Early Events during BK Virus Entry and Disassembly

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BK virus (BKV) is a nonenveloped, ubiquitous human polyomavirus that establishes a persistent infection in healthy individuals. It can be reactivated, however, in immunosuppressed patients and cause severe diseases, including polyomavirus nephropathy. The entry and disassembly mechanisms of BKV are not well defined. In this report, we characterized several early events during BKV infection in primary human renal proximal tubule epithelial (RPTE) cells, which are natural host cells for BKV. Our results demonstrate that BKV infection in RKPE cells involves an acidic environment relatively early during entry, followed by transport along the microtubule network to reach the endoplasmic reticulum (ER). A distinct disulfide bond isomerization and cleavage pattern of the major capsid protein VP1 was observed, which was also influenced by alterations in pH and disruption of trafficking to the ER. A dominant negative form of Derlin-1, an ER protein required for retro-translocation of certain misfolded proteins, inhibited BKV infection. Consistent with this, we detected an interaction between Derlin-1 and VP1. Finally, we show that proteasome function is also linked to BKV infection and capsid rearrangement. These results indicate that BKV early entry and disassembly are highly regulated processes involving multiple cellular components.
elaborate tubular network that is in close proximity to, and/or continuous with, the Golgi apparatus or rough endoplasmic reticulum (ER) (10). Consistent with this observation, labeled BKV has been found to colocalize with an ER marker in cells infected in culture (40). There are still many gaps, however, in our knowledge of the intracellular trafficking pathways utilized by BKV.

A major question that remains unanswered is when and how the BKV capsid disassembles. The very low number of intact intranuclear virions compared to the number of large perinuclear viral aggregates observed by electron microscopy (EM) supports the idea that viral uncoating occurs before nuclear entry (10), although direct evidence is still lacking. For both SV40 and MPyV, the ER compartment is suggested to be the initial disassembly site (16, 29, 35, 41, 48). The ER protein disulfide isomerase or other protein disulfide isomerase-like proteins can cause conformational changes in VP1 molecules, leading to further membrane penetration or virion disassembly (35, 48). Furthermore, proteins involved in the ER-associated protein degradation (ERAD) pathway, which normally moves misfolded proteins from the ER for proteasomal degradation, have been implicated in polyomavirus trafficking (29, 48). This raises the possibility that the ERAD machinery is hijacked by polyomaviruses to escape from the ER prior to the delivery of genomes to the nucleus.

Many early BKV trafficking studies were performed in monkey-derived Vero cells (12). More recently, an in vitro cell culture system was established that allows the study of BKV lytic infection in primary human renal proximal tubule epithelial (RPTE) cells, a major cell type that BKV infects in vivo (33). BKV is able to replicate efficiently and produce infectious viral particles in these cultures (33). Therefore, this system provides an excellent model to study BKV infection in its natural host cell type, which is important because viral trafficking mechanisms are often cell type dependent. For example, both SV40 and MPyV have been shown to enter host cells by either caveola-dependent or -independent pathways, depending on the cell type used (6, 17, 43, 46). There are also contrasting results obtained in different cell types regarding the cytoskeleton requirements for SV40 or MPyV trafficking (17, 18, 44, 46, 49). It is crucial, therefore, to unravel the life cycle of BKV in its in vivo target cells.

In this report, we characterize the early entry and disassembly processes of BKV in RPTE cells and the potential contributions of the host machinery during these events. We show that BKV trafficking is highly regulated, with strict temporal and spatial requirements, including a low-pH step and the involvement of the ER. BKV displays a distinct capsid rearrangement and VP1 cleavage pattern during the course of infection, which is also associated with multiple cellular compartments. Finally, we find that components of the ERAD pathway are involved in BKV infection. Taken together, these results suggest that BKV employs specific trafficking pathways, which rely on various cellular components, to establish a productive infection.
sodium orthovanadate. Protein concentrations were determined by a Bio-Rad protein assay before Western blot analyses.

