Phosphorylation of Ser-80 of VP1 and Ser-254 of VP2 is essential for human BK virus propagation in tissue culture

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BK virus (BKV) infection may cause polyomavirus-associated nephropathy in patients with renal transplantation. Recently, the phosphorylated amino acids on the structural proteins VP1, VP2 and VP3 of BKV have been identified by liquid chromatography–tandem mass spectrometry in our laboratory. In this study, we further analysed the biological effects of these phosphorylation events. Phosphorylation of the BKV structural proteins was demonstrated by $^{32}$Porthophosphate labelling in vivo. Site-directed mutagenesis was performed to replace all of the phosphorylated amino acids. The mutated BKV genomes were transfected into Vero cells for propagation analysis. The results showed that expression of the early protein LT and of the late protein VP1 by the mutants VP1-S80A, VP1-S80-133A, VP1-S80-327A, VP1-S80-133-327A and VP2-S254A was abolished. However, propagation of other mutants was similar to that of wild-type BKV. The results suggest that phosphorylation of Ser-80 of VP1 and Ser-254 of VP2 is crucial for BKV propagation.

INTRODUCTION

Polyomaviruses are non-enveloped small DNA viruses and have a circular dsDNA genome of approximately 5.2 kbp. The polyomavirus family includes murine polyomavirus, simian virus (SV40), human John Cunningham virus (JCV), BK virus (BKV), KI virus, WU virus and Merkel cell polyomavirus (MCV) (Allander et al., 2007; Eash et al., 2006; Feng et al., 2008; Gardner et al., 1971; Gaynor et al., 2007; Kanitakis, 2008; Pérez-Losada et al., 2006). The genomes of these polyomaviruses contain regulatory, early and late regions. The regulatory region includes the replication origin as well as a promoter and an enhancer that regulate the transcription of the early and late genes. The early region encodes the regulatory proteins large tumour antigen (LT) and small tumour antigen, which are associated with replication and the regulation of the early and late genes. The late region encodes three structural capsid proteins, VP1, VP2 and VP3, and the regulatory agonoprotein (Safak et al., 2001). The viral capsid is composed of the major structural protein, VP1, the minor structural proteins, VP2 and VP3, and a viral minichromosome (Christiansen et al., 1977). The viral capsid consists of 72 pentameric capsomeres, including 12 pentavalent and 60 hexavalent capsomeres (Liddington et al., 1991).

The structural proteins of polyomavirus play important roles in the infection life cycle, such as recognition of host receptors, haemagglutination activity (Bolen & Consigli, 1979; Bolen et al., 1981) and encapsidation of the viral genome during maturation (Chang et al., 1993; Gasparovic et al., 2006; Kawano et al., 2006). Furthermore, interactions among the structural proteins are also crucial for virus entry, nuclear translocation, DNA packaging and assembly.
of viral progeny (Chen et al., 1998; Delos et al., 1995; Gasparovic et al., 2006; Gharakhianian et al., 2003; Kawano et al., 2006; Shishido-Hara et al., 2004). However, it has been found that the structural proteins are modified into several subspecies, as demonstrated by two-dimensional gel electrophoresis (Fang et al., 2010; O’Farrell & Goodman, 1976; Ponder et al., 1977). Different post-translational modifications (PTMs) of the same polypeptide may serve different biological functions (Fang et al., 2010; Seo & Lee, 2004; Yan et al., 1998). The PTMs of VP1 of polyomavirus have been demonstrated to have crucial roles in the viral life cycle (Bolen & Consigli, 1979; Bolen et al., 1981; Garcea & Benjamin, 1983; Ludlow & Consigli, 1987a, 1989). SV40 VP1 can be separated into six species by isoelectric focusing and two species have been found to be phosphorylated (O’Farrell & Goodman, 1976; Ponder et al., 1977). The phosphorylation of SV40 VP1 is associated with a structural role and binding to cell receptors (Ponder et al., 1977). The VP1 of polyomavirus can be separated into six distinct isospecies, A–F (Bolen et al., 1981; Marriott & Consigli, 1985; Ponder et al., 1977). Species D, E and F are modified by phosphorylation and their activities have been shown to be connected with the hexon subunit, absorption and the recognition of cell receptors (Anders & Consigli, 1983a, b; Bolen & Consigli, 1979; Bolen et al., 1981; Ludlow & Consigli, 1987a). In addition, species E and F contain tyrosine-O-sulphuration, which has a role in virus attachment to host cells (Ludlow & Consigli, 1987b). Species C and D have been identified as being acetylated (Bolen et al., 1981). Other modifications of VP1 include methylation and hydroxylation (Burton & Consigli, 1996; Ludlow & Consigli, 1989), with hydroxylation affecting the trans- portation of polyomavirus VP1 into the cell nucleus for assembly (Ludlow & Consigli, 1989).

