INTRACELLULAR TRAFFICKING OF BK POLYOMAVIRUS: FROM THE ER TO THE NUCLEUS

by

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DEDICATION

This doctoral thesis is dedicated to my mother, who has always believed in me.

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ABSTRACT

BK Polyomavirus (BKPyV) is a widespread human pathogen that establishes a lifelong persistent infection in the kidneys and can cause severe disease in immunosuppressed patients. BKPyV is a nonenveloped DNA virus whose productive intracellular trafficking pathway requires trafficking through the endoplasmic reticulum (ER); however, it is unclear how BKPyV exits the ER and undergoes nuclear entry. In this dissertation, I elucidated the role of the ER-associated degradation (ERAD) pathway and show that BKPyV traffics through the cytosol en route to the nucleus. Furthermore, I determined the importance of the nuclear localization signal located on the minor capsid proteins VP2 and VP3 during BKPyV intracellular trafficking. These studies were performed in renal proximal tubule epithelial (RPTE) cells, a natural host cell, and provide a relevant model for BKPyV infection.

Using proteasome and ERAD inhibitors, I showed that ERAD is required for productive entry. Altered trafficking and accumulation of uncoated viral intermediates was detected by fluorescence *in situ* hybridization and indirect immunofluorescence in the presence of inhibitor. Additionally, I detected a change in localization of partially uncoated virus within the ER during proteasome inhibition, from a BiP-rich area to a calnexin-rich subregion, indicating BKPyV is accumulating in an ER subcompartment. Interestingly, inhibiting ERAD did not prevent entry of capsid protein VP1 into the cytosol from the ER. By comparing the cytosolic entry of the related polyomavirus SV40, I found that trafficking results varied between different cell types, namely, immortalized CV-1 cells versus primary RPTE cells. By measuring viral DNA in the cytosol,

however, I found that ERAD inhibition led to a decrease in cytosolic viral DNA, regardless of appearance of VP1 monomers, supporting a role for ERAD in ER exit into the cytosol.

To elucidate the nuclear entry mechanism, a nuclear localization signal (NLS) in the basic region of the C-terminus of both minor capsid proteins was first shown to be important for nuclear localization by site directed mutagenesis. The analogous mutation in the genome caused attenuation of infectivity based on an entry defect post-ER trafficking. The role of the NLS in the individual minor capsid proteins was addressed using pseudovirions containing mutations in one or both of the minor capsid proteins. Only mutation of the VP3-NLS led to an attenuated phenotype in 293TT cells. Overall these data are the first to show that BKPyV traffics through the cytosol and the minor capsid protein NLS is important during BKPyV cellular entry in RPTE cells.

CHAPTER I

Introduction

1. POLYOMAVIRUSES

History and Epidemiology

BK Polyomavirus (BKPyV), named for the initials of the patient from whom it was isolated, was one of two of the first human polyomaviruses discovered. BKPyV and the other human virus, JCPyV, were both discovered in 1971 in the urine and brain tissue, respectively, of two different immunocompromised patients (1, 2). Before these human polyomaviruses, only four other polyomaviruses were known: two from mice (mouse polyoma and K virus), one from rabbits (rabbit kidney vacuolating virus), and one originating from monkeys called Simian Vacuolating virus 40. SV40 had been discovered in 1960 as a contaminant in cell cultures used for the polio vaccine, bringing the possible consequences of the DNA tumor virus infection to the consideration of the scientific field (3, 4).

Since initial studies began on the first few polyomaviruses mentioned above, recent efforts toward new virus discovery have uncovered a plethora of new polyomaviruses from various species, including nine new human polyomaviruses. While these viruses share the distinct genome architecture to distinguish them as polyomaviruses, significant differences exist throughout the genome sequences, suggesting the loss or gain of different viral protein features (5). This aspect of polyomavirus evolution will be discussed more in Chapter 4. Polyomaviruses

have been discovered in urine, stool, blood, skin, lesions, or nasopharyngeal tissue and appear to be significant members of the microbiota, with seroprevalence in the population ranging from about 50 – 90% (6). While some were found in normal healthy samples, others were found associated with a disease state. Association with disease tissue does not automatically imply causality, but Merkel Cell polyomavirus (MCPyV) has been firmly linked with the rare but aggressive skin cancer Merkel cell carcinoma, making it the first clear oncogenic human polyomavirus (7). However, the oncogenicity of MCPyV relies on the rare event of viral genome integration into the host cell genome.

BKPyV has approximately 80-90% seroprevalence in the population. It is thought that transmission is likely through a respiratory route in early childhood, since viral DNA has been detected in tonsillar tissue and seroprevalence reaches 65-90% by the age of ten. Other potential routes of transmission have been proposed, including fecal-oral, urino-oral, transplancental, and through blood transfusion. Primary infection is believed to range from asymptomatic to a mild respiratory ailment (8). After initial infection, BKPyV establishes a persistent infection in the kidneys and urinary tract with sporadic replication that can be detected in the urine. It is unknown how the virus comes to persist in kidney cells and whether the viral genome undergoes true "latency" or simply replicates at a low level that is kept under control by an intact immune system. The latter theory is favored as periodic shedding of high virus titers can be detected in healthy persons.

There are two different forms of BKPyV that can be isolated from infected persons, and these forms include archetype virus and rearranged variants. The difference lies in their genomic structure. The circular BKPyV genome is made up of three distinct regions, including the early coding region, late coding region, and non-coding control region (NCCR) (Figure 1.1). The
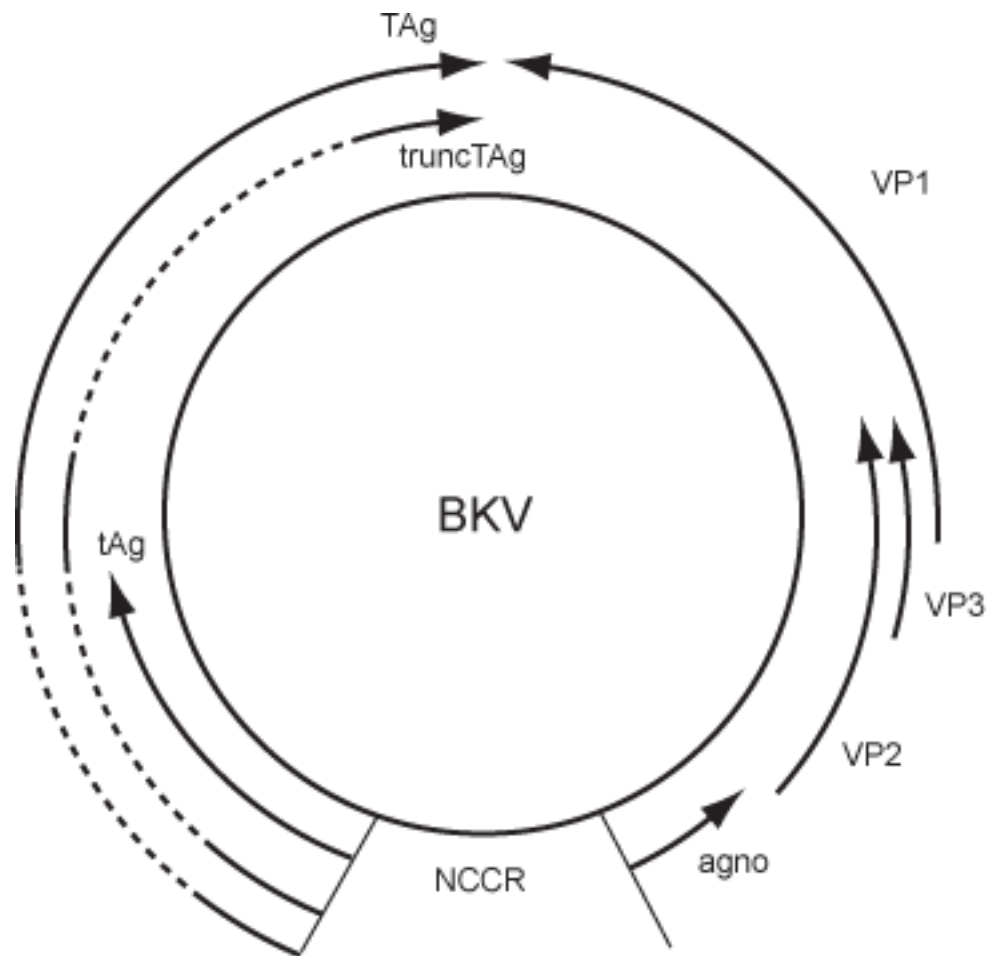
Figure 1.1. Representation of the Polyomavirus genomic structure. The non-coding control region (NCCR), containing the origin of replication and regulatory sequences, is located at the bottom of the diagram. The early region is located on the left and late region on the right. Regulatory proteins large T antigen (TAg), small t-antigen (tAg), and recently identified truncated TAg (truncTAg) are expressed form the early region, with agnoprotein (agno) and structural proteins VP1, VP2, and VP3 expressed from the late region.

rearranged variants have sequence variations distinctly found in the NCCR and are most commonly isolated from the blood serum of diseased patients only. Archetype virus is the most commonly isolated form of BKPyV and has a distinct sequence structure to the NCCR. Archetype is found in healthy as well as diseased patients, suggesting that archetype virus is the transmitted form of BKPyV (8).

BKPyV Structure

BKPyV is a non-enveloped virus, without a surrounding lipid bilayer, and consists of only a protein capsid and DNA genome. The particle is approximately 40 nm in size as determined by electron microscopy. The BKPyV particle consists mainly of three molecules that serve as the building blocks for the structure of the viral capsid: the major structural protein VP1 and less abundant minor capsid proteins VP2 and VP3 (see Figure 1.2). The capsid surrounds the 5 kb double stranded circular DNA genome that is complexed with histones in the form of a miniature chromosome. The outer shell of the virus capsid consists solely of VP1 proteins arranged in pentamers in a T=7 icosahedral lattice, and these pentamers are stabilized by intra- and inter-pentameric disulfide bonds and calcium cations. Sixty of the 72 pentamers, or capsomeres, are coordinated with six adjacent pentamers, and the other twelve of the 72 pentamers are located on the icosahedral vertices of the capsid, coordinated with five surrounding pentamers (9). The importance of these different arrangements within the VP1 shell is likely seen during the capsid disassembly process, as differing inter-pentameric interactions require stepwise uncoating, and this gradual disassembly is used during intracellular trafficking towards the nucleus (10).

On the internal face of each VP1 pentamer, not surface-exposed on the intact viral particle, is one molecule of either VP2 or VP3 (11). The minor capsid proteins are expressed from the same

late mRNA transcript and share a C-terminal amino acid sequence, but VP2 has a unique set of N-terminal amino acids that include a putative myristoylation site. Both SV40 and mouse polyomavirus (MPyV) have been shown to contain myristoylated VP2 (12). The stoichiometric ratio of VP2 to VP3 is not equal, and the functional significance of that ratio is unknown; however, it is believed that each of these minor capsid proteins plays a unique and vital role in cellular entry and assembly based what has been seen with SV40 (13, 14). Together, the features of this simple particle somehow play a role in both stabilizing the virus outside of the cell and enabling the virus to traverse the obstacle course of the cellular interior and eventually enter the nucleus, the site of replication.

2. BK POLYOMAVIRUS –ASSOCIATED DISEASE

As previously mentioned, a normal immune system will keep BKPyV replication at a minimum and prevent the onset of a diseased state. However, under conditions of immuno-deficiency or suppression, BKPyV reactivation in the kidney or urinary tract can go unrestricted and result in lytic infection and destruction of tissue. This occurs most often in bone marrow transplant patients and kidney transplant patients, but can also occur in other immunocompromised individuals including other solid organ transplant recipients, persons with acquired immune deficiency syndrome (AIDS), or sufferers of autoimmune diseases such as systemic lupus erythematosus (15).

One of the more common diseases caused by BKPyV infection is hemorrhagic cystitis (HC) , affecting 10-25% of bone marrow transplant patients sometime after 2 weeks post-transplantation. BKPyV-associated HC is verified based on the presence of decoy cells in the urine, which are urothelial cells with enlarged nuclei and intranuclear viral inclusions, and high

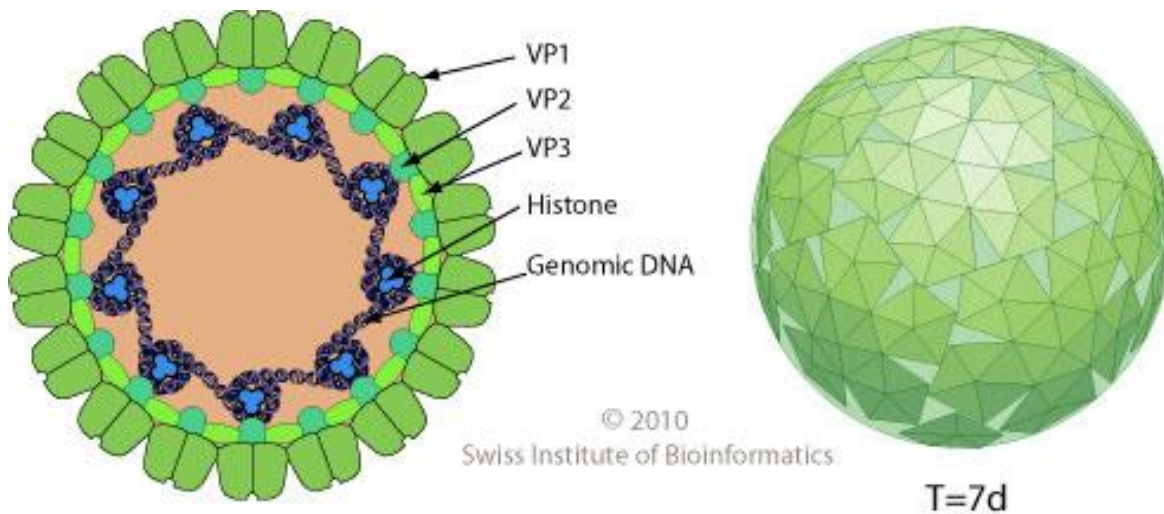**Figure 1.2**. Representation of the Polyomavirus capsid structure, based on SV40. The outer shell of the capsid consists of VP1 pentamers, creating a structure with T=7d icosahedral symmetry (right). Minor capsid proteins VP2 and VP3 reside underneath the pentamers, hidden from surface-exposure, along with the circular 5 kb histone-complexed genome (left). Figure taken from SIB Swiss Institute of Bioinformatics.

BKPyV viruria (or viremia). The symptoms include painful, bloody urination and the only treatment is palliative, with pain-relief, bladder irrigation, and transfusions in severe cases (15).

Another BKPyV associated disease is polyomavirus-associated nephropathy (PVAN), which can arise in kidney transplant patients approximately 10-13 months after renal transplant. PVAN effects up to 10% of kidney transplant patients and can cause graft loss in 90% of those afflicted. Because it occurs most often in the allograft, it is believed that it is typically brought about from a combination of immunosuppression and tissue injury that lead to uncontrolled replication of BKPyV, rather than immunosuppression alone (16). Symptoms of PVAN include increased serum creatinine levels, presence of viruria and decoy cells in the urine, and viremia. Biopsy of the infected kidney is the most straightforward detection of PVAN, in which intranuclear viral inclusion bodies in epithelial cells and virally induced tubular epithelial cell injury and lysis would be evident. Treatment for PVAN is palliative, and the most successful management includes reduction of the immunosuppressive regimen to allow for the host immune system to regain control of the infection (17).

Besides kidney and bladder epithelial cells, viral DNA has been found in other areas of the body and tissue types, including urogenital and brain tissue and peripheral blood mononuclear cells and spleen (16). These other sites of viral detection suggest that there may be an immune cell component in the dissemination of BKPyV. The significance of the presence of virus in these other tissues in terms of latency or disease, however, is unclear. On the other hand, light and electron microscopic evidence from biopsies of PVAN patients shows that BKPyV clearly undergoes lytic infection in renal proximal tubule epithelial (RPTE) cells in vivo (18, 19). Because of this, our lab has established an in vitro cell culture model using primary RPTE cells in order to study the virus in a natural host cell type (20).

3. VIRAL LIFECYCLE

Receptor Binding

In order to establish a persistent or lytic infection and cause disease, BKPyV must be internalized into a host cell type that is permissive to infection. This may include a number of different cell types and different tissues throughout the body. The main site of reactivation and replication, however, is within the kidneys and urinary tract, and thus the cell types commonly used for studies of cellular entry are cell lines or primary cells of renal epithelial origin. For a general overview of the lifecycle, see Figure 1.3.

Successful infection of a cell requires attachment to the cell surface and functional interaction with the surface receptor that will lead to internalization of the virus and targeting to a productive pathway. The presence or absence of a functional receptor often plays a determining role in the tropism of viruses. Initial studies to identify the nature of the BKPyV receptor clearly pointed to gangliosides as an important factor. Hemagglutination was observed in the very first characterization assays for BKPyV, and this could be inhibited by preincubating with gangliosides (21). The first studies were done with Vero cells, a monkey-derived kidney cell line, and were later repeated with the relevant cell culture model of human renal proximal tubule epithelial (RPTE) cells (22). Pretreatment of RPTE cells with neuraminidase, but not proteinase K, abolished binding and infection. Studies with RPTE cells verified the presence of a sialic acid component, and went on to identify the specific gangliosides GD1b and GT1b as binding partners in a sucrose flotation assay. Infection of non-susceptible cells is made possible by the exogenous addition of each of these gangliosides (23). Likewise, infection of RPTE cells with SV40 is not possible unless its ganglioside receptor, GM1, is added to the cells exogenously
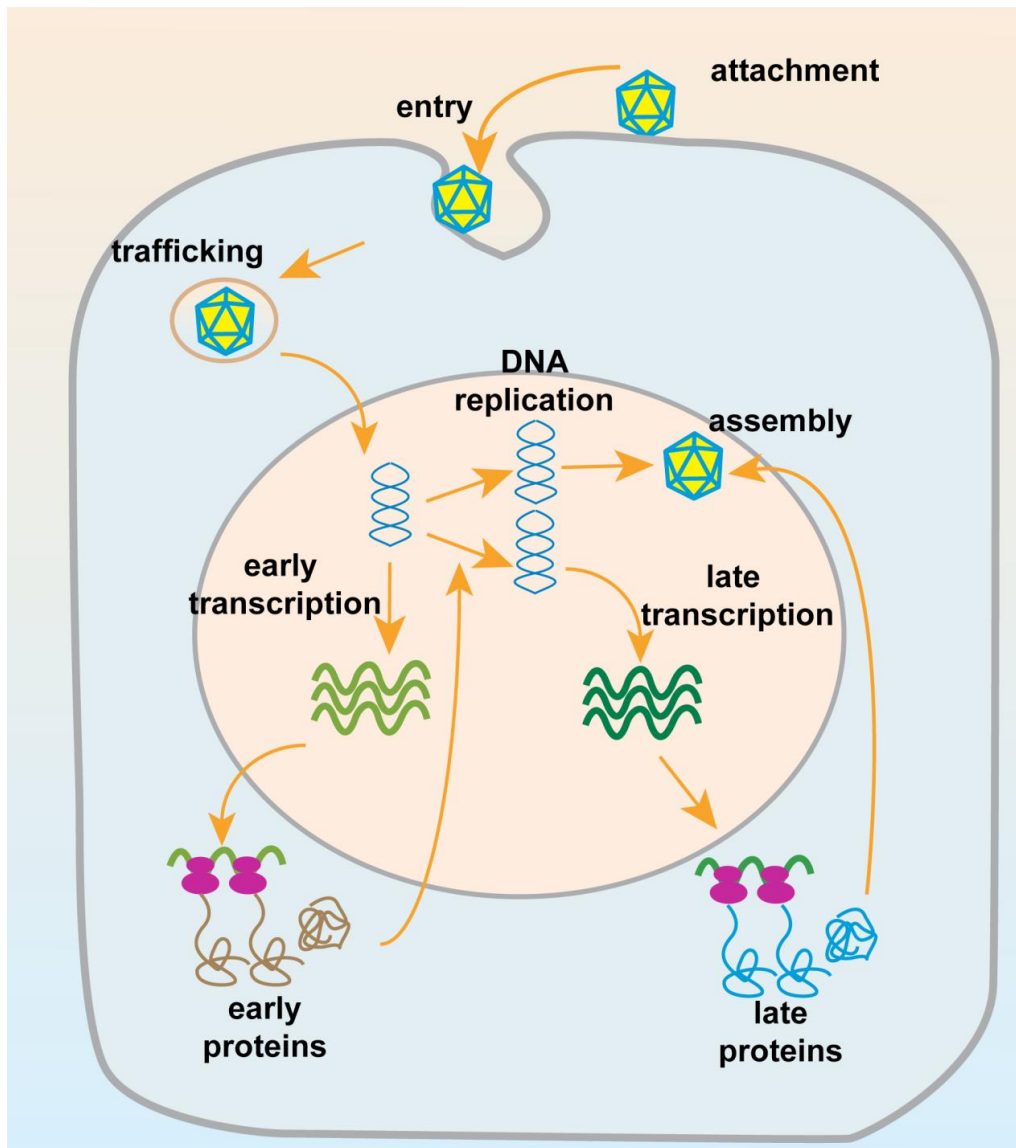
Figure 1.3. General overview of the BKPyV lifecycle from receptor binding to assembly. For details, see text. Figure created by Mengxi Jiang.

(22). A commonality between the GD1b and GT1b gangliosides is an α2-8-linked disialic acid motif, but whether this presents a potential site of interaction between receptor and capsid remains to be determined, and this will require the 3-dimensional structure of the capsid with the receptor.