GST pull-down assay. E. coli cells expressing GST or GST-VP1 were induced with 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at 16°C overnight. The cultures were harvested, resuspended in lysis buffer B (PBS, 0.1% β-mercaptoethanol, 0.1% Triton X-100, 2 mM EDTA, 1X protease inhibitor cocktail), and sonicated twice for 5 s at the setting of 2, using a 550 Sonic Disembrator sonicator (Fisher). The lysates were cleared by centrifugation in a microcentrifuge at maximum speed for 5 min at 4°C and then incubated with 30 μL 50% glutathione Sepharose 4B beads (GE Healthcare) in a total final volume of 500 μL PBS. The proteins were allowed to bind to the beads at 4°C for 1 h by end-over-end rotation, and the beads were pelleted at 500 × g for 5 min. The beads were washed three times with PBS, and the proteins were eluted in 40 μL 2× sodium dodecyl sulfate (SDS) sample buffer (100 mM Tris [pH 6.8], 4% SDS, 20% glycerol, 200 mM dithiothreitol, 0.04% bromophenol blue). The proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE), and the gels were stained with ProtoBlue Safe (National Diagnostics). The amounts of the bacterial lysates bound to the beads were adjusted such that similar levels of GST and GST-VP1 were used in the pull-down reactions. To perform pull downs, RPTE cells were lysed in lysis buffer C (150 mM CH₃COOK, 4 mM MgCl₂, 30 mM Tris [pH 7.6], 10 mM NEM, 1% NP-40, 1X protease inhibitor cocktail) at 4°C for 30 min and cleared by centrifugation at 16,000 × g for 10 min. GST- or GST-VP1-bound beads were incubated with 500 μg of RPTE cell extract by end-over-end rotation at 4°C for 2 h. Beads were then washed three times with wash buffer (150 mM CH₃COOK, 4 mM MgCl₂, 30 mM Tris [pH 7.6], 10 mM NEM, 1% NP-40, 1X protease inhibitor cocktail). Proteins were eluted with 40 μL 2× SDS sample buffer and subjected to Western blot analysis.

Western blotting. Equal amounts of proteins were separated by reducing or nonreducing SDS-PAGE and transferred to a nitrocellulose membrane overnight at 60 V using wet transfer at 4°C. The membrane was subsequently blocked for 1 h at room temperature or overnight at 4°C in 5% nonfat dried milk in PBS containing 0.1% Tween 20 (PBS-T). After blocking, the membrane was incubated with primary antibodies diluted in 5% nonfat dried milk in PBS-T for 1 h at room temperature. The following antibody concentrations were used: PAb416 (1:3,000); PAb240 (1:1,000); PAb242 (1:1,000); PG68 (1:500); PG98 (1:500); PG6484 (Abcam) (1:1,000); anti-β-actin (Sigma) (1:5,000); actinomycin D (Sigma) (1:5,000); anti-glyceraldehyde-3-phosphate dehydrogenase, 1:10,000. The membrane was then washed three times with PBS-T and incubated with horseradish peroxidase-conjugated sheep anti-mouse antibody (Amersham) at a 1:5,000 dilution in 5% nonfat dried milk in PBS-T at room temperature for 1 h. The membrane was washed with PBS-T three times and developed using either enhanced chemiluminescence reagents (GE Healthcare) or luminol (Millipore). In the GST pull-down experiment, the anti-Derlin-1 antibody (4) was diluted 1:2,000 in PBS-T followed by incubation with horseradish peroxidase-conjugated donkey anti-rabbit (GE Healthcare) at a 1:2,000 dilution.

Immunofluorescence microscopy. At 48 h postinfection, BKV-infected RPTE cells grown on chamber slides (Nunc) were fixed in 4% paraformaldehyde (Electron Microscopy Sciences). Following three washes with PBS, cells were permeabilized with 0.1% Triton X-100 in PBS at room temperature for 5 min. Cells were then washed twice with PBS before being blocked with 5% goat serum (Vector Laboratories, Inc.) for 1 h at room temperature. After blocking, samples were incubated with a 1:200 dilution of PAb146 (1:5,000) for VP1, 1:1,000 for VP2, 1:10,000 for GAPDH, 1:1,000 for PG6484 (Abcam) (1:1,000), 1:2,000 for DAPI (4',6-diamidino-2-phenylindole) (Vector Laboratories, Inc.) and examined by standard fluorescence microscopy using an Olympus BX41 microscope with a Plan 10X/0.25 objective. A minimum of seven random fields were counted for each sample from three independent experiments. For the fluorescent focus assay, RPTE cells plated onto chamber slides were infected with 10-fold dilutions of viral lysates for 3 days at 37°C before fixation. The titer was determined by counting nine random fields in at least duplicate wells and is expressed as infectious units per mL (IU/mL). For the Derlin-1-yellow fluorescent protein (YFP) and Derlin-2-YFP transfection/infection experiments, RPTE cells were fixed 48 h postinfection and stained for TAg as described above. YFP- and TAg-positive cells were visualized using an Olympus IX70 inverted microscope with a Plan 40X/0.65 objective. The images were analyzed with the MetaMorph Premier software (Molecular Devices).

