays in gene expression is the best documented. Large T stimulates the cell and causes the cell cycle to proceed to S-phase, where viral replication will occur (15). Large T regulates this process through its interactions with pRb. Large T contains a chaperone J-domain that breaks the interaction between pRb and E2F (41).

Once the cell is in S-phase, the viral genome is copied. Large T also assists in this process. There are large T binding sites within the viral origin of replication (18, 37), which it binds to and recruits additional cellular factors needed for efficient replication. After replication is complete, large T binds to the late promoter region and drives the expression of the late viral proteins agno, Vp1, 2, and 3 (18). There are four late RNAs produced for JCV, unlike SV40 and mouse polyoma. These four are created from alternative splicing (33). SV40 produces two RNA species, which are polycistronic, while mouse polyoma only produces monocistronic RNAs. Agno is the leader for each of the JCV RNA products. One RNA produces Vp2 and Vp3, another produces Vp1, and two RNAs produce splice variants of Vp1 (36).

Agno protein has a regulatory role in the JCV lifecycle (19). It is small and highly basic, giving it an affinity for DNA and a potential role in virus assembly (29). The other late proteins make up the virus capsid. The virion is composed of 72 pentamers of the major protein Vp1 (38). Each pentamer contains a minor protein, Vp2 or Vp3, in the center (6).

To characterize the role of the minor proteins, we used site-directed mutagenesis to remove Vp2, Vp3, both minor proteins, or change the amino acid used for the attachment of the myristoylation moiety. Myristoylation is added to the glycine at the second amino acid position. This glycine was replaced by alanine, glutamine, glutamate, and histidine. These mutated viral DNA molecules were transfected into permissive SVG-A cells and viral protein expression was monitored by indirect immunofluorescence of Vp1. The typical virus lifecycle takes three days, but when originally transfected the viral DNA is given four days to produce Vp1. All mutants were able to produce Vp1 four days post-transfection. All of these mutants, except the Vp2/3 double mutant, had the same discrete nuclear localization and nucleolar exclusion as wild type. The double mutant was partially localized to the nucleus, but also contained Vp1 in the cytoplasm due to its weak nuclear localization signal. JCV infection was then monitored in three-day intervals with indirect immunofluorescence of Vp1. Wild type virus was able to grow rapidly throughout the cell culture: at 19 days post-transfection, 90% of the cells were infected with JCV. However, none of the mutants were able to replicate this phenotype. All of the mutants continued to express Vp1 throughout the infection, but this infection did not spread from those cells originally transfected with viral DNA. These data indicate the minor proteins are both required for JCV. It also shows that even though Vp3 is

identical to a portion of Vp2, they are not able to substitute for each other. These results are consistent with the role of minor proteins in SV40 and mouse polyoma (16, 22, 31). Both SV40 and mouse polyoma require both minor proteins. For many years, it was believed SV40 only required Vp3, but recent evidence proved Vp2 was critical for SV40 infection (10).

The role of the myristoylation site on SV40 has not been fully characterized. In mouse polyoma, the myristoylation is required for both exit and re-entry into cells. The large, glutamate amino acid was able to partially substitute for the myristoylation site. However, having a large positively-charged histidine was the most detrimental to mouse polyoma infection (31). In our system, any substitution to the glycine negatively affected JCV infection.

The minor proteins reside within the capsid and make many important contacts between Vp1 and the viral genome. We evaluated whether their loss changed the structural integrity of the capsid using a DNase protection assay. Wild type, Vp2-, Vp3-, and myristoylation (myr-) mutant genomes were transfected into permissive SVG-A cells. At 4,13, and 22 days post-transfection virus was harvested from the cells. After virus isolation, the samples were treated with DNase or mocked treated with water. The capsids were then removed with proteinase K and the genomes were amplified by PCR. Wild type virus was able to protect its genome from digestion. However, Vp2-, Vp3-, and myr- became sensitive to DNase digestion. This suggests that when the minor proteins are lost or mutated, the capsid integrity is altered.

When this work was conducted, there were no antibodies available to Vp2/3. We were unable to confirm that the loss of Vp2 had no effect on Vp3 and vice versa.

However, this could be determined through complementation using combinations of mutant genomes. The lack of antibodies also prevented us from analyzing other cellular properties. To characterize cellular localization of the minor proteins, we created a variety of constructs that would express the minor proteins individually from a plasmid (Figure 2). These proteins were also engineered with C' terminal tags. The proteins were expressed from their own constructs to avoid interfering with other viral proteins in the genome. These tagged constructs were transfected into SVG-A cells and Cos-7 cells, a highly transfectable cell line. The protein's expression was monitored through indirect immunofluorescence of the tags or epifluorescence of GFP. Unfortunately, these constructs were only able to produce a few transfected cells in each sample. The minor proteins of SV40 are capable of lysing bacteria when they are expressed individually (10). This suggests the minor proteins must be produced with other viral proteins to control their lytic properties. Additionally, the overexpression of the minor proteins could have caused aggregation and cell death due to their overaccumulation.