Internalization and endocytosis

After binding its receptor, BKPyV must enter the cell and successfully traffic through the cytoplasm toward the nucleus, where the uncoated viral genome can utilize the cellular machinery for transcription and its genome replication. Early studies to determine the cellular entry pathway taken by BKPyV were based on transmission electron microscopy (TEM) of infected cells at different times post infection, and these images show virions taken into the cell by small, tight-fitting, non-coated vesicles, trafficking to smooth tubular structures contiguous with rough endoplasmic reticulum (ER), and entering the nucleus as relatively intact particles. Since single virus particles could be seen in the nucleus at a relatively early time before progeny would likely have been produced, and because they appeared smaller than virions at the plasma membrane, these were believed to be partially-disassembled incoming virions (24) (25).

Besides the information from TEM of BKPyV-infected cells, our understanding of the entry pathway of BKPyV was also initially based on inferences made from findings with SV40, and to some extent with the more distantly related mouse polyomavirus (MPyV), due to their earlier discovery and established cell culture models. However, it has recently become clear that interesting and significant differences exist between the biology of BKPyV and other polyomaviruses, including the role of different ganglioside receptors, as mentioned above. Thus,

more detailed analyses of the specific interaction between virus and host cell will be necessary to gain a deeper understanding of how BKPyV establishes an infection.

Following receptor binding, internalization of BKPyV is dependent on a caveolae-mediated endocytic pathway (26). This differs from what was found with JCPyV, which relies on clathrin-dependent receptor-mediated endocytosis (27). Caveolae are a type of specialized microdomain invagination in the plasma membrane that are enriched in cholesterol, and formation is dependent on caveolin proteins such as caveolin-1 (28). Caveolae-mediated entry by BKPyV was discovered from data showing that expression of a dominant-negative caveolin-1 construct decreased infection, and depletion of membrane cholesterol with methyl-B-cyclodextrin had an inhibitory effect, since caveolae are disrupted with cholesterol depletion (29, 30). A knockdown of caveolin-1 also showed its role in BKPyV infection in RPTE cells (29). Additionally, expression of an Eps15 dominant-negative construct, which prevents clathrin-dependent endocytosis by targeting a necessary component in assembly of clathrin pits (31), did not prevent infection in Vero cells (32). Verification of the pathway was also confirmed when co-localization of fluorescently labeled virions with cholera toxin B, which serves as a marker of caveolae endocytosis, was observed during synchronized entry (29, 30) (32). Additionally, BKPyV allograft nephropathy analyzed by EM shows virions close to the plasma membrane in 60-70 nm smooth vesicles whose description is consistent with that of caveolae, confirming this vesicular route in a natural infection setting [51].

Studies show internalization to be relatively slow and nonsynchronized (32, 33). The details of the endocytic pathway taken by BKPyV has not yet been defined. However, it is known that SV40 depends on the classical endocytic route through early and late endosomes towards endolysosomes and relies on acidification and maturation of endosomes for entry (34). It is likely

the route taken by BKPyV as well, and it has been shown that BKPyV requires an acidification

step very early during entry in RPTE cells (33). Although JCPyV enters via a clathrin-mediated

route, it is taken into early endosomes like SV40, and the virus is then directed to an unidentified

compartment by a caveolin-1 dependent step (35). These studies together show that cross-talk

between endocytic pathways allows for differences in the initial entry steps of receptor-mediated

binding and endocytosis between each virus, while later intracellular trafficking steps are likely

more parallel as the virus reaches the ER (36).

Approaches using various pharmacological treatments of infected cells have established the

need for microtubules during trafficking, but apparently not actin (37). Treatment with the drug

nocodazole, which destabilizes microtubules, was found to inhibit infection at an early time

point, as well as tests with the microtubule destabilizing agent colcemid in RPTE cells. (33, 38)

Paclitaxel, a microtubule stabilizer, inhibited infection in RPTE cells as well, which was

surprisingly different from what was seen in Vero cells, and supports the relevancy of an *in vitro*

primary host cell model. Another study addressed the role of dynein, the microtubule motor

protein, using the drug EHNA (erythro-9-[3-(2-hydroxynonyl)] adenine) that inhibits the ATPase

of the dynein complex, and showed independence of BKPyV infection from dynein in RPTE

cells (39). Interestingly, however, this study supports the idea that the Golgi apparatus is

bypassed during the retrograde trafficking route of BKPyV, since EHNA disrupts the

morphology of the Golgi apparatus but did not affect BKPyV infection.

ER trafficking and Virus Uncoating

After following an endocytic trafficking route, the virus reaches the ER. Trafficking through

the ER seems to be a necessary step for all polyomaviruses. This aspect of polyomavirus entry is

unique among DNA viruses, which raises the question of what benefit the ER provides during entry. One main advantage within the compartment of the ER is that it contains chaperones, disulfide isomerases, and reductases of which the virus can make use to facilitate the capsid uncoating and disassembly process. Evidence from SV40 and MPyV points toward involvement of the ER-localized protein disulfide isomerase (PDI) family in providing the reduction and isomerization of the VP1 capsid proteins that provides an initial and vital step in the uncoating process (33, 40, 41). The interactions and subsequent conformational changes that occur within the ER compartment likely also provide the means for the virion to continue on its pathway into the nucleus.

So far, a number of ER resident host factors that are involved in SV40 and MPyV infection have been identified, providing a theoretical framework for the interactions of BKPyV during ER trafficking and uncoating. It was found for MPyV that the PDI family member ERp29 causes a conformational change in VP1, shown by exposure of a trypsin-sensitive site, and that this results in the viral particle being able to interact with a lipid bilayer (42). Other PDI family members PDI, ERp57, and ERp72 were later shown to work in concert for reduction and isomerization of specific disulfide bonds within the MPyV capsid, and these changes are necessary for uncoating [68]. Schelhaas *et al*. had shown that the same scenario and host factors are important for SV40. MPyV was also found to require the ER transmembrane protein Derlin-2 for infection (41, 43, 44).

ER-associated degradation

Involvement of members of the Derlin-family proteins provides an interesting link between infection and the process of ER-associated degradation (ERAD), a cellular quality control

mechanism for sending misfolded proteins out of the ER into the cytosol where they are degraded by the proteasome (45). Derlins have been found to be important for infection with both MPyV and SV40, as well as BKPyV. Derlin-1 was found to be essential for BKPyV infection by transiently expressing a dominant negative construct during infection, and was implicated in SV40 infection through siRNA-mediated silencing (33). A role for the proteasome has also been shown during BKPyV infection, as well as for SV40, and this suggests the importance of the ubiquitin-proteasome system during entry, which is part of ERAD (40, 46).

The mechanism and regulation of the ERAD pathway is an active area of investigation in the field of cell biology. The basic function of the ERAD pathway is to recognize newly translated secretory proteins in the ER that do not undergo proper folding, or it can be used in a regulatory capacity (47). Common features of the pathway shared by all substrates include recognition of the substrate, shuttling of the substrate to the retrotranslocation machinery at the ER membrane, retrotranslocation of the substrate across the membrane and into the cytosol, ubiquitination, and degradation by the proteasome. The degradation of ER proteins was initially believed to occur by an unknown protease within the ER lumen, but later it was discovered that the proteasome, located in the cytosol, was responsible for the degradation of aberrant proteins. This finding was intriguing since it raised an interesting topology question of how the ER-resident proteins made it across the membrane to the cytosol and the proteasome. To date, it is still uncertain if there is a specific protein that serves as a retrotranslocation pore, or if a channel is formed by an assembly of transmembrane proteins. Derlin-1 has been proposed to serve as the channel (48), as has an E3 ligase Hrd1 (49), and the translocation channel Sec61 (50). It has also been proposed that different substrates may use different channels or mechanisms of retrotranslocation. Besides the putative channel components, many lectins and chaperones are involved in the process of

handling and targeting different ERAD substrates. The mechanism and components of the endogenous ERAD pathway are still being described, and many questions remain to be answered.

A few recent studies have identified components of the ERAD machinery to be involved during SV40 trafficking. These studies were able to demonstrate directly that viral trafficking from the ER to the cytosol was inhibited upon disrupting the ER chaperones BiP and its interacting partner DNAJB11, as well as the transmembrane shuttle BAP31 (51, 52). The minor capsid proteins VP2 and VP3 were also shown to be required for this retrotranslocation across the ER membrane by using a pseudovirus consisting of a VP1-only capsid, which was unable to enter the cytosol (51). It has yet to be elucidated what mechanisms are used for BKPyV trafficking from the ER to the nucleus, but evidence suggests that use of the ERAD-associated factors to enter the cytosol is conserved among polyomaviruses.

Nuclear Entry

The pathway used by BKPyV to get into the nucleus is currently unknown. Evidence for SV40 suggests that the virus may use the canonical route through the nuclear pore complex. Since previous work with SV40 implies a model where the virus traffics through the cytosol, it is reasonable to hypothesize that the virus would then use the canonical nuclear import pathway through the nuclear pore for nuclear entry. The full sized virion at ~45 nm (53) would be too big to fit through the nuclear pore, but partial disassembly could allow it to pass through if smaller than 39 nm (54). A nuclear localization signal is located on each of the capsid proteins but none are exposed in the intact particle, so partial disassembly that occurs in the ER and/or cytosol

would be a likely prerequisite for the canonical use of this pathway, especially since evidence

from SV40 suggests that the minor capsid proteins are required for nuclear entry (14).

Canonical Nuclear Import Pathway

Since viruses often co-opt existing cellular pathways, a model where polyomaviruses use the

canonical nuclear import pathway is favored, since this would follow cytosolic trafficking. The

nuclear pore complex is an enormous assembly of proteins that spans both the inner and outer

nuclear membranes. The pore itself consists of 30 different proteins in multiple copies, making

up a macromolecular complex of 500-1000 total subunits (55). The pore size seems to be

flexible, with the largest observed opening at about 39 nm (54). Small molecules and ions can

diffuse through passively, but larger molecules use a string of basic amino acids that make up a

"signal sequence" recognized by specific shuttling proteins that bring it through the pore

complex. It is still unclear exactly how the protein complexes are actively taken through the

pore, but one favored model involves an affinity gradient, where the favorability of the

interactions between substrate and pore proteins continually increases as it goes through the

channel (53, 56).

The classic import pathway first involves recognition of the signal sequence, or NLS, by the

adapter protein importin alpha. Once importin alpha recognizes and binds to an NLS, another

shuttle protein, importin beta, binds importin alpha and takes the complex through the nuclear

pore. Once inside the nucleus, importin beta interacts with RanGTP to release the cargo (53).

Nakanishi *et al.*(57-59) have provided a notable amount of evidence for the use of the canonical

nuclear import pathway by SV40 for nuclear entry through the nuclear pore. SV40 DNA can be

co-immunoprecipitated with importin alpha and beta, and the importins can be co-

immunoprecipitated with VP3, and only during time points early in infection (58, 59). They also

showed that the nuclear localization signal (NLS) shared by the minor capsid proteins VP2/VP3

is vital for nuclear entry, implying that the signal becomes exposed during infection and is used

for nuclear import through the pore (58, 59). Interestingly, it has also been shown that the

nuclear localization signal on VP2 and VP3 is unnecessary for nuclear import in the presence of

functional VP1, since VP1 seems to bring the VP2 and VP3 into the nucleus during production

of progeny virions.

Besides nuclear import, another possible nuclear entry mechanism is for the virus particle to

traverse the inner nuclear membrane directly from the ER, and there are some pieces of evidence

suggesting that this pathway may exist. One recent paper on SV40 shows that there are small

changes in the nuclear envelope and transient perturbations in the nuclear lamina during early

times in infection, and these are associated with cleavage of lamin A/C (60). Another

ultrastructure-based investigation of BKPyV-infected kidney tissue showed some malformations

in the nuclear envelope adjacent to accumulations of virions, and no virions were found near the

nuclear pore (18). Since these ultrastructural observations were made in diseased tissue,

however, it is difficult to determine the stage of the lifecycle occurring in each cell, and whether

the observations are representative of a typical infectious route. To date, there is no evidence

against either of these possible pathways, and there is no indication that they are mutually

exclusive nuclear entry models.

Regulatory proteins

Once the uncoated viral genome is inside the nucleus, BKPyV takes advantage of nuclear

factors to allow the temporal expression of its viral genes. The three regulatory proteins, Large

Tumor (T) Antigen (TAg), small T antigen (tAg), and truncated T antigen (truncTAg) are expressed first, followed by initiation of replication, and then late gene expression [10]. The early and late promoters are located in the NCCR, in opposite orientations like their coding regions, on the 5 kb genome. The NCCR additionally contains the origin of replication, and multiple binding sites for regulatory factors of cellular origin have been identified within this regulatory region (61). The early promoter drives expression of a primary transcript, which is alternatively spliced to allow for expression of the three early proteins. Once the regulatory function of TAg is active, DNA replication begins, followed by expression of the late proteins VP1, VP2, VP3, and agnoprotein (10).

TAg is an impressively multifunctional regulatory protein, responsible for directly interacting with the viral genome to initiate DNA unwinding, as well as exhibiting functions that allow expression of the cellular DNA synthetic machinery that can support viral replication. These functions include binding to the retinoblastoma (Rb) protein and its family members (p107 and p130) to alleviate E2F-mediated transcriptional repression, as well as binding to p53 and inhibiting its downstream tumor suppressor targets to block apoptosis and allow maximal production of viral progeny (62, 63). The tAg shares an N-terminus with TAg, and thus shares the interactions and functions mediated by the domains located in the N-terminus, such as the J-domain (64). One unique function of tAg is its inactivation of protein phosphatase 2A via formation of a complex with the catalytic and regulatory subunits. This leads to increased cyclins D1 and A, and downregulated p27, which are factors in the control of cell cycle progression (65-67). Any unique functions of the truncTAg protein have yet to be elucidated; however, it shares 133 N-terminal amino acids with TAg followed by three unique amino acids, and thus likely serves some redundant roles for TAg functions (68).

The cell cycle-altering functions of the regulatory proteins confer oncogenic properties to BKPyV; however, no convincing evidence of oncogenic transformation has been found in relation to BKPyV infection of human tissue. Injection of BKPyV into rodents does lead to formation of multiple tumors, prompting the question of what host factor interactions lead to specific outcomes during infection (69). The viral genome remains episomal in human cells after infection, while in rodent cell lines it undergoes integration into the cellular DNA (61). Identification of differences in the molecular mechanisms at work in the nuclei of each species may provide insights into the basis for maintenance, latency, and lytic replication.

Replication

After synthesis of the regulatory proteins and establishment of the DNA synthesis-active cellular environment, replication of the viral genome occurs. Replication begins at the origin of replication that sits within the NCCR. TAg binds to the viral origin and assembles into a double hexamer, which leads to its hydrolysis of ATP, initial unwinding of the viral chromatin through its helicase activity, and recruitment of the cellular DNA polymerase α/primase complex (69). Synthesis occurs in both directions from the replication origin until the replication forks meet on the opposite side of the genome. Besides TAg, the minimal molecular requirements for the DNA replication of SV40 include RPA, DNA polymerase α/primase, topoisomerase I, RFC, DNA polymerase delta, and PCNA; this provides a likely scenario for the minimal requirements of BKPyV as well (70).

Replication of archetype BKPyV has previously been a problem to study in cell culture models. Recently, however, Broekema and Imperiale showed propagation of archetype BKPyV in 293TT cells, a human embryonic kidney cell line that overexpresses SV40 TAg (71). The

abundance of this exogenous regulatory factor was able to compensate for the usually undetectable level of BKPyV TAg expression from the archetype virus. Rearranged NCCR BKPyV variants grow readily, and thus far these variants have been used as the experimental laboratory system. The archetype BKPyV NCCR is characterized by a linear O-P-Q-R-S block structure, where O is named for the origin of replication and the blocks that follow are named arbitrarily. The early and late region promoters are located in the P-Q-R-S region. Rearranged variants are characterized by deletions and duplications of these blocks of sequence within the NCCR. It has yet to be determined which differences between the archetype and rearranged NCCR variants affect replication ability, since archetype is usually the form shed from a host. It is possible that the cell type permissive for archetype has not yet been discovered. Some investigations have addressed the levels of replication and progeny production in different NCCR variants, but little information nor a pattern has emerged as to which of the arbitrary blocks, duplications, or deletions affect the ability of BKPyV to replicate in cell culture (61, 72, 73).

Robust late mRNA expression for production of the structural proteins occurs after the regulatory proteins have been produced and begun initiation of DNA replication (61). The capsid proteins VP1, VP2, and VP3 are produced in the cytoplasm and imported into the nucleus, where virion assembly occurs. Progeny virus particles form when capsomeres assemble around newly synthesized genomes. This assembly can also incorporate plasmids of non-viral origin or small cellular DNAs. Assembly can also result in empty particles that contain no nucleic acid (74). New progeny virions accumulate within the nucleus and can be seen clearly by TEM, often in a crystalline lattice arrangement, as a sizeable nuclear inclusion. The means of viral egress for polyomavirus is not clear; however, it had been believed that profuse accumulation of viral

progeny particles within the nucleus cause engorgement and strain on the nuclear envelope and eventual lysis of the cell (24). Recent studies on SV40 have identified a fourth structural protein protein expressed late in infection called VP4 that can form pores in cellular membranes, suggesting a mechanism for egress of SV40 progeny (75, 76). A homologous VP4 protein has not been identified for BKPyV, and although VP2 and VP3 may have membrane-lytic properties that could contribute to cell lysis late in infection, this has not been observed.

4. SUMMARY OF CHAPTERS

A significant question in the field of nonenveloped virus entry is the question of how protein capsids cross the limiting membrane that allows them eventual access to the nuclear machinery. By understanding more details of how BKPyV crosses the ER membrane and enters the nucleus, particularly in a natural host cell type, I hope that a greater understanding of nonenveloped virus trafficking in general can be gained.

The objectives of this thesis work were to further elucidate the intracellular trafficking pathway of BKPyV in RPTE cells. In Chapter 2, I show that the ERAD pathway is involved during infection, and blocking the pathway leads to accumulation of virus in the ER in a quality control subcompartment specific for ERAD substrates. I also showed that BKPyV indeed traffics through the cytosol and that proteasome inhibition leads to a decrease in viral DNA in the cytosol. Chapter 3 shows that a nuclear localization signal is located on the C-terminus of VP2 and VP3 and that this signal is important for optimal entry of the virus during infection, although alternative nuclear entry mechanisms appear to exist as well. In Chapter 4, I discuss the implications of these findings and future investigations that can be done to follow up on our current model of polyomavirus trafficking.

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CHAPTER II

Role of Cell Type-Specific ER-associated Degradation in Polyomavirus Trafficking

BK Polyomavirus (BKPyV) is a widespread human pathogen that establishes a lifelong persistent infection and can cause severe disease in immunosuppressed patients. BKPyV is a nonenveloped DNA virus that must traffic through the endoplasmic reticulum (ER) for productive infection to occur; however, it is unknown how BKPyV exits the ER before nuclear entry. In this study, we elucidate the role of the ER-associated degradation (ERAD) pathway during BKPyV intracellular trafficking in renal proximal tubule epithelial (RPTE) cells, a natural host cell. Using proteasome and ERAD inhibitors, we show that ERAD is required for productive entry. Altered trafficking and accumulation of uncoated viral intermediates was detected by fluorescence *in situ* hybridization and indirect immunofluorescence in the presence of inhibitor. Additionally, we detected a change in localization of partially uncoated virus within the ER during proteasome inhibition, from a BiP-rich area to a calnexin-rich subregion, indicating BKPyV is accumulating in an ER subcompartment. Furthermore, inhibiting ERAD did not prevent entry of capsid protein VP1 into the cytosol from the ER. By comparing the cytosolic entry of the related polyomavirus SV40, we found that dependence on the ERAD pathway for cytosolic entry varied between the polyomaviruses and between different cell types, namely, immortalized CV-1 cells versus primary RPTE cells.

Introduction

BK Polyomavirus (BKPyV) is a human pathogen that is ubiquitous throughout the population. Studies show that up to 90% of adults are seropositive for BKPyV, which is believed to infect individuals during early childhood and establish a persistent subclinical infection for the lifetime of the host (1). While BKPyV does not usually cause disease in healthy individuals, it can lead to severe disease in immunocompromised patients, particularly in bone marrow and kidney transplant patients. Under conditions of immunosuppression, reactivation of BKPyV in the bladder or kidney causes hemorrhagic cystitis or polyomavirus-associated nephropathy (PVAN), respectively. There are currently no effective antivirals against BKPyV, and the current treatment protocol is palliative, or, in renal transplant patients, reduction of immunosuppressive therapy, leaving the patient vulnerable to graft rejection. Graft loss occurs in up to 50% of cases of PVAN (2), either due to the virus or rejection. Before useful antiviral drugs can be developed, a deeper understanding of the BKPyV life cycle is necessary, including the details of intracellular entry. These early interactions between BKPyV and the host cell have yet to be fully elucidated.

In the interest of studying BKPyV in a relevant biological setting, our lab previously established a cell culture model of BKPyV infection using primary renal proximal tubule epithelial (RPTE) cells (3). This is based on the observation of histologic sections and transmission electron micrographs of PVAN patient biopsies, indicating lytic infection by BKPyV in RPTE cells (4-6). We have shown that the intracellular trafficking pathway of BKPyV in RPTE cells begins with binding to the ganglioside receptors GT1b and GD1b, followed by internalization and a pH-dependent step within the first two hours after adsorption. The virus subsequently relies on microtubules (7-9) and traffics through the endocytic pathway

to the ER, where it arrives approximately 8 hours post infection (hpi) (9). Sometime after ER trafficking but before 24 hpi the virus enters the nucleus, where transcription of early regulatory genes occurs, followed by DNA replication and late gene expression. It is unknown, however, how BKPyV gets from the ER to the nucleus. Two possible routes have been proposed: the virus can cross the inner nuclear membrane directly from the ER lumen, or the virus can cross the ER membrane into the cytosol from where it can subsequently enter the nucleus, likely via the nuclear pore complex.