Transfection. Transfections were performed using TransIT-TL1 reagents (Mirus). Transfection complexes were prepared according to the manufacturer's instructions with a DNA-to-transfection reagent ratio of 1:3. Cells were seeded into two-well chamber slides, and each well was transfected with 0.5 μg of DNA. Transfection complexes were removed 12 h posttransfection, and the cells were washed twice with PBS before fresh REGM was added. Cells were infected 24 h posttransfection.

RESULTS
BKV infection in RPTE cells is dependent on low-pH environment and trafficking to ER. To begin our characterization of the intracellular trafficking pathway of BKV in RPTE cells, we took a pharmacological approach by treating cells with drugs that are known to disrupt distinct cellular structures or potential virus trafficking routes. All the drugs used in this study were tested by a cell viability assay to ensure that the dose and the length of treatment did not cause significant cytotoxic effects (at least 80% of the cells were viable compared to the untreated controls) (data not shown). Among the polyomavirus family, it is known that both MPyV and JCV require a low-pH step (3, 28). In contrast, SV40 is known to enter the caveosome, a pH-neutral organelle, and its infection does not rely on an acidification step (3, 43). EM analysis of kidney biopsy samples shows that BKV is associated with caveosome-like structures (10), but in Vero cells, BKV infection is pH dependent (14). To determine the pH dependence for BKV infection in RPTE cells, NH₄Cl was used to treat the cells during the course of infection. NH₄Cl is a lysosomotropic agent that can selectively enter cellular compartments with low internal pH and elevate the pH, thereby disrupting the acidification of these structures. Immunofluorescence microscopy for the early viral protein TAg was performed 48 h postinfection to assay the effects of the drug on BKV infection. Compared to the untreated samples, there was a significant decrease in the number of TAg-positive cells when NH₄Cl was present (Fig. 1). Similar results were obtained when the cells were treated with another lysosomotropic drug, chloroquine (data not shown). The inhibitory effect of NH₄Cl was independent of MOI, as a >80% reduction in infected cells was seen at both MOIs used (Fig. 1B).

We then examined whether BKV infection requires trafficking to the ER, a pH-neutral compartment (23), as has been suggested by previous microscopy studies (10, 40). BFA is a drug that inhibits the formation of COPI vesicles, which are involved mainly in retrograde transport from the Golgi apparatus to the ER (32). Due to cytotoxic effects upon longer exposure, RPTE cells were treated with BFA for only 2 h prior to infection. Even with this short period of treatment, there was a dramatic decrease in the percentage of infected cells, as assayed by TAg immunostaining (Fig. 2). Similar to the effects of NH₄Cl, the inhibition was MOI independent (Fig. 2B). Taken together, these results suggest that an acidic environment and movement to the ER compartment are necessary for BKV infection in RPTE cells.

Time course of BKV intracellular trafficking in RPTE cells. To gain a better understanding of the kinetics of BKV trafficking, a series of time course experiments was carried out in the presence of inhibitors. Infection was synchronized by adsorbing purified virus to prechilled RPTE cells at 4°C, and entry of the virus was initiated by transferring the cells to 37°C. At various time points postadsorption, drugs were added to the cells, and total protein lysates were harvested 48 h postinfection and probed for TAg by Western blotting (Fig. 3). The low-pH requirement occurred very early during infection; by
2 h postadsorption, NH₄Cl was no longer able to inhibit TAg expression (Fig. 3A). The inhibitory effect of BFA was longer lasting, indicating that BKV reaches the ER at ~10 to 12 h postentry (Fig. 3B). Previously, it has been shown that BKV infection requires an intact microtubule network, as nocodazole, a microtubule-disrupting agent, can block TAg expression (13, 40). We therefore also included this drug in our time course experiments (Fig. 3C). The kinetics of inhibition by nocodazole was similar to that of the BFA treatment, suggesting that the virus moves along microtubules to reach the ER.