The phosphorylated residues of VP1 of polyomavirus have been identified at Ser-66, Thr-63 and Thr-156 (Li & Garcea, 1994; Li et al., 1995). Mutation of Thr-156 results in an assembly defect in vivo (Garcea & Benjamin, 1983; Li & Garcea, 1994). Furthermore, Ser-66 of VP1 is the substrate of casein kinase II during phosphorylation (Li et al., 1995). A mutant VP1 with Ser-66 replaced by alanine is poorly phosphorylated and shows both tubular and capsid-pentamer-like structures in vitro (Li et al., 1995). Taken together, the phosphorylation of VP1 seems to play an important role in the replication cycle of polyomavirus. The minor capsid proteins, VP2 and VP3, of polyomavirus and SV40 are also known to have phosphorylation modifications (Ponder et al., 1977; Tan & Sokol, 1972, 1973); however, the phosphorylated amino acid residues and their biological functions have not been investigated as yet.

The post-translational modifications of the BKV structural proteins VP1, VP2 and VP3, were comprehensively identified by liquid chromatography–tandem mass spectrometry (LC–MS/MS) in our previous study (Fang et al., 2010). Amino acids Ser-80, Ser-133 and Ser-327 of VP1, Ser-223, Ser-248 and Ser-254 of VP2, and Ser-129 (VP2-Ser-248) of VP3 were found to be phosphorylated. The effects of these phosphorylation events on viral replication were investigated in this study.

## RESULTS

### Detection of phosphorylation of the BKV structural proteins by Pro-Q Diamond staining

Pro-Q Diamond phosphorylation gel staining was performed to detect phosphorylation of the BKV structural proteins. The purified BKV was resolved by SDS-PAGE and stained with Pro-Q Diamond phosphorylation gel stain. All BK capsid proteins, including VP1, VP2 and VP3, were found to be positive by Pro-Q Diamond staining (Fig. 1b), which indicated that all BKV structural proteins seem to be phosphorylated.

### In vivo 32P labelling of BKV structural proteins

In order to demonstrate that the BKV structural proteins were phosphorylated during the virus life cycle, Vero cells were infected with BKV and cultured with [32P] orthophosphate. The virus was allowed to propagate for 2 weeks. The viral proteins were then separated by SDS-PAGE and the phosphorylated proteins were detected by autoradiography. The results shown in Fig. 2 confirm that BK VP1, VP2 and VP3 are phosphorylated.
Site-directed mutagenesis of the phosphorylated amino acids of the BKV structural proteins

Site-directed mutagenesis by PCR was performed in order to obtain BKV genomes that were mutated at the sites of the phosphorylated amino acids. The genomes of the phosphoamino acid mutations (VP1-S80A, VP1-S133A, VP1-S327A, VP1-S80-133A, VP1-S80-327A, VP1-S133-327A, VP1-S80-133-327A, VP2-S223A, VP2-S248A, VP2-S254A, VP2-S223-248A, VP2-S223-254A, VP2-S248-254A and VP2-S223-248-254A) were cloned into a replication plasmid, pGEM-T-Easy. The primers listed in Supplementary Table S1 (available in JGV Online) were used to construct the site-specific mutations. All mutated nucleotides were verified by DNA sequencing (not shown).