In order for the BKPyV genome to undergo replication and transcription in the nucleus, it must be uncoated and released from the viral capsid. The BKPyV capsid structure consists of three proteins, VP1, VP2 and VP3. The major capsid protein, VP1, oligomerizes into pentamers during virion production and makes up the outer shell of the particle, with 72 pentamers stabilized by inter- and intra-disulfide bonds (10). It is believed that these disulfide bonds become reduced and/or isomerized by host disulfide reductases and isomerases when the virus infects a naïve cell and traffics through the ER (9, 11). One molecule of either minor capsid protein, VP2 or VP3, is associated with each pentamer, and is concealed by VP1 from antibody detection until disassembly begins in the ER (12, 13).

Evidence from previous studies has implicated a role for components of the ER-associated degradation (ERAD) pathway during infection of polyomaviruses (14-17). ER quality control mechanisms of the cell include the ERAD pathway as a means by which secretory proteins in the ER that cannot attain their proper conformation are sent into the cytosol and degraded by the proteasome (18). The feature of ERAD that makes it an enticing host pathway for a non-enveloped virus to co-opt is that it provides a mechanism for ER-localized proteins – in this case the viral particle – to be sent across the ER membrane into the cytosol. ERAD depends on an

intricate collection of chaperones and transmembrane proteins that recognize a misfolded protein, target and shuttle the protein to a retrotranslocation complex, translocate the substrate across the ER membrane into the cytosol where it is ubiquitinated, and send it to the proteasome for degradation (18). One set of ERAD translocation complex proteins, the Derlin family, has been found to be necessary for mouse polyomavirus, SV40, and BKPyV, through experiments with siRNA knockdowns or dominant-negative constructs (9, 14, 17). Proteasome function has also been shown to be necessary for both SV40 and BKPyV infection (9, 14), and treatment with the proteasome inhibitor lactacystin resulted in an increase in disassembly products of the BKPyV capsid (9, 14), suggesting that blocking proteasome function may alter the disassembly and entry pathway of BKPyV.

It was the goal of this study to further investigate the entry route of BKPyV during and after ER trafficking, focusing on the role of the ERAD pathway and the proteasome. This is the first study to examine polyomavirus ER-to-cytosol trafficking in the context of a natural host cell. We show that the proteasome and ERAD system do play a role in the trafficking of BKPyV, as specific inhibitors block an early step during infection. We also show that proteasome inhibition causes a change in the ER localization of BKPyV and an accumulation of detectable VP2/3 protein, indicating disassembled particles in the ER. Additionally we provide evidence that BKPyV enters the cytosol. Interestingly, however, we show that proteasome and ERAD inhibitors do not prevent BKPyV VP1 protein from entering the cytosol. Moreover, our data suggest that differences exist in the trafficking of polyomaviruses in different cell types, as seen with both BKPyV and SV40 in RPTE cells as compared to CV-1 cells. Altogether this report provides new evidence that the ERAD pathway is involved during BKPyV entry, and that there are important differences in the trafficking and entry of polyomaviruses in different cell types.

Results

Productive infection requires the proteasome and ERAD pathway.

Because previous studies indicated that the proteasome may play a role during entry of BKPyV (9, 14), we became interested in further clarifying the function of the proteasome and whether the ERAD pathway is involved during infection. To examine whether the proteasome was involved in an early step during infection coincident with transit through the ER, a time course of drug treatment was performed to determine when infection would no longer be sensitive to proteasome inhibition. We used epoxomicin, which is a potent inhibitor of the chymotrypsin-like activity of the proteasome and also blocks the trypsin-like and peptidyl-glutamyl peptide hydrolyzing activities (19). RPTE cells were subjected to synchronized infection with BKPyV, and samples were treated with epoxomicin at different time points post adsorption, beginning with 0 h. Whole cell lysates were harvested at 24 hpi, resolved by SDS-PAGE, and immunoblotted for the early protein, TAg, as a readout for genome delivery to the nucleus. Compared to untreated cells, samples with epoxomicin added at time points until 18 hpi were inhibited for infection by BKPyV (Figure 2.1, top). These data indicated that BKPyV no longer required proteasome function after approximately 18 hpi. These kinetics fit within the range of time between ER trafficking and nuclear entry; BKPyV traffics through the ER between 8 hpi and 16 hpi (9), and early gene expression can be detected beginning at about 24 hpi (3).

Since the proteasome can function independently from the ERAD system, we decided to address the role of the ERAD pathway during early infection with the ERAD inhibitor, Eeyarestatin I (20). Eeyarestatin I targets the AAA-ATPase p97 that lies upstream of the
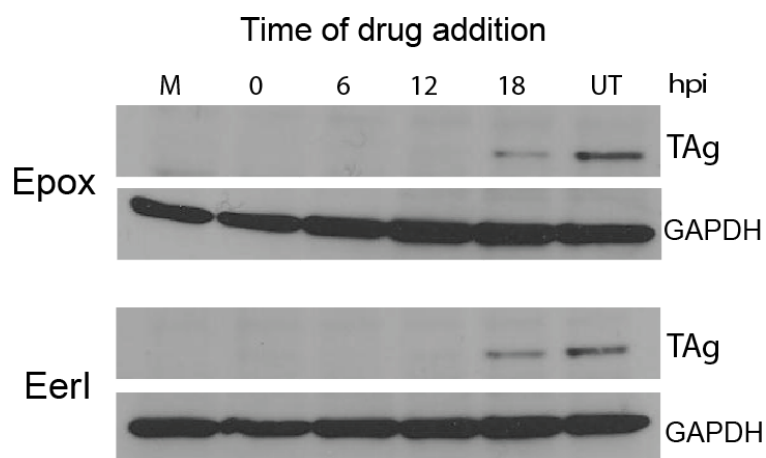
35

Figure 2.1: Timecourse of proteasome and ERAD pathway involvement during infection. RPTE cells were infected at 5 IU/cell at 4° C for 1 hr, moved to 37°C and treated at the indicated timepoints with either 10 μM epoxomicin (Epox) or 10 μM Eeyarestatin I (EerI). M = mock infected, UT = untreated. Whole cell lysates were harvested at 24 hpi, resolved by reducing SDS-PAGE, and probed for TAg and GAPDH. Similar results were obtained for at least 3 independent experiments.

proteasome as a cytosolic component of the ERAD pathway and is thought to provide the driving energy for extraction of ERAD substrates from the ER (21). Interestingly, similar inhibition kinetics were seen with Eeyarestatin I as with epoxomicin, a block in TAg expression until 18 hpi (Figure 2.1, bottom), which supports the possibility that the proteasome may be acting as part of the ERAD pathway to allow BKPyV to exit from the ER. Together these data support a role for the proteasome and ERAD during entry of BKPyV, at a step between ER trafficking and nuclear entry.

Trafficking of BKPyV is altered by proteasome inhibition.

To corroborate results from the drug treatment timecourse, we employed an imaging approach to assess the effects of ERAD inhibition on BKPyV trafficking. RPTE cells were infected with BKPyV and then treated with epoxomicin or the vehicle DMSO at 6 hpi. Cell viability was not affected under these conditions. BKPyV DNA was detected by FISH with a fluorescently labeled probe that recognizes the viral genome. At 24 hpi, FISH signals appeared as puncta throughout the cell, with some signal adjacent to the nucleus. In the presence of epoxomicin, there was a change in the pattern of FISH signals: we saw a slight but reproducible increase in the average area of the puncta, representing an accumulation of the virus, in a juxtanuclear position (Figure 2.2A).

FISH staining labels all viral genomes within the cell, regardless of whether they are following a productive or non-productive pathway. Because of this ambiguity, we became interested in using a viral marker that more likely represents virus that is following a productive infectious pathway. As polyomavirus travels through the ER, the capsid begins to disassemble and rearrange (9, 14). The minor capsid proteins VP2 and VP3 that are normally hidden
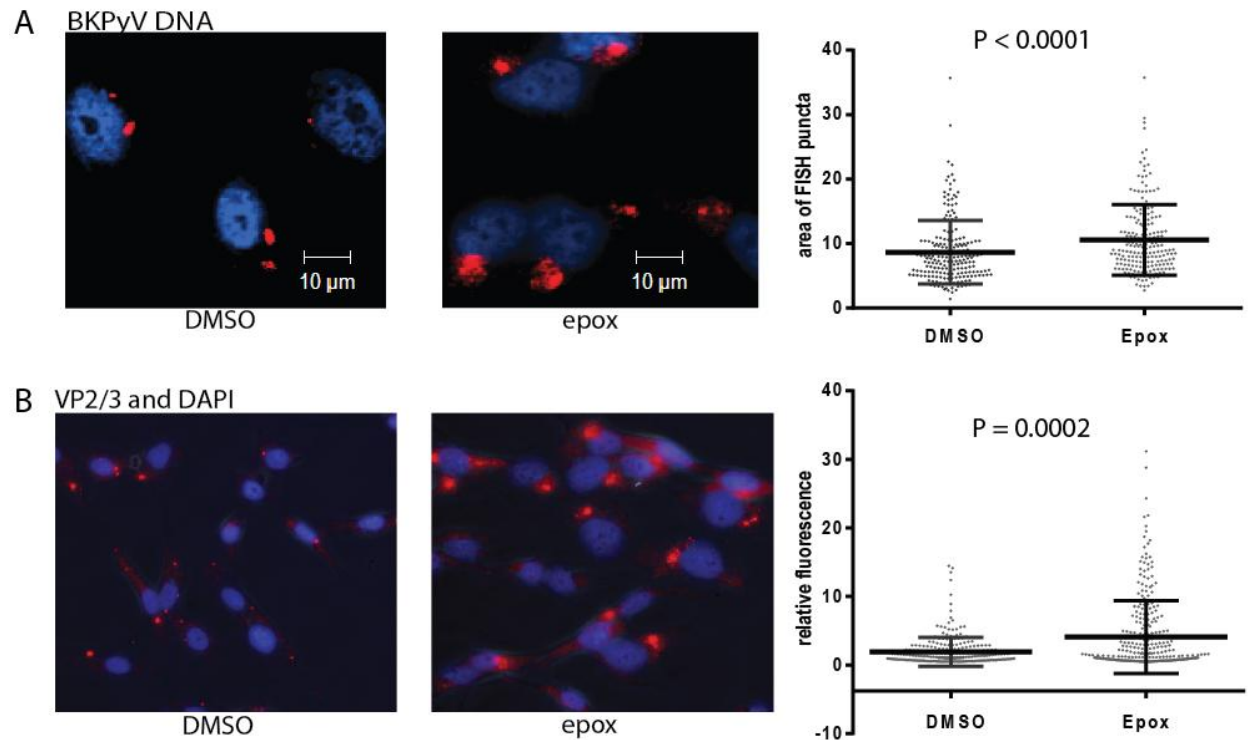
Figure 2.2: BKPyV trafficking is altered when ERAD is inhibited. RPTE cells were infected at 5 IU/cell at 4°C for 1 hr then moved to 37°C. Cells were treated with 10 μM epoxomicin or DMSO at 6 hpi and fixed at 24 hpi. (A) BKPyV was stained by FISH against the viral genome and nuclei were stained with DAPI and then imaged by confocal microscopy. Relative size of fluorescent areas was measured using ImageJ software on >260 puncta from each conditon collected from three independent experiments. (B) Fixed cells were stained for VP2/3 and DAPI and imaged by fluorescence microscopy. Quantitation of relative fluorescence was performed on VP2/3 staining alone per cell using ImageJ software on a total of 200 cells from three independent experiments. Values are corrected total cell fluorescence to normalize for cell size. Statistical analysis in A and B was performed by independent samples T-test using GraphPad Prism software. Scatter plots show each data point value along with the mean and standard deviation.

beneath the outer layer of VP1 become exposed and available for interactions with antibodies (13). Since VP2/3 exposure represents virus that has at least entered the ER and undergone some disassembly, an important prerequisite for infection, we decided to use VP2/3 staining to observe the effects of ERAD inhibitors on viral disassembly. RPTE cells were infected at an MOI of 5 IU/cell for 24 h, with epoxomicin added at 6 hpi, and fixed and labeled by indirect immunofluorescence against VP2/3. Compared to cells treated with DMSO, there was an increase in the intensity of VP2/3 staining in epoxomicin-treated cells (Figure 2.2B). Exposure of VP2/3 to antibody recognition was prevented by Brefeldin A (BFA) treatment, which inhibits ER trafficking of the virus (Figure 2.3). These data showed that proteasome inhibition does not inhibit uncoating, but seems to inhibit a later trafficking step such that it leads to some accumulation of uncoating intermediates.

We hypothesized that if the proteasome was acting in the ERAD pathway, and the ERAD pathway was necessary for BKPyV trafficking out of the ER, then proteasome inhibition may lead to incoming virus becoming trapped within the ER. To address the localization of partially uncoated virus with the ER, we co-stained for VP2/3 and the ER marker BiP. We found that in both untreated cells and epoxomicin-treated cells there was a range of colocalization between VP2/3 and BiP at 24 hpi, between 0 to 100% per cell, with the median colocalization in untreated cells at 82% (Figure 2.4A). Interestingly, however, we noticed that with epoxomicin treatment the pattern of both VP2/3 and BiP staining changed: distinct areas became devoid of BiP, VP2/3 puncta became more localized to a juxtanuclear area, and median colocalization decreased to 53% (Figure 2.4A). Interestingly, a number of reports have described an ER-associated quality control compartment (ERQC) where ERAD machinery becomes concentrated and substrates accumulate (22-24). This subcompartment is induced or exaggerated upon proteasome inhibition,
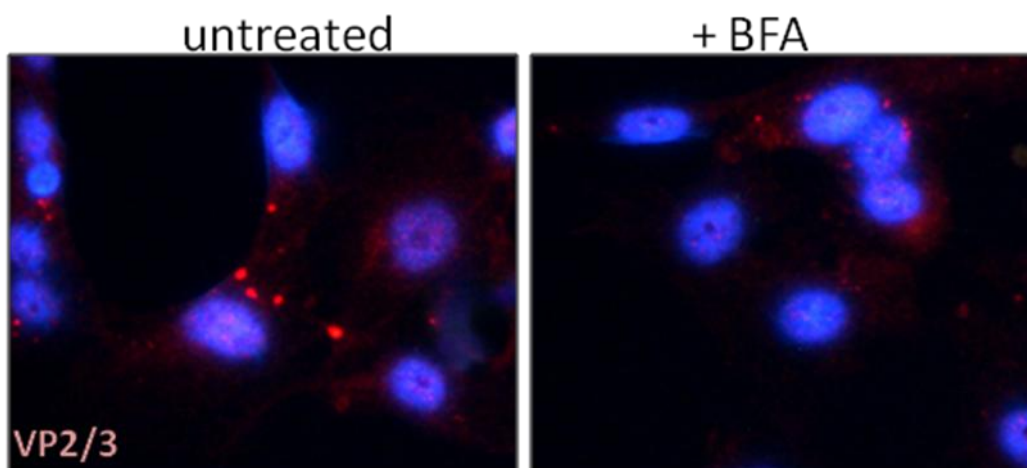
Figure 2.3: VP2/3 exposure is prevented when ER trafficking is inhibited. RPTE cells were infected at MOI 5 IU/cell, treated with BFA at 0 hpi or left untreated, and fixed at 24 hpi. Fixed cells were stained for VP2/3 and DAPI and images were taken with a fluorescence microscope.

A

% colocalization between VP2/3 and BiP

P = 0.0065

DMSO   Epox

VP2/3   BiP   merge

DMSO

epox

10 µm

10 µm

B

% colocalization between VP2/3 and cnx

P = 0.0006

DMSO   Epox

VP2/3   calnexin   merge

DMSO

epox

10 µm

10 µm

41
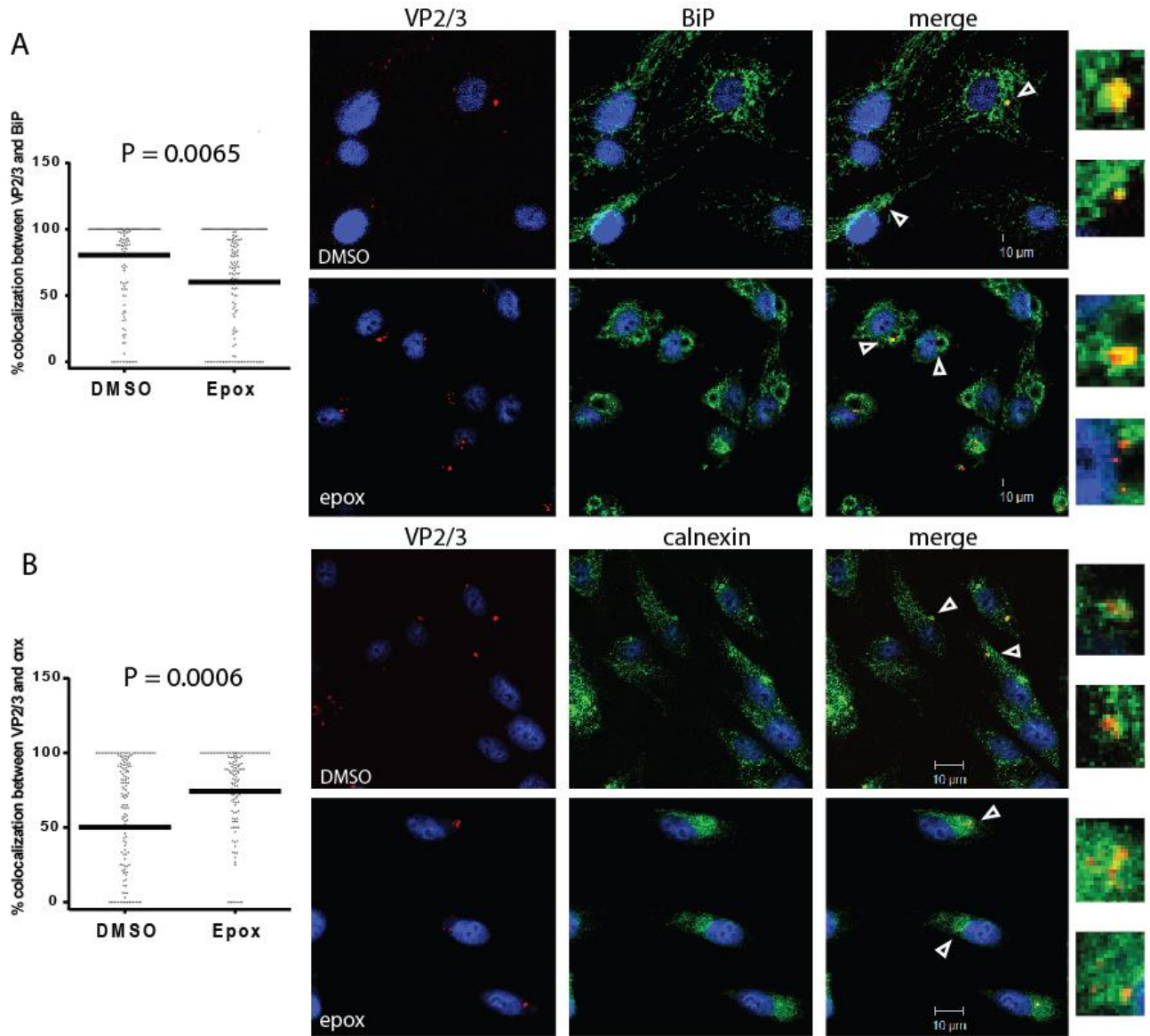
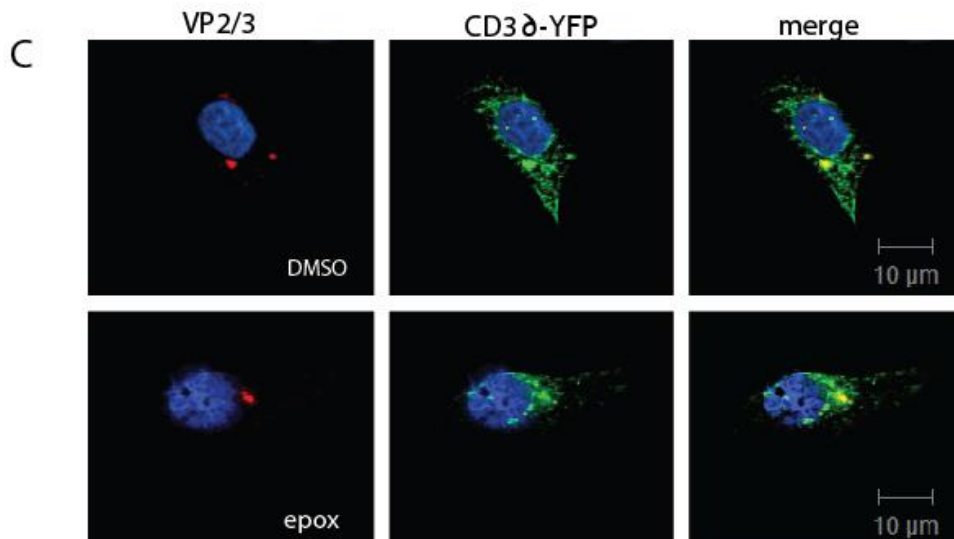**Figure 2.4**: BKPyV behaves like an ERAD substrate. RPTE cells were infected at MOI 5 IU/cell and fixed at 24 hpi. Fixed cells were stained for VP2/3 and the ER markers (A) BiP or (B) calnexin (cnx), and images taken by confocal microscopy. Arrowheads point to enlarged colocalized areas on the right. Colocalization of the individual VP2/3 puncta with calnexin or BiP was measured using MetaMorph software at the Center for Live Cell Imaging at the University of Michigan. Statistical analysis was performed as in Figure 2. The line represents the median colocalization for each condition. (C) RPTE cells were transfected with CD3δ-YFP and the media was changed at 12 h post transfection. Transfected cells were infected at 5 IU/cell at 24 h post transfection, treated with 10 μM epoxomicin at 6 hpi, and fixed at 24 hpi (48 h post transfection). Fixed cells were then stained for CD3δ-YFP with anti-GFP and capsid proteins with anti-VP2/3, and imaged by confocal microscopy.
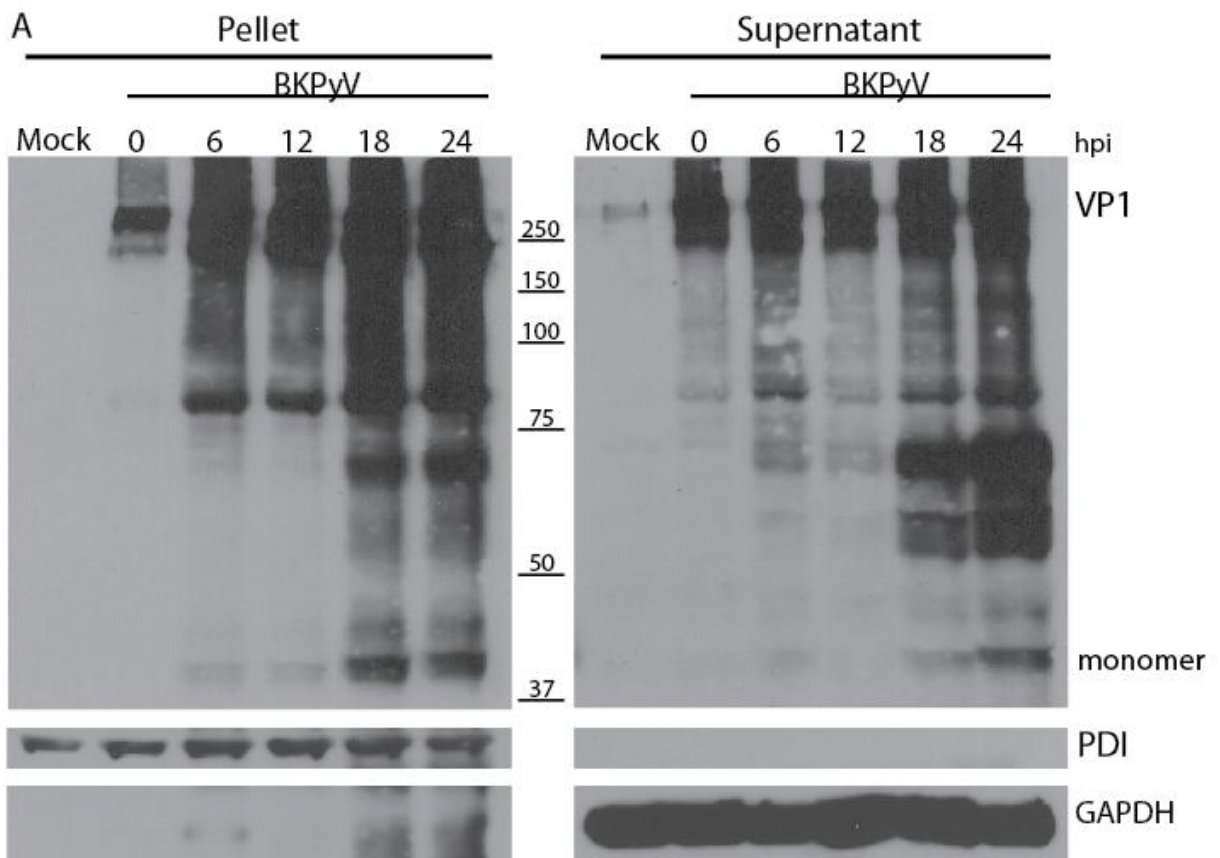
possibly to sequester potentially harmful unfolded and aggregation-prone proteins, and is positive for calnexin while often devoid of BiP (22-24). Therefore, we next looked at calnexin co-staining with VP2/3. In untreated cells, there was again partial colocalization between VP2/3 and calnexin, with the median at 50%. In contrast to the pattern of VP2/3 and BiP colocalization, there was a significant increase in colocalization between VP2/3 and calnexin with epoxomicin treatment, where median colocalization increased to 75% (Figure 2.4B). These data showed that inhibition of the proteasome leads to relocalization of the virus to a calnexin-rich subregion of the ER that is deficient for the chaperone BiP.