To detect the minor proteins in the whole virus, internal V5 tags were added to the proteins (Figure 3A). The tags were added using site-directed mutagenesis. Virus infection was assayed, showing the tags did not affect virus propagation. Vp2 and Vp3 were examined with indirect immunofluorescence of the V5 tag. They were shown to accumulate in a speckled pattern and also shown to be diffuse throughout the nucleus (Figure 3B). During the course of this work, we received antibodies to SV40 Vp2/3, which were able to cross-react with JCV. Using these antibodies, an indirect immunofluorescence assay showed a similar localization pattern. Some samples had diffuse nuclear staining of Vp2/3 while others had a speckled pattern (Figure 3C).

Previous work using non-permissive Cos-7 cells showed Vp2/3 were capable of accumulating in a speckled pattern adjacent to nuclear domains (35). Together, this work suggests Vp2/3 may localize in microdomains at specific times during infection. This localization may be signaled by other events when the virus is ready for assembly and release.

The viral capsid is composed only of three proteins. The minor proteins Vp2 and Vp3 contain many important properties. Both have DNA binding domains, nuclear localization signals, Vp1 interacting domains, and potential transmembrane regions; additionally, Vp2 contains a myristoylation site. This indicates they will be important for many activities within the cell. They are mostly likely the important factors leading to genome release from the ER and assisting in genome import to the nucleus. They are capable of localizing adjacent to PML domains, suggesting they may deposit the viral genome at these sites prior to gene expression. Additionally, they make important structural contacts, which protect the genome from degradation. The minor proteins and their cellular interactions are an excellent example of how viruses have evolved to exploit multiple cell systems for their benefit.

Summary

The goal of this work was to determine the cellular and host-cell factors JCV requires to facilitate delivery of its genome to the nucleus and produce its gene products. Our results show JCV requires a low pH environment during the first two to three hours of its infection. Although this environment is not required for protease activation or conformational change, it is critical to the continued trafficking of JCV. As JCV moves through the cell, it encounters additional environments. We show ER calcium is critical for JCV infection. We also demonstrate JCV infection can be enhanced by destabilizing its capsid with trypsin prior to infection. This is likely due to an increase in the rate of uncoating.

Once inside the nucleus, JCV large T is produced and accumulates in its own viral microdomain. This microdomain is adjacent to ND-10 components PML, Sp100, and Daxx. However, the large T accumulations do not require the presence of these proteins. JCV infection can proceed in the absence of PML protein and is enhanced when it is removed. Additionally, JCV does not actively remove PML during its infection.

Lastly we evaluated viral factors critical for JCV lifecycle. We determined both Vp2 and Vp3 were needed for JCV infectivity. We also showed the myristoylation moiety was critical and could not be replaced by an alternative large, bulky group. The minor proteins are also important for proper packaging of the virus. Additionally, the minor proteins make important contacts inside the virion and will likely be shown to play a critical role in virus uncoating and genome delivery in future experiments.