Propagation of the mutated BKVs determined by measuring LT expression

The BK genomic DNA containing the various mutations was removed from the replicative plasmid. The mutated genomic DNAs were then transfected into Vero cells. Expression of LT was analysed at days 11, 18, 24 and 31 by indirect immunofluorescent assay. Results showed that the LT-expressing cells of the wild-type (WT) BKV genome were approximately 4, 14, 41 and 68% at days 11, 18, 24 and 31 post-transfection, respectively (Fig. 3). Expression of LT with the VP1 mutants S133A, S327A and S133-327A and the VP2 mutants S223A, S248A, S223-248A, S223-254A, S248-254A and S223-248-254A were only slightly decreased compared with WT BKV (Fig. 3). The genomic transfection was performed independently three times and the results were consistent.

Propagation of the mutated BKVs determined by measuring VP1 expression

Expression of the late protein VP1 from BKVs containing the phosphorylated amino acid-mutated sites was examined by Western blotting. The results showed that the expression level of VP1 increased with time in culture (days) after transfection with WT, VP1-S133A, VP1-S327A, VP1-S133-327A, VP2-S223A, VP2-S248A, VP2-S223-248A, VP2-S223-254A, VP2-S248-254A, VP2-S223-248-254A and genomic DNA, and the trends for the VP1-expression increase were similar to those for LT (Fig. 4). In contrast, the expression levels of the VP1 protein of mutants S80A, S80-133A, S80-327A and S80-133-327A, and of VP2 mutant S254A were very low, even at 31 days after transfection (Fig. 4). These results indicated that phosphorylation of Ser-80 of VP1 and Ser-254 of VP2 is crucial for BKV propagation.

Expression of LT and VP1 at days 2 and 5 after transfection of VP1-S80A and VP2-S254A genomes

LT and VP1 expression was barely detected at day 11 after transfection with the VP1-S80A and VP2-S254A mutants (Figs 3 and 4). To examine whether the viral proteins were expressed during the first life cycle, the expression levels of LT and VP1 at days 2 and 5 after genomic transfection were monitored. Results showed that both LT and VP1 were detectable at low levels on days 2 and 5 after transfection of VP1-S80A and VP2-S254A BKV (Fig. 5). The findings suggest that phosphorylation of VP1 Ser-80 and VP2 Ser-254 may be involved in viral assembly or the infection pathway.

Identification of the mutated phospho-serines from BKV progeny by LC–MS/MS

The expression of LT and VP1 of BKV mutants VP1-S133A, VP1-S327A, VP1-S133-327A, VP2-S223A, VP2-S248A, VP2-S223-248A, VP2-S223-254A, VP2-S248-254A and VP2-S223-248-254A indicates that viral progeny were generated (Figs 3 and 4). The virions of these mutants were purified and the presence of the various mutated serines was confirmed by nano-LC–MS/MS. The MS/MS spectra
Fig. 3. Detection of LT expression after transfection with BKV genomes containing mutated VP1 and VP2/3 by using an immunofluorescence assay. Immunofluorescence assays were performed by using anti-LT antibody at days 11, 18, 24 and 31 after transfection with (a) WT, VP1-S80A, VP1-S133A, VP1-S327A, VP1-S80-133A, VP1-S80-327A, VP1-S133-327A and VP1-S80-133-327A BKV genomes, and (b) VP2-S223A, VP2-S248A, VP2-S254A, VP2-S223-248A, VP2-S223-254A, VP2-S223-248-254A and VP2-S248-246-254A BKV genomes. The ratio of LT-positive cells was quantified from the immunofluorescence assays.

in Fig. 6 confirm that all of the phosphorylated serines on VP1 and VP2/3 were substituted by alanine. Viral progeny of mutants VP1-S80A, VP1-S80-133A, VP1-S80-327A, VP1-S80-133-327A and VP2-S254A were not available for LC–MS/MS analysis, but the mutated nucleotides were confirmed by DNA sequencing (not shown). The results demonstrate that phosphorylation of Ser-133 and Ser-327 of VP1 and Ser-223 and Ser-248 of VP2/3 is not crucial for BKV replication, but phosphorylation of Ser-80 of VP1 and Ser-254 of VP2 is essential for BKV replication.