Since the changing colocalization of BKPyV with BiP or calnexin suggested that BKPyV may be handled like an ERAD substrate, we asked whether the virus might colocalize with a commonly studied ERAD-targeted protein. We chose to examine CD3δ, a transmembrane protein subunit of the T cell receptor complex that is sent through the ERAD pathway when it is not assembled into the complex (25). RPTE cells were transfected with a CD3δ-YFP construct, then infected with BKPyV 24 hrs post transfection. Cells were fixed at 24 hpi and stained for VP2/3 and YFP to identify the transfected CD3δ. Confocal microscopy showed that VP2/3 and CD3δ co-localized within the ER at 24 hpi during normal infection, and remained associated with CD3δ in the presence of proteasome inhibition (Figure 2.4C). We could not quantify colocalization with CD3δ-YFP because transfection efficiency is extremely low in RPTE cells. Overall, these data suggest that BKPyV is shuttled to the same location with the ER as a substrate of the ERAD system, possibly due to interaction of BKPyV with the same recognition factors as a bona fide ERAD substrate.

BKPyV enters the cytosol after ER trafficking occurs.

The route taken by BKPyV into the nucleus after trafficking through the ER is unknown. The two possible pathways that have been proposed include crossing the ER membrane into the cytosol, from where the virus then undergoes active transport into the nucleus; or crossing the inner nuclear membrane directly into the nucleus without passing through the cytosol. To address the question of whether BKPyV traffics through the cytosol during infection, a biochemical assay was employed that allows for separation of a cellular lysate into a cytosol-containing fraction and a fraction including membrane-containing cellular components. Low percentage digitonin treatment selectively permeabilizes the plasma membrane by targeting the more cholesterol-rich membrane while leaving organelle membranes intact (26). This assay was previously used for measuring retro-translocation of ERAD substrates and is well-established as a means to address cytosolic entry of polyomaviruses (26, 27).

Employing this fractionation, we first asked whether we could see the appearance of monomeric VP1 capsid protein, which is a product of disulfide bond reduction in the ER, in the cytosol over time (9). To do so, we resolved the protein fractions using non-reducing SDS-PAGE. In this assay, only VP1 monomers, pentamers, or oligomers from viral capsids that have undergone reduction or isomerization in the cell will migrate through the polyacrylamide gel matrix . We harvested infected cells under alkylating conditions, to prevent any post-harvest disulfide bond isomerization, at various time points after infection and isolated the cytosolic fraction via the digitonin permeabilization method. The integrity of the fractionation was confirmed by the absence of the ER protein PDI and presence of GAPDH in the cytosolic fraction (Figure 2.5A). We were able to detect monomer bands as well as higher molecular weight bands in the cytosolic fraction. We observed the appearance of VP1 monomers in the
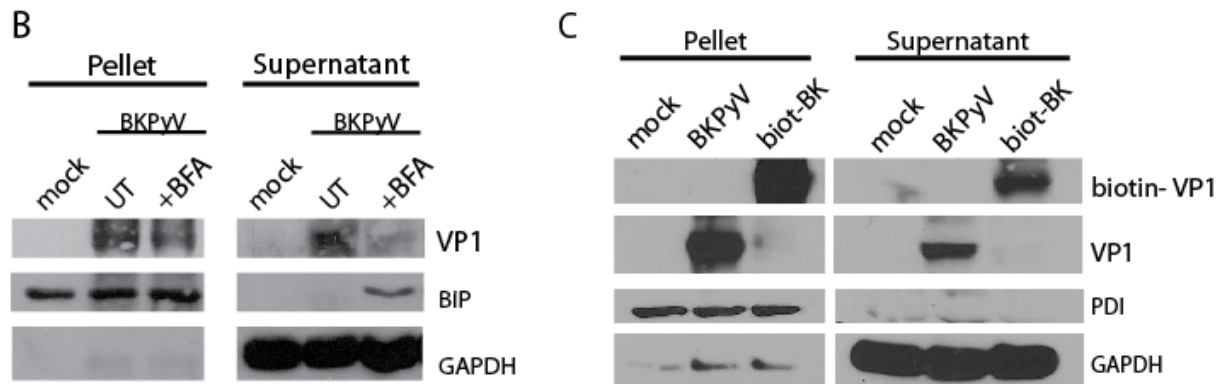
A

	Pellet									Supernatant						

Markers: 250, 150, 100, 75, 50, 37

Supernatant — BKPyV — Mock 0 6 12 18 24 hpi

VP1

monomer

PDI

GAPDH

Figure 2.5: BKPyV enters the cytosol. (A) RPTE cells were infected at 5 IU/cell, harvested at the indicated time points under alkylating conditions, and fractionated into pellet and supernatant fractions. Fractions were separated by non-reducing SDS-PAGE and probed for VP1, ER marker PDI, and cytosolic marker GAPDH. One-third of the protein for the supernatant was loaded for the pellet fractions and the film was exposed for a shorter time. VP1 bands here include monomers and other higher-molecular weight species that have entered the non-reducing gel.(B) RPTE cells were infected at 5 IU/cell and treated at 6 hpi for two hours with 1.25 μg/ml BFA or left untreated, harvested at 16 hpi under alkylating conditions, then separated into pellet and supernatant fractions. Fractions were resolved by non-reducing SDS-PAGE and probed as above but with BiP as the ER marker. "VP1" here represents monomers that have entered the nonreducing gel. (C) RPTE cells were infected with biotinylated BKPyV at 5 IU/cell and harvested at 16 hpi under alkylating conditions, and assayed as in (A). The western blot was first probed with streptavidin-HRP to show the presence of biotinylated VP1 monomer ("biot-VP1") and then probed for VP1, PDI, and GAPDH.

supernatant beginning at approximately 18 hpi, with a slight increase in monomers over time (Figure 2.5A). This timing was consistent with the timing of ER trafficking of BKPyV. As expected, treatment with the ER trafficking inhibitor BFA reduced appearance of monomers in the cytosolic fraction (Figure 2.5B). Monomers were not completely absent in the cytosolic fraction most likely because we could only treat with BFA for 2 hrs before it became toxic. This may have allowed time for some virus to reach the ER early or for retrograde transport to regain function (28). The appearance of ER proteins (BiP or PDI) in the cytosolic fraction of BFA treated samples was seen consistently. This may be explained by the disruption of secretory pathways by BFA and the potential for these ER proteins to undergo export from the ER in small vesicles that may be present in the cytosolic fraction (29).

   To ensure that the VP1 in the cytosolic fraction indeed represented incoming BKPyV rather than VP1 that was newly synthesized during infection, purified BKPyV was labeled with biotin. RPTE cells were infected as above and harvested after 24 hpi. The Western blot was probed with streptavidin-conjugated horseradish peroxidase and showed a band in the cytosolic fraction that ran at slightly reduced mobility than the VP1 in the unlabeled virus-infected fractions (Figure 2.5C). We noted that biotinylation of VP1 prevented recognition by the VP1 monoclonal antibody. The presence of biotinylated VP1 in supernatant indicated that the protein visualized in the cytosolic fraction in Figure 2.5A is indeed from the infecting virus. These data together support our conclusion that the VP1 monomer visualized in the cytosolic fraction is from incoming virus.

Inhibition of ERAD does not prevent BKPyV entry into the cytosol.

The previous experiments suggested that ERAD plays a role in BKPyV exit from the ER. We next wanted to test whether the proteasome inhibitor or ERAD inhibitor would therefore prevent entry of BKPyV into the cytosol, since ER exit and the proteasome are usually tightly linked (18, 30). RPTE cells were infected at an MOI of 5 IU/cell and treated at 6 hpi with epoxomicin or Eeyarestatin I, harvested at 16 hpi, separated into pellet and cytosolic fractions, and proteins were resolved by non-reducing SDS-PAGE and probed for VP1. Unexpectedly, neither inhibitor prevented VP1 monomers from appearing in the cytosolic fraction (Figure 2.6A), and in fact treatment with epoxomicin led to an increase in the VP1 monomer signal in the supernatant fraction compared to cells treated with DMSO alone. We also saw a consistent increase in monomeric VP1 in the pellet fraction with epoxomicin treatment, while there was a decrease with Eeyarestatin I. Because of the discrepancy between VP1 monomers appearing in the cytosol and TAg production, these results suggest that cytosolic VP1 may not represent productive infection.

We also examined whether viral genomes were present within the cytosolic fraction and whether their appearance was sensitive to the inhibitors. Viral DNA was isolated from equal amounts of the supernatant fractions by proteinase K treatment followed by isolation of DNA on a PCR purification column. The proteinase K treatment was performed to release viral genomic DNA that may be still associated with partially disassembled capsids. In agreement with VP1 monomer levels, there was much less viral DNA in the cytosol of infected cells treated with BFA (Figure 2.6B). As opposed to the increase in VP1 monomers in the cytosol of epoxomicin treated cells, however, there was approximately two-fold less viral DNA. In the cytosol of cells treated with Eeyarestatin I, there was no consistent difference in viral DNA levels.
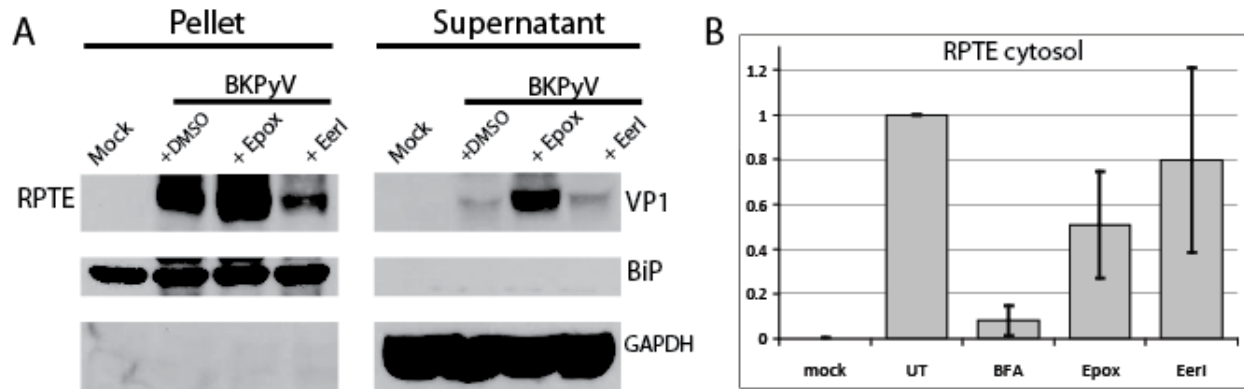
Figure 2.6: ERAD inhibition does not prevent cytosolic entry of BKPyV. (A) RPTE cells were infected with BKPyV at 5 IU/cell at 4°C for 1 h and treated with 10 μM epoxomicin (Epox), 10 μM Eeyarestatin I (EerI), or DMSO at 6 hpi, harvested at 16 hpi under alkylating conditions, then separated into pellet and cytosolic fractions and assayed as in Figure 4. (B) BKPyV genomic DNA isolated from treated and untreated supernatant fractions was measured by QPCR and normalized to untreated levels. Averages from three independent experiments are shown with error bars representing standard deviation. A one-tailed T-test was performed and the significance for BFA treatment was P=0.0008 and epoxomicin treatment was P=0.03. Eeyarestatin I treatment did not cause a significant decrease in DNA.

Trafficking requirements differ between viruses and cell types.

Recent studies have shown that trafficking from the ER to the cytosol of the closely related polyomavirus, SV40, is blocked when the proteasome or ERAD are inhibited, based on appearance of SV40 capsid proteins in the cytosol (15, 16, 27). Since our results with BKPyV in RPTE cells differ from what has been published for SV40 in CV-1 cells, we first decided to examine the trafficking of BKPyV in CV-1 cells. To improve the infectivity of BKPyV, we first incubated CV-1 cells with the BKPyV receptor GT1b. Cells were then infected with BKPyV, harvested at 16 hpi under alkylating conditions, and fractionated into pellet and supernatant fractions. Interestingly, there seemed to be little to no effect of epoxomicin treatment on the appearance of VP1 monomers in the cytosol, and BFA treatment led to a consistent decrease in VP1 monomers (Figure 2.7A). Next we looked at viral DNA levels in the cytosol using qPCR as above. Inhibition of viral genomes from entering the cytosol by both BFA and epoxomicin was similar to what was seen in RPTE cells (Figure 2.7B).

Next we wanted to evaluate the effect of our inhibitors on SV40 infection in both CV-1 cells and RPTE cells. First, we determined whether the inhibitors could prevent infection of CV-1 cells. To allow productive infection of RPTE cells with SV40, cells were incubated with the SV40 receptor, ganglioside GM1, for 24 h before infection (3). Interestingly, epoxomicin treatment strongly inhibited infection in both cell types, as assayed by TAg expression, but Eeyarestatin I only inhibited infection of RPTE cells (Figure 2.7C). As Eeyarestatin I targets p97, and protein expression levels can differ between cell types (31), it may be that there is an indirect effect of p97 inhibition on virus trafficking in RPTE cells, whereas the trafficking pathway in CV-1 cells is less sensitive. Together, the different effects of epoxomicin and
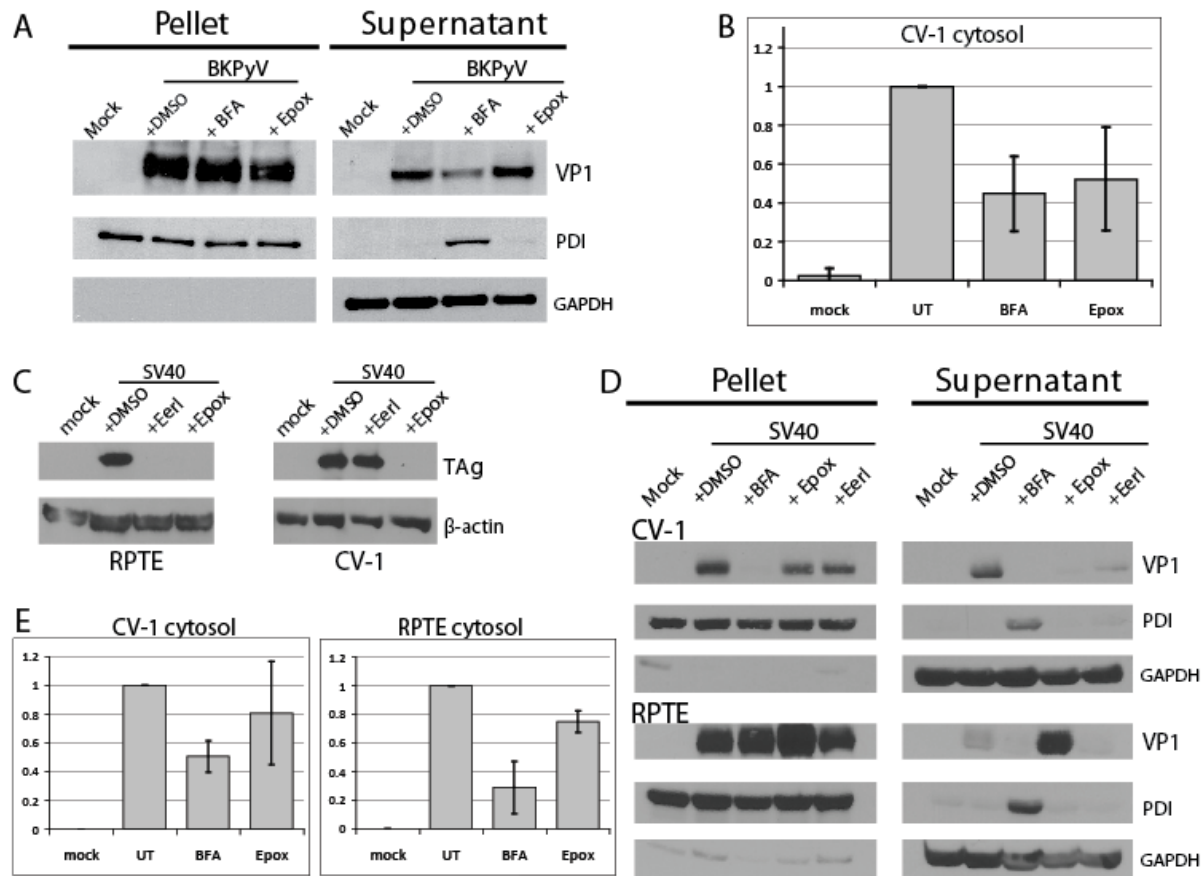
Figure 2.7: Cell type-specific requirements for polyomavirus trafficking. (A) CV-1 cells were infected with BKPyV and treated with BFA, epoxomicin (Epox), or DMSO as in Figure 5A. Pellet and supernatant fractions were separated and assayed as in Figure 4. (B) BKPyV genomic DNA from the CV-1 supernatant fractions was quantified by QPCR and represented as in Figure 4B. A T-test determined significance of the decrease in BFA treatment to be P=0.02 and epoxomicin treatment to be P=0.02. (C) RPTE or CV-1 cells were inoculated with 5 IU/cell SV40 at 4°C for 1 hr then moved to 37°C and incubated in the presence of 10 μM epoxomicin (Epox), 10 μM Eeyarestatin I (EerI), or DMSO. RPTE cells were incubated for 24 h with 3.2 μM ganglioside GM1 prior to infection. Whole cell lysates were harvested at 24 hpi, resolved by reducing SDS-PAGE, blotted, and probed for TAg. (D) As in 4D, RPTE cells were infected with 5 IU/cell SV40 in the presence of 1.25 μg/ml BFA, 10 μM epoxomicin (Epox), 10 μM Eeyarestatin I (EerI), or DMSO. Proteins were harvested under alkylating conditions at 10 hpi and fractions analyzed as in Figure 4. Western blot was probed for SV40 VP1 monomers, PDI, and GAPDH. (E) SV40 genomic DNA was isolated and quantified by QPCR. Values for each condition were normalized to untreated levels and the averages from three independent experiments are represented with error bars showing the standard deviation. A T-test was performed for each condition and the significance in CV-1 cells was P=0.008 for BFA treatment, with no significant decrease for epoxomicin treatment. In RPTE cells significance was P=0.01 for BFA treatment and P=0.01 for epoxomicin treatment.