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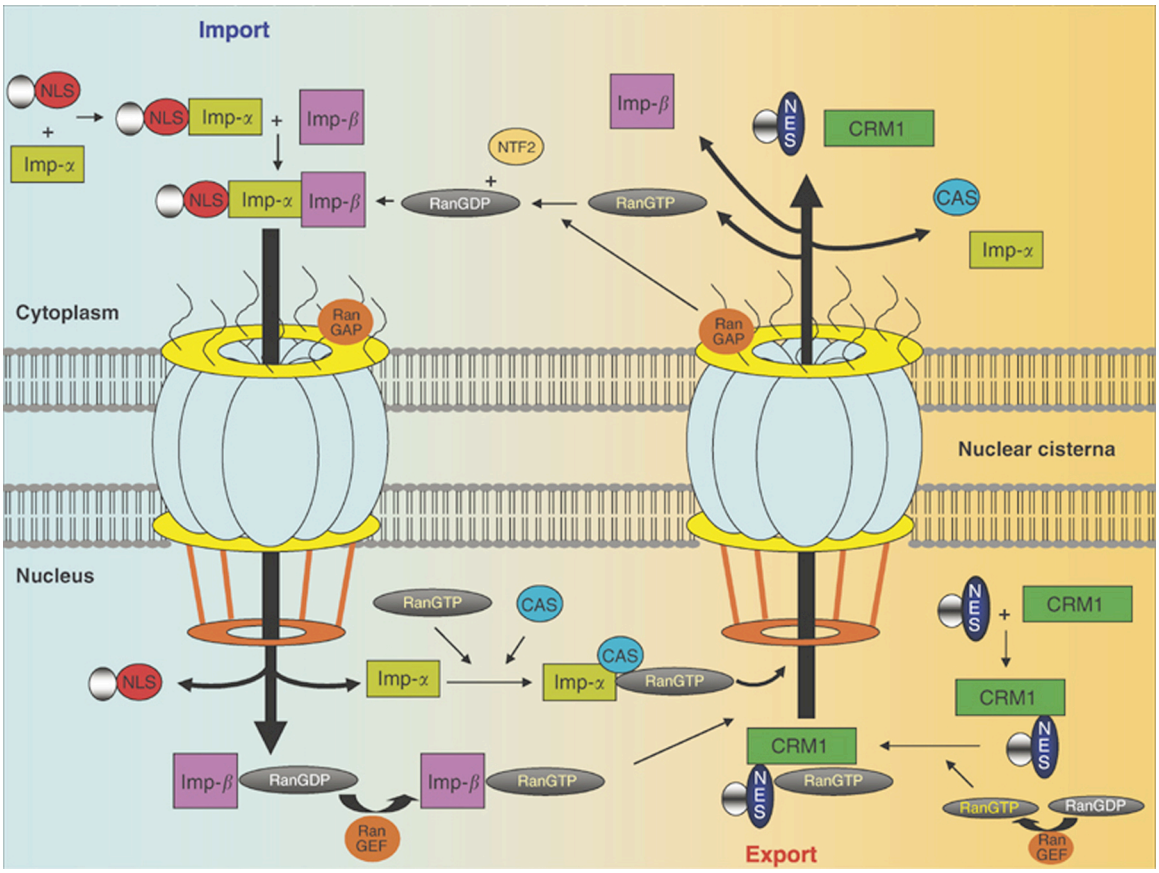

Figure 1: Nuclear Import Pathway. Virus-like particles of JCV Vp1 are imported into the nucleus using the traditional nuclear import pathway. To use this pathway, a cytoplasmic protein must contain a nuclear localization signal (NLS). The NLS is recognized by importin α. Importin β then dimerizes with importin α. This complex binds to the nuclear pore complex and RanGDP, delivering the protein to the nucleus. Inside the nucleus, the import machinery is released from the protein and is recycled back to the cytoplasm. *(Faustino, et al. Clinical Pharmacology and Therapeutics, 2007 and is used with written permission from Nature Publishing Group.)*

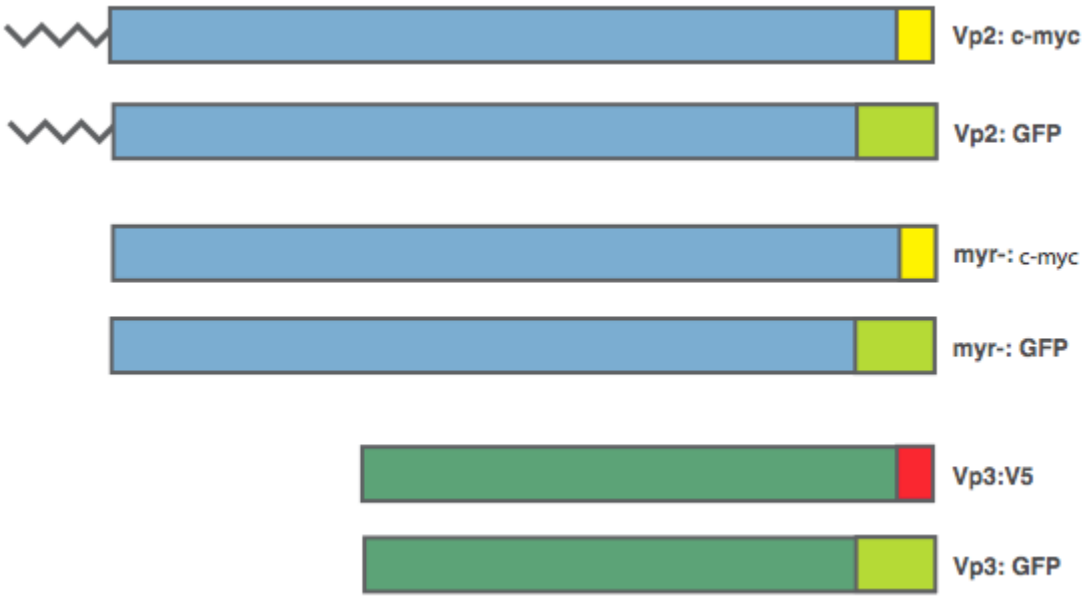

Figure 2: JCV tagged minor proteins. JCV minor proteins were tagged and cloned into pcDNA 3.1. This vector uses a CMV promoter for protein expression. Each construct was transfected into SVG-A or Cos-7 cells and monitored with indirect immunofluorescence or epifluorescence. These constructs were too toxic for cells.