**Time course of BKV capsid rearrangement in RPTE cells.**

Molecules of the capsid protein VP1 are cross-linked through an extensive network of interpentameric and intrapentameric disulfide bonds (27, 48), and VP1 shedding or conformational changes are believed to be the first steps of the polyomavirus disassembly process (35, 48). To monitor the BKV capsid rearrangement process, we harvested protein lysates at different time points postinfection in the presence of an alkylating agent, NEM, which prevents disulfide bond isomerization during sample preparation. The proteins were then separated by either nonreducing or reducing SDS-PAGE, followed by Western blotting for the VP1 protein using a monoclonal anti-VP1 antibody (Fig. 4). At early time points (0 to 8 h postinfection), there was no VP1 signal in the nonreducing gel, whereas full-length VP1 was detected in the reduced samples. This suggests that up to those time points, the BKV virion is still highly disulfide bonded and no VP1 molecules have been released, and therefore, VP1 cannot enter the nonreducing gel. Beginning at 8 to 12 h postinfection, a complex series of bands appeared in the nonreduced samples, and the intensity of the bands increased as the infection progressed. This likely resulted from the reduction and/or isomerization of the disulfide bonds in the capsid. At about the same time, in addition to the full-length VP1 band, two distinct faster-migrating bands were reproducibly detected in the reducing gels (denoted as VP1* and VP1**), implying that VP1 may be cleaved at specific sites during the disulfide bond rearrangement process.

**Effects of NH₄Cl and BFA on BKV capsid rearrangement.** We next asked whether the two inhibitory drugs, NH₄Cl and BFA, had any effects on BKV capsid rearrangement. Infection was carried out in the presence of these two drugs, and total protein lysates were prepared under alkylating conditions at 12 h postinfection, the time point at which VP1 rearrangement and cleavage were both detected (Fig. 4). The samples were subjected to nonreducing or reducing electrophoresis and probed for VP1 to assay disulfide bond rearrangement or cleavage (Fig. 5). NH₄Cl treatment completely blocked the rearrangement process, as no signals were detected in the nonreducing gel nor was either of the putative VP1 cleavage products observed in the reducing gel. Chloroquine treatment had a similar effect on VP1 rearrangement and cleavage (data not shown). When the cells were treated with BFA, however, there seemed to be an overall increase in the level of capsid rearrangement, as evidenced by a series of more intense VP1
bands in the nonreducing gel compared to those in the untreated sample. Correspondingly, there was a dramatic increase in the amount of VP1** in the reduced sample. There was not a dramatic change in the level of VP1*, indicating that the generation of VP1* and VP1** may occur through different routes. These results also indicate that rearrangement and cleavage of VP1 may occur prior to trafficking to the ER.

**ER protein Derlin-1 is important for BKV infection.** Having demonstrated BKV trafficking to the ER, we then wished to determine whether any host factors play a role in aiding BKV to escape from the ER. We first tested if Derlin proteins are involved in this process. The Derlin family proteins are ER transmembrane proteins that are important for targeting certain misfolded proteins from the ER to the cytosol for proteasome degradation, also known as the ERAD pathway (30, 31, 56, 57). Derlin-1 and Derlin-2, two members of this family, have been linked to the ER escape of SV40 and MPyV, respectively (29, 48). To investigate the possible roles of these proteins in BKV infection, RPTE cells were transfected with vectors expressing dominant negative Derlin-1 and Derlin-2 (the addition of a YFP tag converts these proteins into their dominant negative form) (4, 30). The cells were then infected with BKV and analyzed for TAg expression by immunofluorescence microscopy 48 h postinfection. The percentages of TAg-positive cells in transfected (YFP-positive) and untransfected cells were compared (Fig. 6A). A specific inhibitory effect on BKV infection was observed when the cells were transfected with Derlin-1-YFP, but not with Derlin-2-YFP. Neither Derlin-1-YFP nor Derlin-2-YFP inhibited infection of RPTE cells by adenovirus, a virus that does not traffic through the ER (37), confirming that the inhibition seen with Derlin-1-YFP was not due to general cytotoxic effects (data not shown).