Eeyarestatin I on SV40 TAg expression suggest that requirements for infection vary between cell types.

Next we wanted to address SV40 cytosolic trafficking in the RPTE cell system. After incubation with GM1, RPTE cells were infected with SV40 at an MOI of 5 IU/cell and fractionation was performed at 10 hpi to separate membrane bound organelles and cytosolic components. This infection was shorter because SV40 traffics more rapidly than BKPyV, with TAg expression detectable at 14 hpi by Western blot (27). As shown in the top panel of Figure 2.7D, epoxomicin inhibited SV40 VP1 monomers from entering the cytosol of CV-1 cells, as has been previously reported (15, 27). However, appearance of cytosolic SV40 VP1 was not inhibited in RPTE cells: VP1 monomers increased in the cytosolic fraction when cells were treated with epoxomicin (Figure 2.7D, bottom panel) in the same way as seen with BKPyV (see Figure 2.6). For both cell types, Eeyarestatin I prevented SV40 VP1 monomers from appearing in the cytosol, which was interesting since the drug does not inhibit infection in CV-1 cells. In RPTE cells the SV40 VP1 monomers accumulated in the pellet under epoxomicin treatment and decreased in the pellet with Eeyarestatin I treatment, while in CV-1 cells there was not a noticeable change in VP1 monomer levels of the pellet. BFA strongly inhibited VP1 from appearing in the pellet in CV-1 cells, however. Finally, we looked at SV40 DNA levels in the cytosolic fractions of both CV-1 and RPTE cells. Although the appearance of VP1 protein within the cytosol was affected differently by epoxomicin and Eeyarestatin I, we saw a similar decrease in cytosolic viral genomes (Figure 2.7E). Together these data suggest that the appearance of VP1 protein within the cytosol does not correlate with viral genomes, and perhaps the appearance of cytosolic VP1 monomers represents an alternate or nonproductive pathway.

Discussion

Uncovering the mechanism of virus entry and trafficking is important for understanding viral pathogenesis and identifying antiviral targets. Furthermore, since viruses are obligate intracellular pathogens, studies of host-virus interactions often elucidate unknown intracellular pathways and illuminate known pathways, adding to the understanding of basic cellular biology. Previous findings implicated components of the ERAD pathway, including the proteasome, during early events of polyomavirus infection (9, 15, 16, 27). The possibility of ERAD involvement led us to pursue the role of the proteasome during BKPyV infection and whether it was acting in the context of the ERAD pathway and possibly in viral trafficking out of the ER. In this study we have found that BKPyV requires a functional proteasome and ERAD pathway during entry, and we also found that inhibition of these cellular mechanisms leads to a sequestration of BKPyV within an ER-quality control subcompartment. Additionally, we have found that both BKPyV and SV40 capsid proteins and DNA reach the cytosol during infection in both RPTE cells and CV-1 cells. Interestingly, we have found that a number of differences exist between cell types and between viruses, and that measurement of VP1 monomers in the cytosol is not a reliable assay for viral cytosolic trafficking.

We hypothesized that proteasome function was required during BKPyV entry, and we found that the proteasome played a role at an early point during infection by examining sensitivity to epoxomicin treatment. Proteasome function was required before 18hpi, which is a timepoint after ER trafficking as determined previously by treatment with the retrograde trafficking inhibitor BFA (9). In addition, we know that epoxomicin does not inhibit TAg gene transcription when TAg is expressed from a transfected plasmid (Figure 2.8). A role for the ERAD pathway was supported by the treatment timecourse with Eeyarestatin I, which also inhibited TAg
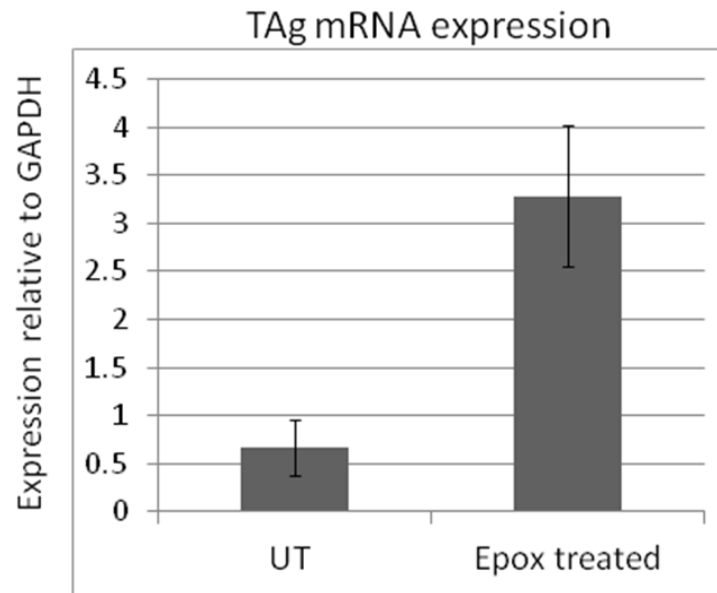
Figure 2.8: Expression of TAg is not directly inhibited by epoxomicin treatment. RPTE cells were transfected with recircularized TU genome and treated with epoxomicin or DMSO immediately after transfection. RNA was harvested at 24 h after transfection, and quantitative RT-PCR was performed for TAg and GAPDH. Results are from three independent experiments.

expression when added before 18hpi. We also found that reduction of p97 protein, the target of Eeyarestatin I, by siRNA led to a reduction in infection (Figure 2.9); however, cytotoxic effects were observed in the p97 knock down cells, causing us to view this result with caution.

Since inhibition of the ERAD pathway abrogated infection, we were interested in visualizing where the blockage was occurring within the cell. We hypothesized that if BKPyV was unable to traffic to the nucleus without the ERAD pathway, we might see an accumulation of virus outside the nucleus. In support of this, we saw a reproducible increase in the amount of virus genome accumulating, much of it in a juxtanuclear position. We then visualized partially uncoated virus particles by staining for the minor capsid proteins VP2/3, which become available to antibody recognition only after capsid disassembly begins. Interestingly, we observed a change in the morphology of the ER following epoxomicin treatment, as ER-resident proteins BiP and calnexin showed different staining patterns, suggesting their partitioning to different ER subdomains (Figure 2.4). BKPyV localization within the ER also changed, colocalizing more with calnexin under proteasome inhibition than in untreated conditions (Figure 2.4B). A number of recent studies describe an ERQC subcompartment that is enriched in certain ERAD and ERQC factors including calnexin, but is devoid of other ER proteins such as BiP (22-24). This subcompartment is exaggerated upon proteasome inhibition, and ERAD factors and substrates such as CD3δ accumulate within this area (23). If BKPyV is handled like an ERAD substrate, an increase in the concentration of viral disassembly factors within the ERQC compartment may explain the accumulation of partially disassembled virus in the epoxomicin-treated cells, as shown by the increase in total VP2/3 immunofluorescence. In further support of BKPyV acting as an ERAD substrate, we detected colocalization between BKPyV and CD3δ, and this colocalization was still detected under proteasome inhibition.
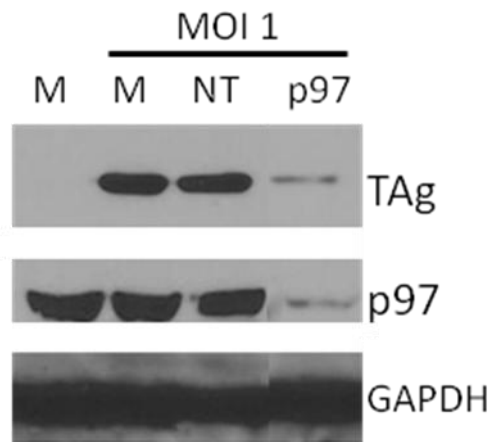
Figure 2.9: Knockdown of p97 reduces infection. RPTE cells were transfected with non-targeting (NT) or p97 siRNA for 2 days, then infected with BKPyV at MOI 1. Whole cell lysates were harvested at 24 hpi and resolved by SDS-PAGE and probed for TAg, p97, and GAPDH. M = mock transfection.

Recent findings with SV40 support the role of ER-localized ERAD components during polyomavirus passage into the cytosol. These factors include DnaJ chaperone family proteins and the abundant ER chaperone BiP, all discovered in unbiased siRNA screens for modulators of SV40 infection (16). These factors were shown to be necessary after partial disassembly and VP2/3 exposure but before cytosolic entry of SV40. Another ERAD protein, Bap31, was also found to be necessary for entry of SV40 into the cytosol, and these authors found that an interaction between VP2 and Bap31 was necessary for cytosolic entry (15). Interestingly, Bap31 has been described as a shuttle for ERAD substrates to the ERQC compartment (22). Proteasome inhibition may increase the concentration of other ERAD substrates, titrating Bap31 away from the virus and thus decreasing ER exit of the virus particle. In addition, this may cause BiP to move away from the ERQC compartment and BKPyV.

In support of a role for an ER-to-cytosol entry step, we detected VP1 monomers in the cytosol at a timepoint that follows ER trafficking. Detection of VP1 protein by non-reducing SDS-PAGE supports the conclusion that the capsid protein in the cytosol has first passed through the ER where it has undergone reduction and/or isomerization by oxidoreductases, especially since VP1 monomers are diminished in the presence of BFA. The amount of VP1 is just above the limit of detection by Western blot, suggesting that a very low percentage of input virus particles reach the cytosol. Because of detection issues, we were not able to use VP2/3 as a readout for virus in the cytosol by Western blot.

The appearance of BKPyV capsid protein in the cytosol, along with ERAD involvement, points to a co-opting of the ER-to-cytosol retrotranslocation pathway by the virus. Surprisingly, however, we were not able to prevent appearance of VP1 monomers with ERAD inhibition by epoxomicin or Eeyarestatin I in RPTE cells. Rather, we saw an accumulation of VP1 monomers

in the cytosol in the presence of epoxomicin, and no difference with Eeyarestatin I. Since the ERAD inhibitors prevent infection as measured by TAg expression, these data seemed to go against the hypothesis that inhibition of the proteasome and ERAD pathway would prevent trafficking of BKPyV out of the ER. For this reason we asked whether the viral genome was also reaching the cytosol, since transport of the viral genome to the nucleus is necessary in any potential productive pathway. Interestingly, in the presence of epoxomicin, there was a decrease in BKPyV genomes present in the cytosol. These changes in viral DNA levels within the cytosol support the possibility that some infectious virus particles may be trapped within the ER when proteasome function is inhibited, even though VP1 protein can still enter the cytosol. It may be that retro-translocation of VP1 monomers, disassembly by-products, normally occurs independent of proteasome function, followed by proteasome degradation, allowing for the accumulation we see in the presence of inhibitor. This scenario does not seem to occur during SV40 infection. The inconsistent effects of Eeyarestatin I treatment on viral DNA levels in the cytosol may suggest that the p97 inhibitor is targeting other trafficking pathways besides ERAD that lead to inhibition of TAg expression.

A number of recent studies have evaluated ER-to-cytosol trafficking of SV40 based on the presence of capsid protein VP1 in the cytosol. Our studies, however, seem to suggest that VP1 and viral genomes may represent different events or pathways, separate or overlapping. Importantly, these events differ between cell types and even between viruses. Compared to infected RPTE cells, BKPyV-infected CV-1 cells show a different phenotype of VP1 entry into the cytosol, where there seems to be no difference between epoxomicin-treated and untreated cells. And as has been previously published (15, 27), SV40 VP1 does not enter the cytosol in epoxomicin-treated CV-1 cells, while in RPTE cells it undergoes the same accumulation as

BKPyV. While the variation in levels of cytosolic VP1 in the presence of trafficking inhibitors suggests that virus trafficking may differ between cell types, the effect on cytosolic viral genomes is similar in each cell type and between viruses.

The level of viral genomes in the cytosol with epoxomicin treatment ranged from about 50% of untreated for BKPyV to about 80% for SV40. Although an inhibitory effect of epoxomicin treatment is consistent with proteasome function being required for ER exit, the fact that TAg expression is much more strongly inhibited in the presence of the inhibitors than the cytosolic entry of viral DNA raises the question of whether the viral genomes in the cytosol are part of the infectious pathway. If cytosolic trafficking is a productive pathway, perhaps the proteasome acts after transport of the infectious particle across the ER membrane. On the other hand, our results could be explained by non-specific effects of proteasome inhibition: i.e., aggregates or other misfolded obstructions within the ER lumen would prevent effective passage of virus through the ER to its next proper destination. This nonspecific effect is supported by the change in ER morphology and accumulation of disassembled virus that we observe under proteasome inhibition. This result is consistent with a possible model in which the cytosolic viral components represent a non-productive or dead-end pathway. Originally, inhibition of SV40 VP1 from entering the cytosol by epoxomicin in CV-1 seemed to support cytosolic trafficking as a productive pathway since it correlated with a block in TAg expression. However, SV40 VP1 monomers are also inhibited by Eeyarestatin I even though the drug does not inhibit overall infection. Moreover, in RPTE cells, VP1 monomers from both viruses can enter the cytosol under either drug treatment, while TAg expression is prevented with both treatments.

This study raises an interesting question about differences in the biology of the cell types used for studying polyomavirus entry. It is well accepted that protein expression levels (31), cell cycle

regulation (32), and other aspects of cell biology differ between cell types, and these discrepancies should be considered when studying cell biology of a viral infection. We also cannot rule out species-specific behavior of cells. The experiments in this report were performed in primary cells that are a natural host cell of BKPyV, and are more likely to represent the biology of BKPyV lytic infection in the natural host.

Previous results also questioned whether SV40 travels through the cytosol as its productive route of infection. Some studies have shown transient disturbances in the nuclear membrane that occur around the time of nuclear entry (6, 33), suggesting that trafficking from the ER to the nucleus occurs by crossing the inner nuclear membrane through temporary disruption in the lipid bilayer. This model cannot yet be ruled out to describe BKPyV nuclear entry. One interesting thing to consider is the hydrophobic nature of the minor capsid proteins. Both VP2 and VP3 of SV40 have been previously shown to have membrane lytic properties and to be able to at least partially insert into ER membranes (34). If the virion can penetrate the ER membrane to enter the cytosol, then it is reasonable to believe that it may also be able to penetrate the inner nuclear membrane to gain direct access to the nucleus. Penetration may be limited to a membrane that is contacting the cytosol if specific cytosolic proteins are required for the process. Also, even though there is no precedent for an ERAD substrate to be translocated into the nucleus, an inner nuclear membrane–localized ERAD E3 ligase has been identified in yeast and may suggest a way for the ERAD pathway to be combined with direct nuclear entry (35). Another possibility that would further support cytosolic trafficking as a productive pathway is if a disassembly step occurs within the cytosol. A better understanding of BKPyV disassembly will be required before the process of nuclear entry can be fully elucidated.

In conclusion, this study demonstrates involvement of the ERAD pathway during BKPyV entry and infection, and provides evidence that there are complex interactions between polyomaviruses and the host cell machinery. Additionally, the data presented here regarding the observed cell type differences in polyomavirus trafficking call for re-examination of the current paradigm for cytosolic trafficking of polyomaviruses. This study emphasizes the relevance of primary cell models of infection and provides avenues for further investigation to determine how polyomaviruses enter the cell nucleus.

Materials and Methods

Cell Culture. RPTE cells were grown in renal epithelial basal growth medium (REGM) with SingleQuots Bulletkit from Lonza at 37°C and 5% CO_2 and passaged up to six times as previously described (36). The CV-1 cell line (African Green Monkey Kidney Cells, ATCC) was grown in Dulbecco's modified Eagle's medium with 10% heat-inactivated fetal bovine serum (HyClone), 100 U/ml penicillin, and 100 µg/ml streptomycin (Cambrex).

Transfections. The CD3δ-YFP plasmid was obtained from Addgene, Cambridge, MA (plasmid 11951) (37). Transfection complexes were prepared according to manufacturer's instructions, using *Trans*IT®-LT-1 (Mirus) reagent at a 1:6 ratio of DNA-to-transfection reagent. Cells were seeded on glass coverslips in a 12 well plate and transfected with 0.5 µg plasmid per well at approximately 70-80% confluency. Media was changed 12 hours post transfection and infections were initiated at 24 hours post transfection.

Infections. Purified BKPyV (TU variant) was propagated and purified as previously described (36). SV40 was a gift from Billy Tsai (University of Michigan). The viral titer was measured by fluorescence focus assay as previously described (9). Infections were performed when RPTE cells or CV-1 cells were approximately 70-80% confluent. Cells were first pre-chilled for 15 minutes at 4°C. Purified virus was mixed with cold REGM or serum-free DMEM for RPTE cells or CV-1 cells, respectively, and virus was bound for one hour at 4°C. Cells were washed once with cold media and then switched to warmed full media and incubated at 37°C until the desired time. For ganglioside receptor pre-treatment, cells were incubated 24 hrs before infection with either GM1 or GT1b, then washed 3 times with media. Biotinylated BKPyV was prepared by incubating virus particles with EZ-Link Sulfo-NHS-LC-Biotin (Thermo), according to the manufacturer's instructions.

Drug Treatments. Epoxomicin (Sigma, Enzo Lifesciences) and Eeyarestatin I (Santa Cruz Biotech) were each dissolved in DMSO and used at 10 µM. Brefeldin A (Sigma) was dissolved in ethanol and used at 1.25 µg/ml. A WST metabolic assay (Roche) was used to ensure that drug treatments did not cause significant cytotoxicity under the conditions used. Ganglioside GM1 or GT1b (Santa Cruz Biotech) was reconstituted in water and used at 3.2 µM.

Preparation of cell lysates. Whole cell protein lysates were harvested in E1A buffer (50mM HEPES pH 7, 250 mM NaCl, 0.1% NP40, with protease inhibitors 5 µg/ml PMSF, 5 µg/ml aprotinin, 50 mM NaF, 0.2 mM Na-orthovanadate, and 5 µg/ml leupeptin added right before use). Protein concentrations were determined using a Bradford assay. For cytosolic fractionation, a 100 mm plate or 2 wells of a 6-well plate cells were infected with virus at an MOI of 5

infectious units per cell (IU/cell) with BKPyV or SV40. Cells were harvested at 16 hpi for

BKPyV and 12 hpi for SV40 by treating with 0.25% trypsin for approximately 1 minute or until

cells detached from the plate. Trypsin was inhibited with equal volume 1 mg/ml soybean trypsin

inhibitor, and cells were collected in 4 ml cold PBS. Cells were pelleted at 300 x g for 5 min at

4°C and then resuspended in 1 ml 20 mM N-Ethylmaleimide (NEM) (Sigma) in PBS for 45 min

on ice. After alkylation, cells were again pelleted and resuspended in 100 µl of 0.01% digitonin

(for RPTE cells) or 0.05% digitonin (for CV-1 cells) in HCN buffer (150 mM HEPES pH 7.5, 50

mM NaCl, 2 mM CaCl$_2$, 20 mM NEM and protease inhibitors at 5 µg/ml PMSF, 5 µg/ml

aprotinin, 50 mM NaF, 0.2 mM Na-orthovanadate, 5 µg/ml leupeptin added right before use) for

10 min on ice. The cytosolic fraction was separated from the pellet fraction by centrifugation at

16,000 x g, 10 min, 4°C. The supernatant (cytosolic) fraction was removed and the pellet was

washed with 500 µl 1x PBS. The pellet fraction was solubilized by resuspension in 100 µl of

E1A lysis buffer, and then clarified by centrifugation at 16,000 x g, 5 min. To isolate either

BKPyV or SV40 genomic DNA from cytosol-enriched supernatant fractions, equal amounts of

lysate based on protein concentration (150 ng) from each sample was treated with 5 µg

proteinase K (Qiagen) for 1 hr at 37°C. DNA was then isolated from the samples by PCR

Purification kit (Qiagen).

Western Blots. Equal amounts of protein were resolved by SDS-PAGE, (8% for TAg, 10% for

all other proteins), under reducing or nonreducing conditions and transferred overnight onto a

nitrocellulose membrane at 60 V by wet transfer. Membranes were blocked in 2% nonfat dry

milk in PBS containing 0.1% Tween 20 (PBS-T). Primary and secondary antibodies were diluted

in 2% milk in PBS-T as follows: TAg was detected with mouse monoclonal antibody pAb416

(38) at 1:1000; BKPyV VP1 was detected by mouse monoclonal antibody P5G6 at 1:5000; ER proteins were detected by mouse anti-BiP (BD Biosciences) at 1:1000 and rabbit anti-PDI (Enzo) at 1:2000; SV40 VP1 was detected by mouse monoclonal anti-VP1 (a gift from Billy Tsai, originally from Dr. W. Scott) at 1:2000; rabbit anti-β-actin (Cell Signaling) was used at 1:10,000 and mouse anti-GAPDH (Abcam) was used at 1:5000; HRP-conjugated ECL™ anti-mouse and anti-rabbit IgG secondary antibodies (GE) were used at 1:2000- 1:5000; streptavidin-conjugated horseradish peroxidase (GE) was used at 1:1000.