Fig. 4. Detection of VP1 expression after transfection with BKV genomes containing mutated VP1 and VP2/3 by Western blot analysis. Western blot analysis was performed by using anti-BK VP1 antiserum at days 11, 18, 24 and 31 after transfection with (a) WT, VP1-S80A, VP1-S133A, VP1-S327A, VP1-S80-133A, VP1-S80-327A, VP1-S133-327A and VP1-S80-133-327A BKV genomes, and (b) VP2-S223A, VP2-S248A, VP2-S254A, VP2-S223-248A, VP2-S223-254A, VP2-S223-248-254A and VP2-S248-246-254A BKV genomes. The percentage of expression level of VP1 was quantified after Western blotting.
DISCUSSION

The LC–MS/MS method has demonstrated that Ser-80, Ser-133 and Ser-327 of VP1, Ser-223, Ser-248 and Ser-254 of VP2, and Ser-129 (VP2-Ser-248) of VP3 are phosphorylated (Fang et al., 2010). The biological functions of phosphorylation of the BKV structural proteins were further investigated in this study. The presence of phosphorylation of BKV VP1 and VP2/VP3 was demonstrated by using Pro-Q diamond phosphoprotein gel stain in vitro and [32P]orthophosphate labelling in vivo. Site-directed mutagenesis of the BKV genome was performed to change the serines that may be phosphorylated to alanines. The results of transfection of the phosphorylated mutants indicated that mutations at Ser-80 of VP1 and Ser-254 of VP2 abolished BKV replication. In addition, mutations at Ser-133 and Ser-327 of VP1, Ser-223 and Ser-248 of VP2 and Ser-129 (VP2-Ser-248) of VP3 have no effect on BKV replication. Therefore, our findings suggest that phosphorylation of Ser-80 of VP1 and Ser-254 of VP2 is crucial for BKV propagation.

Sequence alignment analysis of VP1 between murine polyomavirus and other polyomaviruses, SV40, BKV, JCV, KI, WU and MCV, shows that these proteins have a relatively low degree of sequence similarity, namely 41, 41, 46, 10, 7 and 51 %, respectively (data not shown). In addition, the phosphorylated Ser-63, Thr-66 and Thr-156 residues of murine polyomavirus VP1 are not conserved in the VP1 proteins of the other polyomaviruses. Thus, although these viruses belong to the same family, their VP1 sequences have low similarity and therefore modifications of the capsid proteins may affect various biological functions, such as host range, tissue tropism and pathogenesis, in different ways (Freund et al., 1991; Mezes & Amati, 1994). In the current study, the phosphorylated residues of BK VP1, including Ser-80, Ser-133 and Ser-327, are distinct from those of polyomavirus VP1, where phosphorylation occurs at Ser-63, Thr-66 and Thr-156. The Ser-80 residue of BK VP1 is conserved in JCV and SV40 VP1, while the Ser-327 residue of BK VP1 is conserved in SV40 VP1 (Fang et al., 2010). The similarity as well as the variation in phosphorylated amino acids across the different VP1 of polyomaviruses may be associated with viral physiology.