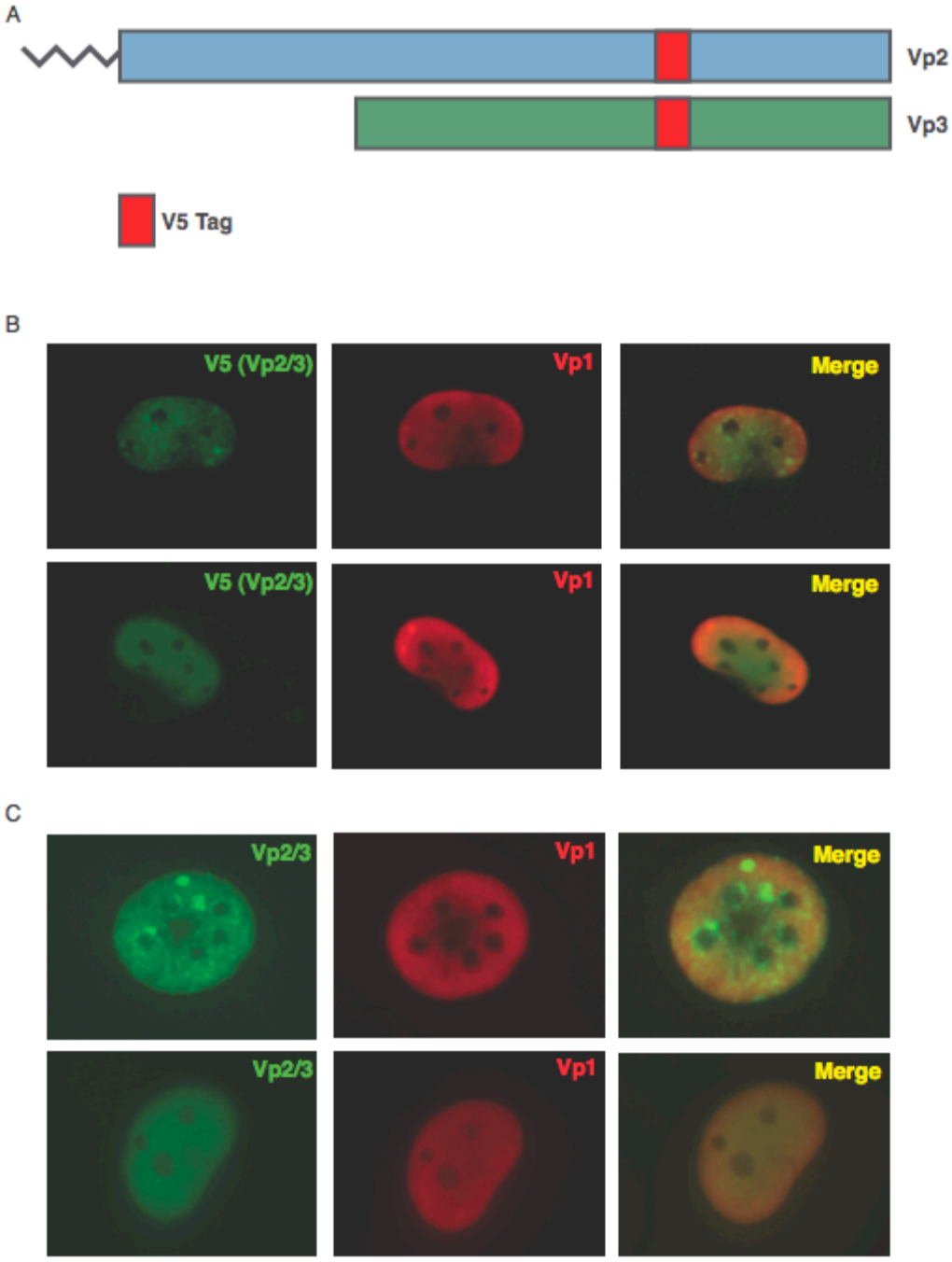

Figure 3. JCV minor protein expression. A. Location of V5 tag. The V5 tag was added to the overlapping region of the minor proteins using site-directed mutagenesis. It was added to the proteins in the viral genome, not in individual plasmids. B. Indirect immunofluorescence of the V5 tag and Vp1. The minor proteins can appear in a speckled pattern, as well as diffuse throughout the nucleus. C. Indirect immunofluorescence of the Vp2/3 and Vp1. Vp2/3 were detected using an SV40 antibody that cross-reacts to the JCV minor proteins. The minor proteins can appear in a speckled pattern, as well as diffuse throughout the nucleus.