Flourescence *In Situ* Hybridization (FISH). RPTE cells were grown on coverslips and infected as described above, and fixed at 24 hpi using cold 95% EtOH/ 5% acetic acid. Coverslips were treated with RNAse for one hour at 37°C to avoid any RNA background staining. Coverslips were then pre-hybridized by incubation in hybridization buffer (50% formamide, 10% dextran sulfate, 4% SSC) for at least 30 min at 37°C. The fluorescently labeled DNA probe against the viral genome was prepared using nick translation kit (GE) with Cy3-dCTP and pBR322 plasmid containing the TU BKPyV genome. Unincorporated nucleotides were filtered using a Bio-Rad Quickspin column. The hybridization step was performed by first diluting the probe in hybridization buffer, denaturing the probe and coverslips at 95°C for two minutes, and immediately incubating the denatured coverslips with the denatured probe overnight at 37°C in a humidified chamber. The next day, the coverslips were washed 1x with 2x SSC at 60°C, 1x with 2x SSC at room temperature, and 1x with 1x PBS, at room temperature. Coverslips were then co-stained for indirect immunofluorescence or mounted on slides using Prolong Gold Antifade with DAPI (Invitrogen).

Immunofluorescence microscopy. At the desired time post infection, cells were fixed and permeabilized with 95% ethanol/ 5% acetic acid. Coverslips were blocked with 5% goat serum in PBS and then probed with primary antibody diluted in 5% goat serum. Antibodies used were mouse anti-calnexin (BD Biosciences) at 1:40, mouse anti-BiP (Santa Cruz) at 1:200, and mouse anti-GFP at 1:100 (Santa Cruz) dilutions. Anti-VP2/3 was raised by Bethyl Laboratories, Inc. against the peptide sequence CKTGRASAKTTNKRRSRSSRS and obtained as affinity-purified antibodies after processing from hyperimmune sera from immunized rabbits. Secondary antibodies Alexa-Flour® 594 goat anti-rabbit IgG or Alexa Fluor® 488 goat anti-mouse (Invitrogen) were used at a 1:200 dilution. Coverslips were mounted onto slides using Prolong Gold Antifade with DAPI (Invitrogen). Imaging was done on a Zeiss LSM 510-META Laser Scanning Confocal Microscope using a C-Apochromat 63x objective and 1.0 µm optical section at the University of Michigan Microscopy and Imaging Analysis Laboratory.

Quantitative Polymerase Chain Reaction (qPCR). Before qPCR, samples were diluted 100-fold. Primers used to quantify BKPyV were 5' TGTGATTGGGATTCAGTGCT 3' and 5' AAGGAAAGGCTGGATTCTGA 3', and to quantify SV40 were 5' AAGCAAAGCAATGCCACTTT 3' and 5' CACTGCAGGCCAGATTTGTA 3' (Invitrogen). Both pairs amplify a region of the TAg open reading frame.

Notes

This work was reprinted and modified with permission from Bennett, S.M., Jiang, M, Imperiale, M.J, Role of Cell-Type Specific ER-Associated Degradation in Polyomavirus

Trafficking, *J Virol*, 2013, 87(16): 8843-8852. Mengxi Jiang helped with experimental design and performed initial time-course and imaging experiments with proteasome inhibitors.

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CHAPTER III

The nuclear localization signal on VP2/3 is important for BK Polyomavirus nuclear entry

BK Polyomavirus (BKPyV) is a small nonenveloped virus that is ubiquitous in the human population and can cause severe disease in immunocompromised patients. It is believed that BKPyV traffics through the ER and then into the cytosol during entry in renal proximal tubule epithelial cells. It is unclear, however, how the virus reaches the nucleus. In this study, we elucidate a role for the nuclear localization signal located on the minor capsid proteins VP2 and VP3 during entry. We show that site directed mutagenesis of a single lysine in the basic region of the C-terminus of the minor capsid proteins will abrogate their individual nuclear localization, and the analogous mutation in the genome caused attenuation of infectivity based on an entry defect sometime after ER trafficking. The role of the nuclear localization signal (NLS) in the individual minor capsid proteins was addressed using pseudovirions. Mutation of the VP3-NLS led to an attenuated phenotype in 293TT cells, but not in RPTE cells. Overall these data are the first to show the importance of the NLS of the minor capsid proteins during BKPyV infection.

Introduction

BK Polyomavirus (BKPyV) is a widespread human pathogen that establishes a persistent subclinical infection in the majority of the population. However, reactivation can occur in imm

unosuppressed individuals, particarly bone marrow and kidney transplant patients (1). Currently there are no specific antivirals and the only treatment is palliative or the reduction of immunosuppression, respectively, leaving renal transplant patients open to organ rejection. Understanding the life cycle of the virus within the host will provide much needed information for future development of targeted therapies to lessen BKPyV reactivation and disease.

The BKPyV particle is small, nonenveloped, and consists of three structural proteins, including the major capsid protein VP1 and minor capsid proteins VP2 and VP3. The structural proteins encapsidate the 5 Kb circular double stranded DNA genome, which takes the form of a minichromosome associated with histones (2). The outer layer of the particle is made up of 72 VP1 pentamers stabilized by intermolecular disulfide bonds (3). Associated with each pentamer is approximately one molecule of either VP2 or VP3, which are not exposed to the surface of the particle until disassembly begins within the endoplasmic reticulum (ER) of the host cell. VP2 and VP3 are expressed from the same reading frame and share an identical C-terminus, but VP2 contains a unique N-terminal sequence that can be myristoylated (2). Our lab has previously shown in renal proximal tubule epithelial (RPTE) cells that BKPyV reaches the ER at ~8 – 10 hpi (4), then traffics through the cytosol ~16 hpi (5), followed by nuclear entry and early gene expression beginning ~24 hpi (6). Data suggest that BKPyV and other polyomaviruses exit the ER to reach the cytosol by using components of the ER-associated degradation pathway. It is currently unknown how the virus reaches the nucleus from the cytosol. It is reasonable to hypothesize that the partially disassembled virus could enter the nucleus through the nuclear pore, since disassembly would allow the particle to be small enough for import through the pore.

Previous studies have provided conflicting evidence for the nuclear entry mechanism of polyomaviruses. Studies from Nakanishi *et al.* have shown an interaction between importins α

and β and SV40 genomes in the cytosol, implying the use of the canonical nuclear import pathway for nuclear entry of SV40 (7, 8). Additionally, it was shown that the basic amino acids in the C-terminus of VP2/3 are necessary for the interaction with the importins, suggesting that nuclear entry of SV40 is mediated by interactions between a nuclear localization signal (NLS) on the minor capsid proteins VP2/3 and the canonical nuclear import machinery, which would mediate import through the nuclear pore complex (7, 8). However, studies by Butin-Israeli *et al.* have provided evidence that infection may lead to breakdown of the nuclear lamina and disruption of the nuclear envelope, allowing the virus to pass from the ER lumen directly into the nucleus (9).

In this study we have evaluated the role of the nuclear localization signal of the minor capsid proteins during entry of BKPyV. We have found that a vital lysine in VP2 and VP3 is important for localization to the nucleus of the BKPyV minor capsid proteins, and that mutation of this lysine in the virus attenuates entry at a point after ER trafficking. Additionally, we have found that the NLS of VP3 is more important during infection than the NLS of VP2. These findings provide more details of the trafficking route of BKPyV, and they provide a model where polyomavirus enters the nucleus from the cytosol through the nuclear pore, using the NLS of VP3 as the import signal.

Results

Lysine 319 on VP2 and Lysine 200 on VP3 are critical for nuclear localization of the minor capsid proteins.

The cellular localization of the minor capsid proteins of BKPyV, when expressed individually, has not been determined to date. Because BKPyV VP2 and VP3 share homology

with SV40, especially in the C-terminal basic amino acids determined to be important for nuclear localization of the SV40 proteins (10, 11) (Figure 3.1A), we hypothesized that VP2 and VP3 of BKPyV would also possess a nuclear localization signal that mapped to these residues. To test this, we expressed VP2 or VP3 alone in RPTE cells by transfection with an expression plasmid. In RPTE cells, both VP2 and VP3 showed localization in the nucleus, colocalizing with cellular DNA as measured by DAPI staining (Figure 3.1B, first columns). We next wanted to determine the amino acids necessary to confer this nuclear localization. It has been previously shown that the Lysine 320 on SV40 VP2 (and the same amino acid on VP3) was vital for nuclear localization of the SV40 minor capsid proteins (11). Therefore, we targeted the conserved lysine on BKPyV – 319 of VP2 and 200 of VP3 – for mutagenesis (Figure 3.1A, arrow). Site-directed mutagenesis was used to convert the lysine to a threonine, and the mutant constructs were transfected into RPTE cells to test localization of the proteins. Indeed, VP2K319T and VP3K200T no longer localized to the nucleus (Figure 3.1B, second columns).

For SV40, it had been shown that co-expression of VP1 would allow for nuclear import of nuclear localization-defective minor capsid proteins (12, 13). To test whether this was the case for BKPyV as well, we cotransfected a VP1 expression vector with each of the VP2K319T and VP3K200T expression vectors. The presence of VP1 led to nuclear localization of both VP2K319T and VP3K200T (Figure 3.1B, third columns). This observation suggests that the nuclear localization signal on VP1 allows for import of the VP1-VP2 or VP1-VP3 complex. These results suggest that assembly of capsids, which takes place in the nucleus, should not be affected by the presence of a mutation in the minor capsid protein NLS.

The VP2/3 NLS is important for entry in RPTE cells

A

```
  BKPyV   MLPLLLGLYGTVTP--ALEAYEDGPNQKKRRVSRGSSQKAKGTRASAKTTNKRRSRSSRS 232
  JCPyV   MLPLLLGLYGTVTP--ALEAYEDGPNKKKRRK--------EGPRASSKTSYKRRSRSSRS 225
  SV40    MLPLLLGLYGSVTS--ALKAYEDGPNKKKRKLSRGSSQKTKGTSASAKARHKRRNRSSRS 234
  mPyV    MLPLILGLYGDITPTWATVIEEDGPQKKKRRL---------------------------- 204
```
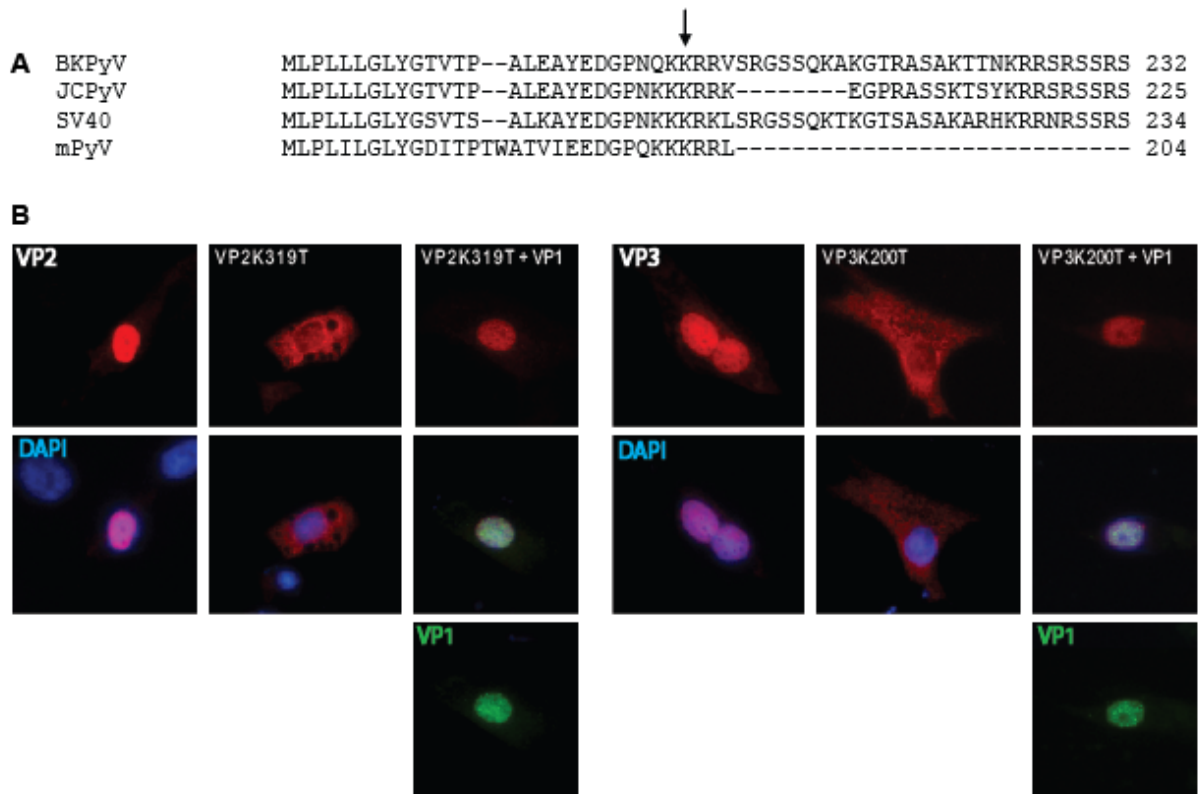
B

Figure 3.1: Lysine 319 in VP2 and 200 in VP3 is required for nuclear localization. (A) BKPyV, JC Polyomavirus (JCPyV), SV40, and mouse polyomavirus (mPyV) C-terminal sequences were aligned to compared homology. The lysine we chose to mutate is indicated with the arrow. (B) RPTE cells were transfected with a VP2 expression plasmid (left) or VP3 expression plasmid (right), the plasmid with a point mutation, or both the mutant and VP1 expression plasmids, then fixed at 24 h post transfection. VP2 and VP3 were stained with red, VP1 with green, DAPI in blue.

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To evaluate the role of the minor capsid protein NLS during infection, a point mutation was created in the viral genome to convert the vital lysine of VP2 and VP3 to a threonine as was done in the expression plasmids. The mutation was created in both the Dunlop and TU variant genetic backgrounds. The genome was excised from the plasmid, and recircularized mutant and wild type genomes were transfected into RPTE cells. The transfected cells were harvested and used as a crude stock of virus to infect a new set of RPTE cells. Those infected RPTE cells were then harvested after two weeks and the quantity of viral genomes in the crude stock was measured using qPCR. Of note, there were similar levels of viral genomes in both purified and mutant stocks of virus. PCR and sequencing was performed to ensure that the mutation was still present in the crude stocks (data not shown).

To test infectivity of the mutants, equal numbers of genomes were used to infect RPTE cells. Lysates were collected at 24 hpi, resolved by SDS-PAGE, and probed for the early protein large T Antigen (TAg). Compared to wild type viruses, mutant Dunlop and TU were both inhibited in TAg expression (Figure 3.2A), suggesting a possible defect in entry.

To assess whether early entry events were disrupted by the introduction of the mutation, we evaluated disassembly and ER trafficking. As the virus traffics through the ER, the disulfide bonds between the VP1 pentamers become reduced. The disassembly process can be detected beginning at about 12 hpi and shows increasing levels of VP1 monomers and oligomers over time (4). This can be detected by SDS-PAGE under non-reducing conditions, as compared to virus that has not begun disassembly. To compare mutant and wild type virus, RPTE cells were infected with equal genomes and harvested at 0 hpi and 24 hpi under alkylating conditions. The lysates were run under non-reducing conditions and probed for VP1. At 0hpi, there is an absence
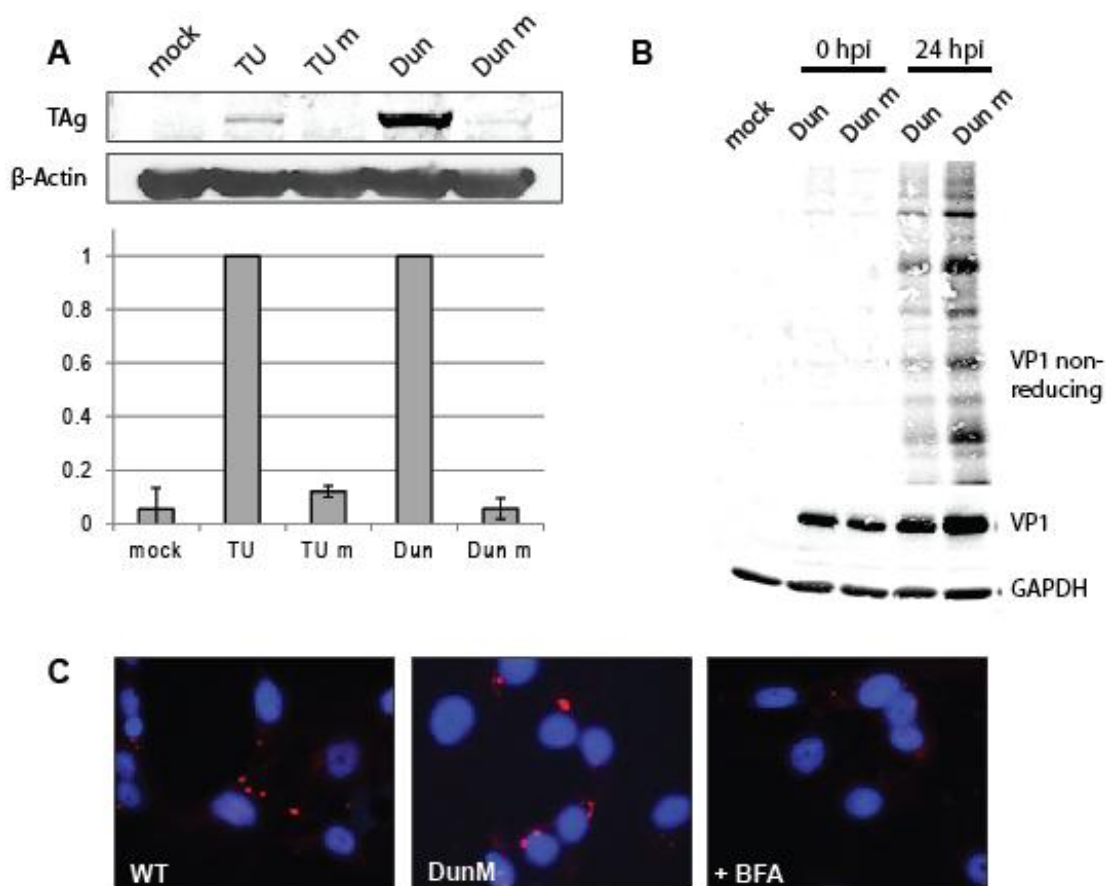
Figure 3.2: The minor capsid protein NLS is important during infection of RPTE cells. (A) RPTE cells were infected with 10 thousand genomes/cell of mutant or wild-type virus in the Dunlop (dun) or TU backgrounds then harvested at 24 hpi, run on SDS-PAGE and the western blot was probed for TAg. The TAg levels from three independent experiments were measured using the Odyssey system and displayed on the graph (below). (B) RPTE cells were infected with 1000 genomes of mutant or wild type purified Dun virus and then harvested under alkylating conditions at 0 hpi or 24 hpi, and resolved by non-reducing or reducing SDS-PAGE, probing for VP1. (C) RPTE cells on coverslips were infected with 10 thousand genomes/cell of purified mutant or wild-type Dun virus and fixed at 24 hpi followed by staining for VP2/3 (red). One set of cells was infected with wild-type Dun and treated with BFA for 2 hpi immediately following adsorption of the virus.

of VP1 that enters the gel because all of the virus is still fully assembled. The VP1 disassembly intermediates were seen for both the wild type and mutant virus at 24 hpi (Figure 3.2B), suggesting that the mutant is able to disassemble in the ER similar to wild type virus. As another way to test trafficking of the mutant virus through the ER, the exposure of the minor capsid proteins was assessed. As the virus particle becomes partially disassembled in the ER, VP2 and VP3 become accessible to antibody recognition, and thus immunofluorescence detection of the minor capsid proteins is another readout for disassembly. For both mutant and wild type BKPyV, staining against VP2/3 showed similar levels of puncta throughout the cells, in contrast to cells treated with the trafficking inhibitor, Brefeldin A (BFA) (Figure 3.2C). Together these data show that disassembly and ER trafficking is still occurring during entry of the NLS-mutant virus. Therefore, the defect is likely occurring at a step between disassembly and nuclear entry.

NLS mutation of the minor capsid proteins does not inhibit assembly of pseudovirions

Our next interests were in determining the role of the NLS of the individual minor capsid proteins during infection. Because the minor capsid proteins are encoded in the same reading frame, any mutation in the C-terminal sequence of one minor capsid protein will be shared by the other. To be able to manipulate the sequences of the minor capsid proteins individually, we turned to a pseudovirus system previously developed for the study of BKPyV(14). Pseudovirus particles are produced by over-expression of individual capsid proteins in 293TT cells, where they form capsid structures similar to wild type viruses, and can encapsidate a co-transfected reporter plasmid. It has been reported that pseudovirions of JC Polyomavirus, a closely related virus, behave identically to authentic virus during cellular entry and trafficking (15). Because the capsid proteins are expressed from separate expression plasmids, they can be individually

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A

B

VP1	wt	wt	wt	wt
VP2	wt	K319T	wt	K319T
VP3	wt	wt	K200T	K200T

VP1

VP2

VP3
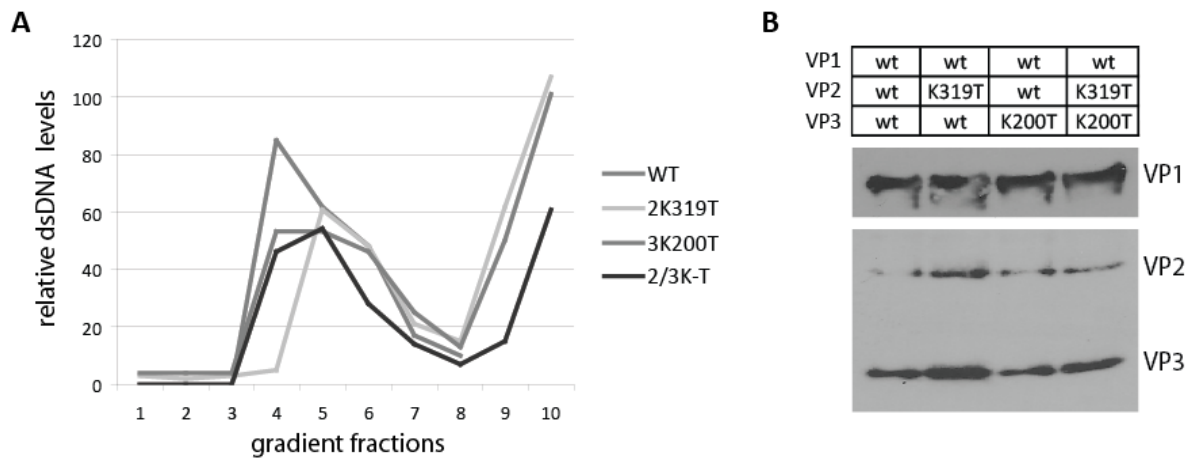
Figure 3.3: Wild-type and pseudovirion variants with NLS mutations form similar, non-empty particles. (A) After pseudovirions were purified by optiprep gradient, a picogreen assay was performed on each fraction from the gradient to measure double-stranded DNA. (B) Equal genomes of purified pseudovirion variants were resolved by reducing SDS-PAGE and probed for each of the capsid proteins.

manipulated. We transfected combinations of wild-type and NLS-mutant capsid expression

vectors into 293TT cells along with a GFP reporter plasmid, and then we harvested and purified

the pseudovirus particles by optiprep gradient. Wild-type, VP2 NLS-mutant, VP3 NLS-mutant,

and double VP2/VP3-NLS mutant variants were created. During purification, each pseudovirus

showed a band at approximately the same density in the optiprep gradient (data not shown), and

the fractions from each gradient showed a peak of DNA at similar densities (Figure 3.3A),

implying the reporter plasmid was encapsidated into pseudovirus particles. Additionally, the

purified pseudovirion variants showed similar levels of each capsid protein by SDS-PAGE when

equal genomes were loaded on the gel (Figure 3.3B). These data show that the defects in nuclear

localization of the minor capsid proteins do not affect pseudovirus particle production.