Previous studies have suggested that phosphorylation of VP1 of murine polyomavirus and SV40 is associated with viral structure, the subunit capsomers and cell recognition (Bolen & Consigli, 1979; Bolen et al., 1981; Ponder et al., 1977). In addition, the phosphorylated residues Thr-63 and Thr-156 of polyomavirus VP1 are located in the B–C and D–E loops, respectively. These two threonine residues have been shown to be exposed on the exterior viral surface (Li & Garcea, 1994). The construction of a virus with a Thr-156 to alanine mutation resulted in virions with an assembly defect (Li & Garcea, 1994). The phosphorylated residues Ser-80, Ser-133 and Ser-327 of BK VP1 were known to be located in the B–C loop, D–E loop and C-terminal region (Dugan et al., 2007; Liddington et al., 1991). The B–C loop of polyomavirus has been suggested to be involved in cell receptor binding and haemagglutination (Freund et al., 1991). In this study, we proved that the viral proteins, LT and VP1 were detectable at days 2 and 5, but not day 11, after transfection of the mutated DNA of VP1-S80A and VP2-S254A. Previous studies have reported that replication of BKV may reach secondary infection after 10 days of propagation (Acott et al., 2006; Flaegstad & Traavik, 1987; Olsen et al., 2009). The current study demonstrated that secondary infection of VP1-S80A and VP2-S254A BKV was abolished 11 days after transfection. Therefore, phosphorylation of VP1 Ser-80 and VP2 Ser-254 of BKV may play a role in infection. Furthermore, genotypes of BKV isolated from patients with interstitial nephritis have been analysed. Substitution of Ser-77 with aspartic acid, glutamic acid, glutamine or asparagine was found to be associated with nephritis (Randhawa et al.,...
Fig. 6. Confirmation of the presence of mutated serines on the mutated BKV structural proteins by MS/MS spectroscopy. The mutated BKV structural proteins were digested by trypsin/ chymotrypsin and analysed by LC–MS/MS. (a) The mutated phospho-serine, VP1-S133A, substituted for alanine (underlined), was identified within the peptide NLHAGAQK with a double charge precursor ion at a mass/charge ratio \((m/z)\) of 419.7282. (b) VP1-S327A was identified within the peptide VDGQMYGMEAQVEEV with a double-charge precursor ion at \(m/z\) 5969.9398. (c) VP2-S223A was identified within the peptide SDLAPIRPSMVR with a double-charge precursor ion at \(m/z\) 447.9713. (d) VP2-S248A was identified within the peptide AIDDADSIEEVTQR with a double-charge precursor ion at \(m/z\) 781.3719. (e) VP2-S248-254A was identified within the peptide AIDDADIEEVTQR with a double-charge precursor ion at \(m/z\) 773.3762.
et al. on the B–C loop of BKV VP1 may be involved in viral infectivity.

In a previous study Fang et al. (2010) demonstrated that the Ser-223, Ser-248 and Ser-254 residues of BK VP2 were phosphorylated. From sequence alignment analysis of VP2 we know that Ser-223 and Ser-254 are conserved in BKV, JCV and SV40 but not in polyomavirus. Ser-248, on the other hand, is conserved in BK and JC. The biological functions of phosphorylation of the polyomavirus minor capsid proteins have not been reported. In a previous study on polyomavirus, the numbers of the VP1 subspecies were changed upon co-expression with VP2/3, which indicates that VP2/3 may be involved in VP1 modification (Dean et al., 1995; Forstová et al., 1993). Furthermore, hydrophobic interactions between C-terminal residues 214–318 of VP2/3 and the VP1 pentamer of polyomavirus have been demonstrated (Chen et al., 1998). For SV40, the C terminus of VP2/3, from residues 119 to 272, is essential for enhancing VP1 pentamer assembly (Kawano et al., 2006). Our previous study also provided evidence that phosphorylation does occur at the C terminus, namely at Ser-223, Ser-248 (VP3-Ser-129) and Ser-254, of VP2/3 (Fang et al., 2010). In this study, we demonstrated that Ser-254 of VP2 is crucial for BKV replication; however, it is still not clear if this phosphorylation affects viral assembly and/or entry into the replication life cycle. We have also proved that mutation of the conserved VP2 serine residue of BKV, JCV and SV40, Ser-254, will abolish BKV replication. However, BKV replication was only decreased and not completely abolished with the double mutants S223-254A and S248-254A and the triple mutant S223-248-254A, although the mutated nucleotides and amino acids have been confirmed by DNA sequencing and LC–MS/MS. It is unclear how mutation of two other serine residues rescues the phenotype caused by the mutation of Ser-254 of VP2. Nevertheless, the molecular mechanism of how phosphorylation of VP proteins contributes to regulation of BKV replication still needs to be investigated further.