To investigate a possible association between BKV and Derlin-1, a GST pull-down assay was performed to see if Derlin-1 interacts with the viral capsid protein VP1. GST or GST-VP1 was immobilized on glutathione beads and incubated with RPTE cell extracts. Complexes recovered from the beads were resolved by SDS-PAGE and probed for VP1 and GAPDH.
specifically pulled down by GST-VP1, but not by GST. These results suggest that Derlin-1 may directly participate in transporting BKV out of the ER.

**Involvement of the proteasome in BKV infection and capsid rearrangement.** The association between BKV and Derlin-1 led to the hypothesis that the proteasome may be involved in the infectious pathway of BKV, as Derlin-1 has been found in complexes with many of the components of the ERAD pathway (30, 31, 56, 57). Moreover, it is also possible that the proteasome participates in the disassembly process of BKV, as the ER and ERAD components have been suggested to function in the capsid rearrangement and ER escape of SV40 and MPyV (29, 48). To test this hypothesis, RPTE cells were treated with a specific proteasome inhibitor, lactacystin, which covalently binds to the \( \beta_9 \) subunit of the 20S core particle and irreversibly inhibits proteasome activity (19). Infection was assayed by Western blotting for TAg (Fig. 7A), and capsid rearrangement was determined by blotting for VP1 under either nonreducing or reducing conditions (Fig. 7B). Lactacystin caused a significant reduction in TAg expression, suggesting that proteasome function is part of the infectious pathway. The degree of virion rearrangement, however, as judged by the amount of VP1 entering the nonreducing gel, was greatly increased in the presence of lactacystin. There was also a dramatic increase in the level of VP1* in the reducing gel, but the level of VP1** remained relatively unchanged. These results indicate that VP1 rearrangement or cleavage is not dependent on proteasome activity.

**DISCUSSION**

Virus entry is a complex process that involves interactions with multiple cellular compartments, transport to and from them, and different sorting pathways. A productive infection by a virus requires a proper association with its receptors, internalization into the host cell, and the delivery of its genomic content to the site of replication, all of which can be influenced by the cell types that the virus infects (36). Therefore, it is important to dissect the entry mechanism of a virus in its natural host cell type to gain the most relevant knowledge about the infectious course. In this study, we characterize several intracellular trafficking events of BKV in RPTE cells, the cell type in which the virus persists and reactivates in vivo (8). Pharmacological experiments demonstrate that BKV infection requires a low-pH step, an intact microtuble network, and trafficking to the ER. A more-detailed time course reveals the kinetics of these events, as follows: BKV travels to an acidic compartment soon after internalization, which is followed by movement along the microtuble network, presumably within a vesicular compartment, to reach the ER (Fig. 8, steps A to C). Disassembly is another important step during polyomavirus entry, as the highly structured virion needs to be dissociated to allow entry of the genome into the nucleus. We show that the extensive disulfide bond network in the BKV capsid starts to be disintegrated at about 8 to 12 h postinfection. Interestingly, at similar times, we begin to detect two distinct, smaller fragments of VP1 that likely represent specific proteolytic cleavage products. To our knowledge, this is the first time that cleavage...
BKV trafficking is still unknown. Treatment with NH$_4$Cl can take part in BKV transport prior to the caveosome (Fig. 8, that an unidentified endosomal/lysosomal compartment may virus and papillomavirus infections (42, 45, 50). It is likely the endosomal compartments has been observed in both poly-

results clearly show that BKV infection requires a low-pH step. The caveosome is a pH-neutral organelle (43); however, our BKV passes through the caveosome en route to the nucleus. Major component of the caveosome (39), supports the idea that the colocalization between labeled BKV and caveolin-1, a ma-

of the capsid protein VP1 of a polyomavirus has been observed during the course of infection. At this time, we do not know the sites of cleavage, and the epitope that the P5G6 monoclonal antibody detects is not known. Furthermore, whether the re-

The endocytic events associated with BKV trafficking in RPTE cells are far from being completely understood. BKV is seen within caveosome-like structures by EM analysis (10), and the colocalization between labeled BKV and caveolin-1, a ma-

Our results show a unique pattern of BKV capsid protein rearrangement and cleavage. This observation raises several questions. First, the cellular location(s) of VP1 rearrangement and cleavage is also possible that NH$_4$Cl treatment traps the virus in a compartment that prevents it from further trafficking to the site of disassembly.