The VP3 NLS but not the VP2 NLS is important in 293TT cells

To evaluate the role of the individual minor capsid protein NLS during entry, we infected

293TT cells with each of the pseudoviruses at equal GFP genomes per cell, then harvested

protein at 48 hpi to measure GFP production. Because the reporter plasmid has an SV40 origin

and the 293TT cells overexpress SV40 TAg, low levels of the GFP reporter are amplified,

making the signal easier to detect. Interestingly, GFP levels as measured by western blot showed

that pseudovirus with the VP2K319T mutation were as infectious as wild type, whereas the

variants with the VP3K200T mutation and the double VP2/3KtoT mutations were attenuated by

about 70% (Figure 3.4A). These data suggest that the NLS on VP3 is important for entry, while

the NLS on VP2 is dispensable.

Next we were interested in validating this finding in RPTE cells. We infected RPTE cells

with equal genomes of pseudovirus and harvested lysates at 48hpi, followed by assessment of
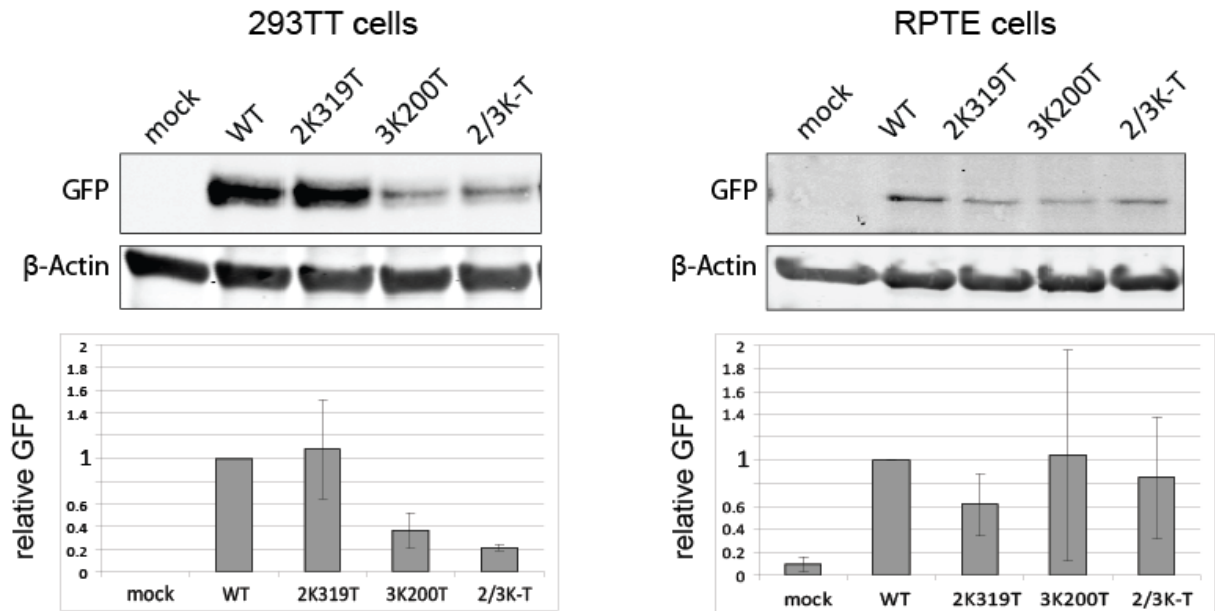
Figure 3.4: Pseudovirion variants with a VP3 NLS-mutation are defective for entry in 293TT cells. (A)293TT cells were infected with 1000 genomes/cell of pseudovirion variants and harvested at 48 hpi. Whole cell lysates were resolved and probed for GFP. A representative western blot is shown, and average GFP levels averaged from three independent infections and measured using the Odyssey system are graphed below it. (B) RPTE cells were infected with 10 thousand genomes/cell of pseudovirions and whole cell lysates were harvested at 48 hpi. GFP levels from western blotting were measured using Odyssey, averaged from three independent experiments, and graphed below a representative western blot.

GFP expression by western blot. Interestingly, GFP levels, while near the limit of detection, were similar for all four pseudoviruses (Figure 3.4B). This result is in contrast with the data from 293TT infection and the mutant virus, suggesting there might be cell type differences in trafficking and nuclear entry during pseudovirion infection.

Discussion

Polyomaviruses are abundant human pathogens whose infection can range from persistent and asymptomatic in healthy individuals to manifesting into devastating disease in immunocompromised patients, and at least one recently discovered polyomavirus is oncogenic (16). The cellular entry pathway of polyomaviruses is only partially understood, and many details remain to be elucidated. Previous studies have provided evidence that the virus is internalized and travels through endocytosis to the ER, where disassembly begins. After ER trafficking, the virus must get into the nucleus, and we and others have provided a model in which polyomaviruses exit the ER into the cytosol before nuclear entry, using the host pathway of ER-associated degradation. However, the mechanism of polyomavirus nuclear entry, and specifically BKPyV nuclear entry, has so far remained ill-defined. In this report, we have provided evidence that the nuclear localization signal on the minor capsid proteins of BKPyV is important during entry, suggesting that after the virus particle exits the ER, the minor capsid proteins play a vital role in the process of nuclear import.

Extensive work has previously been done on the nuclear localization properties of SV40 viral proteins, since the canonical NLS was first discovered on SV40 large TAg. Each of the capsid proteins separately were shown to localize to the nucleus and the necessary amino acid sequences were identified to include a QPNKKRR sequence (8). BKPyV has extensive

homology with SV40 and a number of conserved motifs, but the localization properties of BKPyV VP2 and VP3 have never been assessed, particularly in the context of infection and in a natural host cell, such as RPTE cells. We have demonstrated the nucleophilic property of both BKPyV VP2 and VP3 during overexpression in RPTE cells, and subsequently identified a lysine in the basic C-terminal sequence that was vital for this localization. Of note, expression of VP2 or VP3 alone was low in RPTE cells, but this is likely due to their lytic properties. This idea is supported by the observation that more VP2/3 positive cells were detected at 24 h after transfection than at 48 h.

The identification of a lysine required for nuclear localization allowed us to create a mutation in the genome, changing the amino acid in both VP2 and VP3, to test the importance of the NLS of the minor capsid proteins during entry. Because the presence of wild-type VP1 allows for nuclear import of nuclear localization-deficient VP2 or VP3, we could not assess the localization defect during expression from the viral genome, since VP1 is expressed concurrently with VP2 and VP3. However, we concluded that there was an obvious defect during entry, as determined by decreased levels of TAg expression from the mutant at 24 h, which is prior to the onset of viral DNA replication and capsid protein expression (unpublished data). Therefore, cause of lowered TAg levels would most likely be at the step of nuclear entry of the genome. We know the defect is not at an earlier trafficking step because VP2/3 exposure in the ER still occurs, and the capsid becomes reduced in the same way as wild-type.

The effect of the analogous mutation in the double-mutant pseudovirions in 293TT cells supports a role for the minor capsid protein NLS during entry as well, since pseudovirions only undergo single-round infection. Interestingly, results from the 293TT cells infection support a role specifically for the VP3 NLS during entry, since the mutation in VP2 alone had no

significant effect on GFP expression. However, the result of infection in RPTE cells with the pseudovirion variants complicates this conclusion, since there were no obvious differences in GFP expression levels between wild-type and mutant. An explanation for this may lie in potential differences between virus particles and pseudovirus particles; even though they are morphologically similar (17), since pseudovirion particles are formed under different conditions than in a normal infection, there could be variations in inter- and intra-pentameric disulfide bonds and/or Ca^{2+} ion stabilization. These differences could lead to different disassembly steps and subsequent trafficking into the nucleus in RPTE but not 293TT cells. Many differences exist between the two cells types, since 293TTs are immortalized and RPTEs are differentiated primary cells, and it is likely that nuclear import factors vary as well (18, 19). Additionally, 293TT cells over-express SV40 TAg, leading to replication of the GFP reporter plasmid and amplification of any differences; thus, slight differences in nuclear entry in RPTE cells may just be below significance in this case.

A possibility that we cannot rule out is that the basic lysine residue in the NLS is needed for early gene transcription after the genome reaches the nucleus. It is unclear whether any or all of the capsid proteins accompany the viral minichromosome into the nucleus, although it has been shown for SV40 that the minichromosome alone is insufficient for nuclear import (20). Also, micro-injection of anti-VP3 antibodies into the cytoplasm or the nucleus of infected cells prevented infection with SV40 (20), suggesting that one or both minor capsid proteins play a role in nuclear import and/or post-nuclear entry steps.

The data in this report also suggest the possibility of an alternative nuclear entry pathway, since the NLS mutation only led to attenuated infection. One alternative way for cytosolic viruses to get into the nucleus is during mitosis, when nuclear envelope breakdown occurs. This

pathway could be evaluated by testing entry during inhibition of cell division. Additionally, it is possible that the virus still has use of an NLS. It is known that VP1 contains an NLS in addition to VP2 and VP3 (21, 22), but this motif is thought to be buried within the particle such that entry would rely on the other capsid proteins. However, it is possible that this NLS is available on BKPyV particles during entry in RPTE cells, or that another NLS exists in the context of the virus structure that does not exist when VP2 and VP3 are expressed individually.

In summary, this report shows the relevance during infection of a specific basic lysine in the C-terminus of the minor capsid proteins that is vital for nuclear import of the individual minor capsid proteins. Additionally, we show here the first evidence of a requirement for the VP3 NLS specifically, suggesting the VP2 NLS is redundant and VP2 serves additional functions during trafficking, upstream of nuclear import. Our data suggest a model where partially disassembled BKPyV exits the ER into the cytosol, where the exposed NLS of VP3 is used to bring the viral genome into the nucleus through the nuclear pore complex.

Materials and Methods

Cell Culture. RPTE cells were grown in renal epithelial basal growth medium (REGM) with SingleQuots Bulletkit from Lonza at 37°C and 5% CO_2 and passaged up to six times as previously described (23). The 293TT cell line was maintained and not passaged more than 25 times in DMEM with 10% heat-inactivated fetal bovine serum (HyClone), 100 U/ml penicillin, and 100 μg/ml streptomycin (Cambrex) in the presence of 40μg hygromicin to maintain selection.

Transfection. The VP2 and VP3 expression plasmids were obtained from Addgene, Cambridge, MA (plasmid #s 32109, 32110). The VP1 expression plasmid was obtained from Christopher Buck, NCI, Bethesda, MD. RPTE cells in a 12-well were transfected using 3μl Mirus LT-1 tansfection reagent, and 1 μg plasmid DNA following manufacturer's instructions. LT-1 was mixed with 100μl Opti-mem and incubated 15 min before adding the DNA and incubating 25 min at room temperature. To create pseudovirions, a T75 flask of <50% confluent 293TT cells were transfected with 13μg VP1 plasmid, 13μg of GFP reporter plasmid, 6.5μg of VP2 plasmid, and 6.5μg of VP3 plasmid using 85μl lipofectamine 2000. DNA and transfection reagent were incubated in 2ml Opti-mem separately for 25 min, then combined and incubated 45 min at room temperature before adding to the cells.

Pseudovirion production. 293TT cells were harvested 3 days post transfection and pseudovirions were purified using a slightly modified version of the previously published method. Briefly, transfected cells were trypsinized, pelleted, and resuspended in DPBS-Mg in a siliconized tube. The cell pellet was treated with 1 U/ml of neuraminidase for 15 min at 37°C, followed by treatment with 1/20th volume triton X-100 for 15 min at 37°C to lyse the cells. Then 1/40th volume 1M ammonium sulfate pH 9 was added along with 0.1% Benzonase and 0.1% Plasmid Safe and incubated about 24 h at 37°C for maturation. Following maturation, lysate was chilled on ice 5 min, 0.17 volumes of 5M NaCl were added, and lysate was incubated 20 min on ice. The lysate was clarified by centrifugation and extraction step repeated with 2 volumes 0.8M NaCl-DPBS, combined with first clarified lysate and re-clarified. The lysate was then layered on an optiprep gradient of 27%, 33%, 39%, and spun for 4.75 h at 49,000 rpm. Approximately ten

fractions were collected from each gradient. Picogreen assay was performed using manufacturer's instructions and 20 µl of each optiprep fraction.

Infections. Purified virus BKPyV (Dunlop variant) was propagated and purified as previously described (23). Crude TU, Dunlop, and mutants consisted of RPTE lysates that were infected for two weeks with a blind passage of RPTE cells that had been transfected with re-circulared viral genomes and harvested after 10 days. Before titering, crude stocks were freeze-thawed three times. Infections were done in REGM or serum-free DMEM at 4°C for one hour, and then the inoculum was washed off and replaced with warm media with serum and placed at 37°C for the indicated time frame.

Quantitative PCR (qPCR) titering of genomes. To calculate genomes per volume, 5 µl of virus or pseudovirus was treated with 2 µl DNAse for 1 h at 37 C, then treated with 50µg proteinase K for 1 h at 37, followed by isolation of the viral genomes by a PCR purification column. Dilutions of the isolated DNA were used for qPCR and final concentrations were back-calculated to get genomes per ml. For BKPyV, qPCR was done using primers 5' TGTGATTGGGATTCAGTGCT 3' and 5' AAGGAAAGGCTGGATTCTGA 3'in the TAg open reading frame. For pseudovirions, primers 5'AGAACGGCATCAAGGTGAAC 3' and 5'TGCTCAGGTAGTGGTTGTCG 3' against the GFP open reading frame were used.

Preparation of cell lysates. Whole cell protein lysates were harvested in E1A buffer (50mM HEPES pH 7, 250 mM NaCl, 0.1% NP40, with protease inhibitors 5 µg/ml PMSF, 5 µg/ml aprotinin, 50 mM NaF, 0.2 mM Na-orthovanadate, and 5 µg/ml leupeptin added right before

use). For alkylating conditions, 20mM N-Ethyl Maleimide (NEM) was added to the E1A buffer.

Protein concentrations were determined using a Bradford assay after clarifying the lysate.

Western Blots. Equal amounts of protein were resolved by SDS-PAGE on a 10% gel (8% for

TAg), under reducing or non-reducing conditions and transferred overnight onto a nitrocellulose

membrane at 60 V by wet transfer. Membranes were blocked in 2% nonfat dry milk in PBS

containing 0.1% Tween 20 (PBS-T). Antibodies were used at the following concentrations

diluted in PBS-T: anti-TAg 1:3000, anti-VP1 1:10,000, anti-VP2/3 1:3000, anti-GFP 1:2000,

anti-B-Actin 1:5000, anti-mouse 1:5000, anti-rabbit 1:5000. For western blots analyzed by the

Odyssey system, membranes were blocked in Odyssey blocking buffer and probed with the

indicated primary antibodies or anti-mouse or anti-rabbit secondary antibodies diluted in

Odyssey blocking buffer with 0.1% Tween 20.

Immunofluorescence microscopy. At 24 hours post transfection, cells were fixed with cold

95% EtOH/ 5% acetic acid. Cells were blocked with 5% goat serum in PBS, then coverslips were

co-stained for indirect immunofluorescence with 1:500 dilution of anti-VP2/3 in goat serum.

Secondary antibodies Alexa-Flour® 594 goat anti-rabbit IgG or Alexa Fluor® 488 goat anti-

mouse (Invitrogen) were used at a 1:200 dilution. Cells were imaged with an inverted

fluorescence microscope.

Notes

Cathy Bosard performed site-directed mutagenesis reactions, standard PCR, and assisted with other aspects of the experiments. This work will be submitted for publication as part of a larger manuscript in early 2014.

Acknowledgments

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CHAPTER IV

Discussion

Summary and Model of BKPyV trafficking

The findings presented in this dissertation answer many questions about how BKPyV reaches the nucleus and provide a more solid model for the BKPyV intracellular trafficking pathway in RPTE cells (Figure 4.1). I have provided evidence for a trafficking pathway where BKPyV travels to the ER and uses the ERAD system to get out of the ER and into the cytosol. From there, the virus is imported into the nucleus by way of the NLS on the minor capsid proteins. I also have evidence that multiple other trafficking pathways may exist, including non-productive pathways and alternative nuclear entry pathways. These experiments were carried out in RPTE cells, a cell culture system that involves primary kidney cells, a cell type known to be infected with BKPyV in diseased patients, making these results translatable to lytic infection *in vivo* (1).

Polyomavirus ER membrane penetration

In Chapter 2, we investigated the role of ERAD machinery and the proteasome during the course of BKPyV infection in RPTE cells. Before these studies began, there was much evidence for involvement of ER proteins as factors in the uncoating of polyomaviruses. As reviewed in chapter one, some of these factors were chaperones known to also play a role in ER quality control mechanisms, leading to the investigation of the ERAD pathway during infection. A role
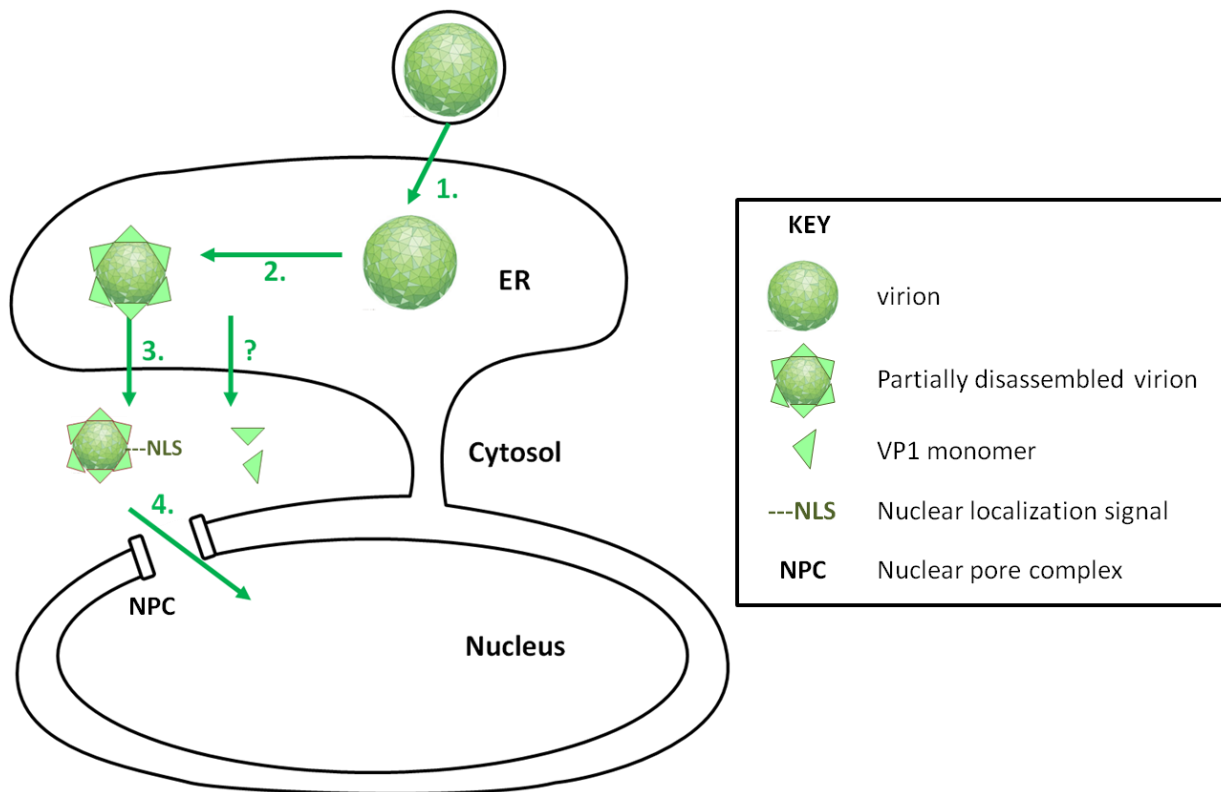
Figure 4.1. Model for BKPyV trafficking from the ER to the nucleus. First, (1) BKPyV traffics to the ER where (2) partial disassembly occurs, including reduction of disulfide bonds within and between VP1 pentamers, and conformational changes that expose VP/3 for antibody staining. After partial disassembly, (3) the particle interacts with yet-unknown ERAD components that allow it to cross the ER membrane into the cytosol. A proteasome-independent retrotranslocation mechanism also allows for VP1 protein to enter the cytosol that does not come from productive virus particles. Once in the cytosol, (4) the NLS on the minor capsid protein is used for nuclear import of the virus through the NPC, though it does not seem to be the only nuclear import mechanism.

for the ER-associated degradation pathway during polyomavirus infection was initially implied from studies of the derlin proteins and the proteasome during the course of infection (2-4).