In conclusion, we have demonstrated that the structural proteins of BKV are phosphorylated at Ser-80, Ser-133 and Ser-327 of VP1, and Ser-223, Ser-248 and Ser-254 of VP2/3 and that phosphorylation of Ser-80 of VP1 and Ser-254 of VP2 is required to complete the infectious life cycle. Clinically, BKV infection is the major cause of polyomavirus-associated nephropathy (PVN) (Dörries, 1998; Jiang et al., 2009; Nickelet et al., 2000). Recently, PVN has increasingly become an important reason for renal dysfunction among renal transplant patients (Binet et al., 1999). However, little is known about the molecular mechanism of BKV replication. Our findings provide important information that will help the elucidation of the mechanism of BK virus replication and aid in establishing a mechanism for viral pathogenesis.

**METHODS**

**Propagation and purification of BKV.** Vero cells (ATCC no. CCL-81) were grown in a humidified 37 °C, CO2 incubator in Eagle’s minimal essential medium (Invitrogen) supplemented with 10% heat-inactivated FBS (Biological Industries) and 1% penicillin/streptomycin [penicillin-streptomycin solution (10 000:1 0000); Invitrogen]. BK virus, the UT strain (BK-UT), was a gift from Walter Atwood (Brown University, Rhode Island, USA) and was propagated in Vero cells (Eash & Atwood, 2005; Fang et al., 2010). The process of virus purification was previously described (Fang et al., 2010).

**Detection of phosphorylated protein by using Pro-Q Diamond phosphoprotein gel stain.** Phosphoprotein staining with Pro-Q Diamond dye (Invitrogen) was performed according to the manufacturer’s instructions (Agrawal & Thelen, 2005; Goodman et al., 2004). Two micrograms of the purified BK viral protein was treated with SDS sample buffer. The mixture was resolved by SDSPAGE (12.5% acrylamide gel) after being boiled for 7 min. After electrophoresis at 100 V for 2 h, the gel was treated twice with a fixing solution (50% methanol and 10% acetic acid) with gentle agitation, for 30 min each time. The gel was then soaked in water for 10 min and incubated with Pro-Q Diamond phosphoprotein dye in the dark for 60 min. Afterwards, the gel was treated with a destaining solution (20% acetonitrile, 50 mM sodium acetate, pH 4.0) and finally washed with water. Fluorescent images were obtained by using a Typhoon fluorescence scanner (Amersham Biosciences). A total protein gel profile was obtained by staining with SYPRO Ruby dye (Molecular Probes). The image of the stained total proteins was also detected using the Typhoon scanner.

**Phosphorylation of BKV in vivo.** Vero cells were grown in 100 mm-diameter plates and infected by BKV. At 14 days after infection, the infected cells were cultured with 1 mCi (37 MBq) of [32P]orthophosphate in 1 ml of phosphate-free Eagle’s minimal essential medium with 2% calf serum for 72 h. The cells were then harvested by centrifugation at 1500 g for 30 min. The cell pellet was washed twice with cold TBS (10 mM Tris/HCl and 150 mM NaCl, pH 7.4) and then resuspended in TBS. The cells were lysed by freeze–thawing three times. Neuraminidase (0.025 mg ml⁻¹; Sigma) was added to the mixture to release virus and the mixture was incubated for 16 h at 37 °C. The enzyme was then inactivated at 56 °C for 30 min. After centrifugation at 1500 g for 30 min, the supernatant containing the virus was analysed by SDS-PAGE and autoradiography.