All the polyomaviruses that have been examined so far enter the ER compartment (10, 29, 40, 41, 43, 45); the BFA inhibi-

tion data of BKV infection are consistent with this. The ER Berlin family proteins have been implicated in the ER escape of SV40 and MPyV (29, 48). Our results show that, similar to SV40, Derlin-1 is important for BKV infection in RPTE cells. Moreover, we are able to demonstrate, for the first time, an interaction between the major capsid protein VP1 and Der-

The involvement of the proteasome during BKV infection is also intriguing. A specific proteasome inhibitor, lactacystin, reduces the infectivity of BKV but does not block BKV capsid rearrangement or VP1 cleavage. Proteasome function has been associated with multiple entry events of different viruses, including endosomal penetration and nuclear translocation (7, 22, 47, 58), and the specific step at which it is required for BKV infection remains to be determined. Since Derlin-1 is closely associated with ERAD components (30, 31, 56, 57), it is pos-

table that BKV may be transported to the proteasome from the ER via Derlin-1 (Fig. 8, step E). Alternatively, the effect of lactacystin on BKV infection may be indirect by impacting Derlin-1 function.

Our results show a unique pattern of BKV capsid protein rearrangement and cleavage. This observation raises several questions. First, the cellular location(s) of VP1 rearrangement and cleavage is not clear. BFA treatment results in an increased level of overall disulfide bond network breakdown and an accumulation of the VP1** molecule, suggesting that the ER is not the site of either event. Lactacystin treatment has a similar effect on capsid rearrangement and VP1 cleavage, and both drugs have an inhibitory effect on TAg expression. These results imply that the presence of either drug may reroute the virus to a pathway that is more favorable for capsid rearrange-

ment (Fig. 8, step D). Second, specific cleavage products of VP1 are seen during infection (Fig. 4), indicating that cleavage

FIG. 8. Proposed model of BKV entry in RPTE cells. (A) BKV enters an acidic compartment following caveola-mediated endocytosis, where the low pH triggers conformational changes within the capsid, beginning the disassembly process. (B) BKV proceeds to the pH-neutral caveosome. (C) BKV is transported along microtubules to the ER, where it interacts with Derlin-1 to escape out of the ER. Certain conformational changes (indicated by dashed virion) may occur in the ER to aid in the escape process. (D) BKV traffics to an unknown compartment, where VP1 disulfide bond rearrangement and cleavage occur. BFA or lactacystin treatment redirects BKV toward this pathway. Whether the virus enters the proteasome through the ERAD pathway (E) remains unknown.
is not a random event. Identification of the cleavage sites on the VP1 molecules may help us elucidate the mechanism of capsid rearrangement. Third, what triggers the disassembly process remains elusive. Low pH certainly plays a significant role, but other signals such as the presence of host factors or certain cellular environments may also contribute. Our results indicate that the breakdown of the disulfide network and VP1 cleavage occur at similar times, and it would be interesting to examine whether these processes are interdependent. Finally, additional genetic and biochemical analyses are warranted to determine whether the capsid rearrangement process and/or VP1 cleavage detected by our assays represent part of an infectious or noninfectious pathway of BKV infection.

In conclusion, our results show that BKV entry in RPTE cells is a highly regulated process, engaging coordinated interactions between viral structural and cellular components. We demonstrate that BKV infection and capsid rearrangement involve low-pH activation, trafficking to the ER, and components of the ERAD pathway. Understanding these steps at the detailed molecular level will expand our insight on general virus entry mechanisms. Moreover, it may help identify important immune regulators, such as the innate immune receptors that are involved in BKV infection, and offer potential intervention targets for BKV-related diseases.

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transport to the endoplasmic reticulum, where the virus disassembles. J. Virol. 76:5156–5166.