Our investigations of proteasome function during BKPyV infection revealed that the virus relied on the ER quality control pathway of which the proteasome is a part. Inhibiting the proteasome caused an accumulation of virus within a specialized compartment within the ER called the ER quality control compartment (ERQC), normally characterized by ERAD substrates. The data suggest that the virus becomes sequestered to this compartment under conditions where the ERAD pathway is blocked. Since it must be recognized like an ERAD substrate, there is likely a hydrophobic patch or perhaps a protein-binding motif recognized by ERAD chaperones. Geiger *et al.* found that glutamic acid 17 in the N-terminal sequence of SV40 VP2 was essential for transport of the virus into the cytosol (5), and suggested that this negatively charged reside may be recognized by ERAD factors. It was also shown that SV40 requires the ER proteins BiP and Bap31 in order to get out of the ER, and these proteins colocalize with the virus in distinct puncta during infection (5). Bap31 has also been implicated in the transport of quality control substrates to the ERQC, so perhaps Bap31 is the factor that sequesters the virus in the subcompartment after proteasome function is inhibited (6). Since BiP has been shown to be required for retrotranslocation of polyomaviruses, the re-localization of the virus to the ERQC during proteasome inhibition may provide an explanation for why infection is inhibited. BiP is excluded from the ERQC, and without the required factors, polyomavirus retrotranslocation is blocked.

How the virus actually crosses the ER membrane, especially as a large intact particle, remains a mystery. The prevailing theory states that the membrane lytic properties of the minor capsid proteins allow for penetration of the lipid bilayer, and this may be assisted by the ER associated

degradation machinery and putative retrotranslocation pore to allow for the virus to pass through into the cytosol. It has been shown that both VP2 and VP3 can interact with membranes (7, 8), but since the ER membrane is not damaged during infection, it is likely that membrane insertion may be one regulated step in the translocation process. There are multiple forms of ERAD depending on whether the substrate is soluble (ERAD-L) or membrane integrated, and whether the structural irregularity is located on the lumenal (ERAD-M) or cytosolic side (ERAD-C) of the ER membrane (9). These different ERAD pathways utilize different sets of factors. It seems likely that integration of VP2 into the ER membrane might lead to recognition of the virion as a membrane-integrated substrate. The membrane-integrated viral particle may then rely on the assistance of ERAD-M. Unfortunately, this pathway is less understood than ERAD-L, and thus further progress in this field of cell biology would benefit the field of polyomavirus trafficking.

The particle that exits the ER would be larger than any normal ERAD substrate, since capsid proteins are likely to be transported along with the genome. Since the viral substrate is larger than usual, the mechanism of crossing the membrane would likely be different than that of a normal substrate. Perhaps the viral proteins facilitate the assembly of a pore-like opening that consists of both cellular and capsid protein components to make it custom size. Evidence presents a scenario where many more virus particles accumulate inside the ER lumen than eventually make to the cytosol or nucleus, so it is possible that the capsid proteins from some virus particles act as part of the pore, while a different one makes it across the membrane. Additionally, evidence shows that SV40 VP3 has the capacity to oligomerize compared to VP2 (8), so while VP2 serves the function of membrane integration, VP3 may allow penetration into the cytosol.

Another interesting question is where the energy comes from to pull the virion across the ER membrane. For normal ERAD substrates, it is believed that the process of ubiquitination and deubiquitination and the ATPase activity of p97 pull the substrate out of the membrane towards the proteasome (9, 10). Ubiquitination of viral capsid proteins has yet to be detected, however, and we found that inhibition of p97 by the small molecule inhibitor Eeyarestatin I did not prevent viral DNA from entering the cytosol. These findings suggest that the virus must rely on another mechanism for favorable movement of the particle into the cytosol, yet to be described.

An important issue that exists is the discrepancy between the appearance of VP1 monomers and viral DNA in the cytosol of different cell types. One explanation may be that disassembled VP1 monomers that dissociate from the virion during uncoating in the ER end up undergoing ERAD. With inhibition of the proteasome, perhaps in RPTE cells the VP1 monomers, likely soluble ERAD substrates, can still undergo the retrotranslocation process but are not degraded, resulting in cytosolic accumulation of the monomers. This would not be difficult to reconcile with the inhibition of retrotranslocation of the larger particle, since different machineries seem to be used. It is interesting that this does not occur for SV40 in CV-1 cells, but perhaps the ERAD machinery and requirements are different in that cell type. It is obvious that cell type differences exist, and one of these differences may be at the step of membrane penetration. A study with MCPyV showed that VP2 is not important for infection with certain cell types, while in some cell types it increases the infectivity of the virus (11). MCPyV proteins exhibit much less homology to BKPyV and SV40, and it has been shown for MCPyV that VP3 is unnecessary for infection, even though it is still encoded in the genome. It was hypothesized that the MCPyV VP1 had perhaps evolved to take over some functions of the C-terminal motifs of VP2 and VP3, making VP3 unnecessary. The VP2 of MCPyV still contains homology to BKPyV and SV40 in

the N-terminus, with the myristoylation still present, so perhaps the function of membrane integration is a cell-specific necessity, while the functions provided by VP3 are required for all cell types. This was supported in the report by showing that BKPyV VP2 and VP3 were important for all cell types, since VP1 of BKPyV has not gained the VP3-specific function (11).

While the favored model involves a mechanism where the virus particle crosses the ER membrane and enters the cytosol, another scenario may also exist where the productive virus particle penetrates the ER membrane to become a membrane-integrated complex, but does not actually enter the cytosol. Cytosolic viral components may then be only non-productive side products of a transiently permeabilized ER membrane. There is currently no evidence that the ER membrane becomes simply "leaky" based on western blots of ER proteins in the cytosolic fractions of infected cells, but it may be at an undetectable level. The membrane-associated particle would still rely on the nuclear pore complex in order to make it inside the nucleus, but transport would occur in the plane of the ER membrane, interacting with transmembrane factors that are used for trafficking of inner nuclear membrane proteins. We tested whether one of the factors, sec61β, implicated in ER membrane - to - inner nuclear membrane transport of epidermal growth factor receptor, was involved during BKPyV infection using siRNA knockdown (12). However, there was no negative effect on TAg expression when sec61β protein levels were decreased (Figure 4.2). This result may rule out the sec61β-mediated route during infection, or it may be that low levels of sec61β protein provide enough function to allow infection.

Penetration of the limiting membrane is a shared obstacle for all nonenveloped viruses. Adenovirus must escape the endosome into the cytosol, and the current model for this is the release of a viral protein that acts as a lytic factor, disrupting and ultimately bursting the
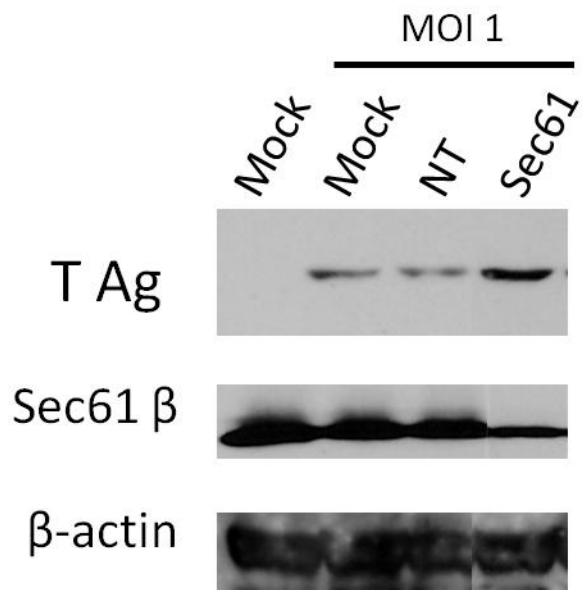
Figure 4.2. Sec61B knockdown does not block BKPyV infection. RPTE cells were tranfected with non-targeting (NT) or sec61β siRNA or mock transfected for 2 days, then infected with BKPyV at MOI 1 for 24 h. Whole cell lysates were harvested and resolved by SDS-PAGE and probed for TAg, Sec61 β, and β-actin. Results are representative of three independent experiments.

endosome (13). This large disruption is necessary because the partially disassembled adenovirus particle is relatively large at 90 nm. Since endosomes are smaller transient organelles, using this system to cross the membrane would not harm the cell. Disrupting the ER membrane would be quite harmful for a cell and would not be beneficial for a virus like BKPyV that takes at least 48 hours to produce progeny. Other potential mechanisms of membrane disruption include the formation of pores or transient local disruption of the lipid bilayer. Parvovirus is believed to cause a transient membrane disruption using the phospholipase 2 domain in its capsid protein, allowing penetration of the virus without leakage of any other substances from the compartment (13). However, the mechanism has not been fully described. Understanding the mechanics of viral protein membrane penetration is an area where there is much still to be understood.

Nuclear entry mechanism

In Chapter 3, I showed that mutation of a residue in the minor capsid proteins important for nuclear localization led to an attenuation of virus infectivity, implying a role during entry. This finding complements a model where BKPyV traffics through the cytosol, interacts with the canonical nuclear import machinery using the NLS on the minor capsid proteins, and is imported through the nuclear pore complex. If this pathway was vital during infection, it would suggest that the direct ER–to–nucleus alternative trafficking route could be ruled out, unless both pathways exist in order to provide the most opportunity for the virus to reach the nucleus. However, neither pathway can be ruled out at this time, since the mutations that we created caused only an attenuated phenotype and did not prevent infection. One possible explanation for this is that the NLS can still be used but to a lesser degree. Since the virus particle would likely still consist of multiple copies of the minor capsid proteins, together they may act to increase the

nucleophilic potential of the entire particle. Another explanation may be that there is another NLS that exists. This possibility was ruled out when the cytoplasmic localization of the mutants was verified by transfection, but another NLS may exist in the structural context of the particle. VP1 has an NLS, but it is believed it is not available during entry based on conclusions from studies of SV40 (14). Perhaps in BKPyV, this NLS is more available to interaction with importins. A third explanation may be that another pathway to the nucleus exists that is independent of the use of an NLS. Dividing cells experience nuclear envelope breakdown, such that any cytosolic virus could access the nucleus during the process of mitosis. Additionally, a route directly across the inner nuclear membrane has not been ruled out, and this would be an NLS-independent mechanism.

The use of an NLS implies that importin β is involved, and interestingly, importin β has been implicated during retrotranslocation of ERAD substrates as it was found to interact with the ERAD machinery (15). This finding may suggest that the trafficking of BKPyV from the ER to nucleus is coupled in a way to provide some kind of favorable movement out of the ER into the nucleus. If we consider the possibility that VP3 plays a separate role from VP2 membrane integration, then the attenuation of the VP3 NLS-mutant pseudovirion variant in 293TTs might be explained. Perhaps the VP3 NLS is required for interaction with the nuclear import machinery because VP2 is performing an upstream function within the ER. The function of the VP3 NLS could be simply for nuclear import, or perhaps it interacts with importin β in the context of ERAD to pull the virus out of the ER. It is unclear, however, why the pseudovirion variants did not exhibit these differences in RPTE cells.

Other viruses that must access the nucleus have shown us that use of the NPC or other aspects of the canonical import pathway do not imply that all aspects of the pathway are used. Hepatitis

B virus (HBV) was found to use the canonical nuclear import pathway, including importin β mediated nuclear transport, but not RanGTP. HBV capsid protein interacts with Nup153, which displaces the interaction with importin β in the same way as normally occurs with RanGTP interaction with importin β (16). Adenovirus uses the nuclear pore complex, but not importins; nuclear entry was found to be combined with final steps of genome uncoating, such that disassembly of the capsid is combined with removal of certain NPC components. Ultimately, nuclear envelope permeability is increased, allowing access of the adenovirus genome into the nucleus (17).

We attempted to test the importance of the canonical nuclear import pathway during entry by treating infected cells with the small molecule inhibitor Importazole, which was identified in a screen and prevents the interaction of importin β and RanGTP (18). Interestingly, treatment with the inhibitor prevented infection, but at an early step in entry preceding ER trafficking. Additionally, we found that adding Importazole any time after 6 hpi was no longer inhibitory, implying that the early step was the only step in which the drug was preventing the infectious route. Since there have been relatively few studies with this new small molecule inhibitor, this finding suggests that there may be off-target effects besides the RanGTP-importin β interaction. Alternatively, a very early signaling event necessary for infection and reliant on RanGTP-importin β is perhaps being inhibited. If RanGTP is not required during infection, this may provide evidence against the canonical nuclear import pathway, or it could be that a BKPyV-specific nuclear import pathway exists like that of adenovirus or HBV.

Alternative and non-productive trafficking routes

From the presence of VP1 cleavage products over time, cytosolic VP1 monomers appearing in a disproportionate levels with cytosolic DNA, and a high particle to infectivity ratio, it is obvious that there are a considerable number of non-productive paths during BKPyV infection of RPTE cells. It seems that there is a limiting step during the trafficking of the virus towards the nucleus, since we detect such low levels of viral DNA in the cytosol. Some virus seems to accumulate as a result in the ER, and we have evidence of this since we can see VP2/3 puncta staining throughout the cell even after DNA replication has started at 48 hpi. It is unclear what occurs with this lingering virus once the minimum infectious viral genomes reach the nucleus. Can late-arriving viral genomes continue on to nuclear entry to follow the normal infection cycle if the infection-specific changes have begun in the nucleus? If the ERAD pathway already recognizes viral proteins, the viral particles may just continue to undergo retrotranslocation into the cytosol. Once in the cytosol, viral proteins may be degraded by the proteasome or continue on to the nucleus. Additionally, cytosolic viral aggregates may be cleared by autophagy.

The other mechanism of degradation and cleanup used by the cell is lysosomal degradation. It seems very likely that a portion of incoming virions may end up degraded by the lysosome, since the endocytic pathway used by polyomaviruses is thought to continue to the late endosomes and endolysosomes (19). If the endocytosed virions do not reach the ER, then they likely go on to be degraded in the lysosome. This conclusion is supported by our trafficking studies of BKPyV using the less-specific proteasome inhibitor MG132, which is known to also inhibit certain cysteine proteases located in the lysosome. Treatment with MG132, compared to other specific proteasome inhibitors epoxomicin or lactacystin, causes a larger amount of VP1 monomers to appear in both the cytosolic and pellet fractions after cytosolic fractionation. This may be due to escape of virions from lysosomal degradation. There is no corresponding increase in infection,

however, likely due to the additional inhibition of proteasome activity. Previous efforts by the lab to identify the source of the VP1 cleavage products initially implicated cathepsins, but no increase or decrease in infection occurred under cathepsin inhibitor treatment.

Future directions

One of the biggest issues in the field has been to determine the infectious route to the nucleus, and it has yet to be determined whether the apparent cytosolic pathway is the one and only route. It has been attempted to address trafficking questions through EM studies of infected cells, looking for visual clues as to the route of virus entry. One ultrastructural study of a nephropathy biopsy showed that there are many virus particles in the ER and virus aggregates in the cytoplasm, and there are disturbances in the nuclear membrane, but no sign of viruses entering directly through the nuclear envelope or through the nuclear pore (20). These observations from EM studies have yet to be reconciled with results from biochemistry and cell biology studies. Theoretically it should be possible to gain insight from direct visualization of infecting viruses, but with the high amount of nonproductive pathways followed by BKPyV virions, it creates the task of finding a needle in a haystack. Biochemical methods have the problem of teasing apart results from productive versus nonproductive pathways. In the future, EM studies could benefit from correlative fluorescence and electron microscopy, merging what we know about the cell biology of infection with visualization of the virus particles on a cellular level to minimize the proverbial haystack. For example, we can stain for VP2/3 and BiP or Bap31, since they are believed to represent areas of retrotranslocation based on what was shown with SV40 (5). After finding areas of colocalization, we can zoom in using EM to see if one or multiple viral particles are creating a disturbance in the membrane. Additionally, we can compare what the viral

particles look like in proteasome-treated cells compared to untreated, to see if there is a difference in their pattern or structure. For treated cells, we would have to focus on virus that was not colocalized with BiP, to see how those particles that are not part of active retrotranslocation complexes interact differently with the cell. More colocalization studies may need to be done first on proteasome inhibitor-treated cells before subsequent correlative EM is used for comparison with untreated. Besides the ER membrane penetration step, other aspects of trafficking can be addressed using correlative microscopy such as nuclear entry. Co-staining can be done for VP2/3 or viral genomes along with nuclear pores or envelope markers, and any proximal or co-localizing areas can then be imaged by EM to attempt to capture the process of nuclear entry.

The mechanistic details of how BKPyV crosses the ER membrane are very intriguing and would be an area of high technical difficulty but great reward for the field of nonenveloped viruses. So far, our understanding is limited partly because the mechanism of ERAD is also not clearly understood. As has been done to elucidate the important factors in retrotranslocation of model ERAD substrates, the development of an *in vitro* membrane penetration assay for polyomaviruses would be ideal (21). Necessary cytosolic components could be identified by isolating microsomes from infected cells, and then incubating with full cytosolic extract or extract with components depleted, such as importins, other chaperones, etc. In order to evaluate the necessity for different ER lumenal or transmembrane components, the microsomes would have to be isolated from infected knockdown or knockout cells. This would be technically difficult but extremely informative.

A number of details can be attained through development of more *in vitro* assays. It would be valuable to develop a nuclear import assay in order to gain a better understanding of the nuclear

entry mechanism of BKPyV. This can be done using a previously developed nuclear import

assay where cells are grown on coverslips and permeabilized with digitonin, which will leave the

nuclear envelope and pores intact (22). Then different BKPyV substrates can be tested for import

into the nucleus, using the visualization of VP2/3 or viral genomes by fluorescence as a readout

for nuclear entry. Many questions can be asked using this assay, starting with the structure of the

particle that undergoes import. Purified virus would be treated with ER lumenal extract to mimic

the uncoating process within the ER. These disassembled particles would then be used to show

whether or not nuclear import can occur, and whether it occurs through the nuclear pore complex

by treating with the nuclear pore complex inhibitor wheat germ agglutinin. Additionally, the

cytosolic cellular factors that are required for nuclear import can be evaluated by selective

removal from the transport buffer in which the assay takes place. This can be done to evaluate

the need for importin α or β, or any other cytosolic factors that may play a role in nuclear entry.

Another interesting avenue that can be explored is the difference in trafficking between

different cell types. This could be informative in the context of *in vivo* infection. We found a

number of differences between the trafficking of BKPyV and SV40 in RPTE cells versus CV-1

cells, which differ in both species origin as well as transformed versus primary cell properties. It

may be important to look at the trafficking of BKPyV in other primary cells that may be infected

in the body, such as urothelial cells or other kidney cell types. This could be done using the same

assays as used in this thesis so that results could be directly compared. Trafficking differences

between cell types could mean differences in innate immune recognition or responses.

Interestingly, our lab found that there was no obvious immune response from BKPyV infection

at 24, 48, or 72 hpi in RPTE cells through microarray analysis (23). Perhaps BKPyV cleverly

evades immune detection in that cell type but does not in others. It would be important to

determine since it has been suggested that the immune responses, although it was adaptive immunity that was implicated, are thought play an important role in pathogenesis in a mouse model of PVAN (24). The findings from this study were interesting because high virus titers did not correlate with disease state, suggesting that the virus itself is not enough to cause the damage seen in diseased tissue. Besides the differences in cell entry or immune responses, different cell types may be important to study for the sake of understanding persistence and reactivation of BKPyV in vivo.

Significance for the Field

Before this work began, one of the major questions in the field was whether polyomaviruses indeed traveled through the cytosol and if this was a productive pathway. Previous studies with SV40 had suggested that the virus could traffic from the cytosol into the nucleus, since microinjection of virus into the cytoplasm was able to result in infection, and injection of antibodies into the nucleus prevented this infection (25). Since the injected virus would theoretically not have undergone the disulfide bond reduction and isomerization within the ER, it was curious how the viral genome would have come to be uncoated for transcription and replication. Development and advancement in biochemical approaches has provided more techniques for addressing intracellular trafficking questions and applying these questions to viruses. The digitonin-based retrotranslocation assay, first developed to show retrotranslocation of ERAD substrates (26), allowed a number of groups including us to show the presence of polyomavirus in the cytosol, a long sought-after question. There is no doubt that more questions will be cleared up about polyomavirus entry with more sophisticated research techniques.

Besides the contributions this work has provided about polyomavirus trafficking in general, this is the first analysis of BKPyV trafficking through the cytosol in a natural host cell type. Most of the previous data from which we have made inferences came from studies of SV40 in transformed cell lines. For that reason, this study includes the most relevant data about polyomavirus cytosolic trafficking in a natural host cell, as well as the most current data about BKPyV-specific trafficking. Furthermore, the different results attained in RPTE cells as compared to CV-1 cells and 293TT cells have demonstrated that important differences exist in different cell types, and these differences represent not only important variables to consider for experiment design, but also that different cell types in the body may play different roles during viral persistence and reactivation in the host.

It could be said that many more questions were raised from the work in this dissertation than were answered. This work verified the cytosolic trafficking of BKPyV at the same time as other work was published showing the cytosolic trafficking of SV40, along with confirmation of the ERAD pathway's involvement during entry and the potential role for the NLS of the minor capsid proteins. Additionally, the findings and discussions throughout these chapters raise important issues of cell type differences in the trafficking pathways and the always present question of whether it is a productive pathway that is being examined. These are vital points that are important for the field of polyomavirus trafficking and will allow future research to move forward more quickly towards a better understanding of polyomavirus infection.

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