**Construction of the phosphorylated amino acid mutants.** The genomes of the phospho-amino acid mutants (VP1-S80A, VP1-S133A, VP1-S223A, VP1-S248A, VP1-S254A, VP1-S80-133A, VP1-S80-327A, VP1-S80-133-327A, VP2-S223A, VP2-S248A, VP2-S254A, VP2-S223-248A, VP2-S223-254A and VP2-S223-248-254A) were constructed by two-step PCR. Seventeen primers (Supplementary Table S1) were used to construct the site-specific mutations. For the first PCR, a WT forward primer (S) and a mutated reverse primer (AS) were used to generate a 5’-terminal VP1 or VP2/VP3 DNA fragment. In parallel, a mutated forward primer (S) and a WT reverse primer (AS) were used to generate the 3’-terminal VP1 or VP2/VP3 DNA. For the second PCR, the WT forward and reverse primers were mixed with the 5’- and 3’-terminally mutated products to generate full-length mutated VP1 or VP2/VP3 DNA sequences. The mutated DNA products were then cloned into a replication vector, T-Easy (Promega), at the XbaI site. The mutated VP1 DNA with an XbaI site was used to replace a 1400 bp fragment in the viral genome of BK-UT to generate the mutated VP1-containing genomes. In a similar way, MfeI sites in the BK-UT genome were used to replace WT sequences with mutated VP2 DNA sequences to generate the VP2 and VP3 mutated genomes. All mutated nucleotides were verified by DNA sequencing after genomic cloning.
Genomic transfection of the mutants. Vero cells (3 × 10^5) in 35 mm-diameter culture dishes were transfected with 3.9 × 10^11 copies (approx. 5 μg) of WT or mutant BK genomic DNA with Lipofectamine (Invitrogen). The viral proteins were detected at days 11, 18, 24 and 31 post-transfection. The VP1 protein was detected by Western blotting. LT protein was detected by an indirect immunofluorescence assay.

Detection of LT by indirect immunofluorescence assay. Vero cells transfected with a BKV genome were fixed with cold methanol and acetone at a ratio of 2:1. The cells were washed three times with PBS and incubated with normal horse serum (Hyclone) and cells staining with Evans Blue solution were counted. The cells were then incubated with mAb against SV40 LT (Calbiochem) for 1 h at RT. After washing three times with PBS, the cells were incubated with goat anti-mouse IgG conjugated with FITC (Sigma) for 30 min at RT. Finally, the cells were washed with PBS three times and the coverslips mounted on glass slides with anti-fading fluorescence mounting medium (Dako). The cells were observed by confocal microscopy (LSM 510; Carl Zeiss). Five hundred cells were counted to determine a positive or negative result.

Detection of BKV VP1 by Western blot. Thirty micrograms of cell lysate was treated with SDS sample buffer. The mixture was separated by SDS-PAGE (12.5 % acrylamide gel). The separated proteins were transferred onto a PVDF membrane (Pall Corporation). The membrane was blocked with 5 % non-fat milk powder in Tris buffer (50 mM Tris/HCl, 150 mM NaCl, pH 7.4) and then incubated with rabbit anti-BK VP1 antiserum at a dilution of 1:2000. Goat anti-rabbit IgG conjugated with biotin, and avidin conjugated with HRP (Vector) were used to detect BK VP1.

Analysis of the mutated amino acids by nano-LC–MS/MS. Vero cells (3 × 10^5) in 35 mm-diameter culture dishes were transfected with 4 μg of mutant BK genomic DNA by using Lipofectamine. The cell lysates containing mutant viruses were harvested 24 days after transfection and used to infect Vero cells in 100 mm-diameter culture dishes. The viruses were then purified 14 days after infection. The process of virus purification was as previously described (Fang et al., 2010). The proteins from 10 μg of the purified mutant viruses were separated by SDS-PAGE (12.5 % acrylamide gel). Mutated VP1, VP2 and VP3 were extracted and protease digested. The mutated amino acids were verified by nano-LC–MS/MS using the same procedures as in our previous study (Fang et al., 2010).

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