Detection and characterization of a novel polyomavirus in wild rodents

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Received 11 October 2010
Accepted 17 December 2010

To investigate polyomavirus infection in wild rodents, we analysed DNA samples from the spleens of 100 wild rodents from Zambia using a broad-spectrum PCR-based assay. A previously unknown polyomavirus genome was identified in a sample from a multimammate mouse (Mastomys species) and the entire viral genome of 4899 bp was subsequently sequenced. This viral genome contained potential ORFs for the capsid proteins, VP1, VP2 and VP3, and early proteins, small t antigen and large T antigen. Phylogenetic analysis showed that it was a novel member of the family Polyomaviridae, and thus the virus was tentatively named mastomys polyomavirus. After transfection of the viral genome into several mammalian cell lines, transient expression of the VP1 and large T antigen proteins was confirmed by immunoblotting and immunocytochemical analyses. Comparison of large T antigen function in mastomys polyomavirus with that in rhesus monkey polyomavirus SV40 and human polyomavirus JC virus revealed that the large T antigen from mastomys polyomavirus interacted with the tumour suppressor protein pRb, but not with p53.

INTRODUCTION

Polyomaviridae is a family of non-enveloped viruses that carry circular, dsDNA genomes. At present, eight human and 18 non-human polyomaviruses are known (Groenewoud et al., 2010; Krumbholz et al., 2009; Misra et al., 2009; Schowalter et al., 2010; van der Meijden et al., 2010). Most mammalian polyomaviruses cause subclinical infections with life-long persistence in their natural immunocompetent hosts. However, when host immunity is compromised, the virus can reactivate and cause disease. In humans, infection of healthy individuals by JC virus (JCV) or BK virus (BKV) is asymptomatic, but opportunistic reactivation of these viruses can result in severe disease. JCV is the causative agent of progressive multifocal leukoencephalopathy (PML), a fatal demyelinating disease of the central nervous system (Padgett et al., 1971), and reactivation of BKV in kidney allografts leads to polyomavirus-associated nephropathy (PVAN), with the consequent risk of allograft loss (Comoli et al., 2006). In recent years, large-scale molecular screening techniques have identified several novel human polyomavirus species. For example, the KI and WU polyomaviruses were characterized from patients with respiratory tract infections, although their role as a causative agent has not yet been confirmed (Allander et al., 2007; Gaynor et al., 2007). Merkel cell polyomavirus (MCPyV) was found to be specifically linked to Merkel cell carcinoma (MCC), a rare but aggressive form of skin cancer of neuroendocrine origin (Feng et al., 2008). The DNA sequences of MCPyV are present in about 80% of MCC tumour specimens, mostly in a clonally integrated form. More recently, human
polyomavirus-6 (HPyV6) and HPyV7, in addition to MCPyV, were identified from healthy human skin swabs (Schowalter et al., 2010). As for non-human polyomaviruses, most of these were identified as cell culture contaminants, from cell-free preparations or in laboratory animals. Simian virus 40 (SV40) is a well characterized polyomavirus and was identified in 1960 as a vacuolating virus contaminating rhesus monkey cell cultures used to produce the poliovirus vaccine (Sweet & Hilleman, 1960). SV40 leads to a persistent, asymptomatic infection in its natural host, but has transforming properties in a broad range of cell types and induces tumours after inoculation into newborn rodents (Pipas, 2009). SV40 has been detected in human tissues, but its prevalence and relationship to human cancer are still controversial. Using a broad-spectrum PCR approach, recent studies discovered new polyomaviruses in zoo animals or wild animals such as chimpanzees, squirrel monkeys, bats and orang-utans (Johne et al., 2005; Verschoor et al., 2008; Misra et al., 2009; Groenewoud et al., 2010). These studies indicate that there could still be many unknown polyomaviruses in wild animals. In this study, we investigated the presence of polyomavirus in wild rodents.

RESULTS

Screening of rodent samples for polyomavirus

A total of 100 wild rodents including 45 rodents belonging to Mastomys spp. was collected in the vicinity of houses within the Namwala, Lusaka and Mfuwe areas of Zambia. DNA isolated from the spleens of these rodents was screened by using a nested broad-spectrum PCR technique to detect the polyomavirus VP1 region (Johne et al., 2005). A unique PCR product of approximately 250 bp was detected in a single rodent sample collected in Namwala. Species identification using genomic analysis revealed that this sample was derived from Mastomys spp., which is closely related to Mastomys natalensis. A BLAST search showed the PCR product to have 82% similarity at the nucleotide level with the VP1 region of myotis PyV. This unknown virus was tentatively named mastomys polyomavirus (MasPyV).

Genome and phylogenetic analyses of the deduced amino acid sequences

The entire MasPyV genome was amplified from the mastomys spleen sample by using inverse primers designed using the nucleotide sequence of the VP1 fragment; subsequently, the entire 4899 bp genome sequence was determined. Analysis of the DNA sequence revealed that the MasPyV genome had a typical PyV genome organization, including putative ORFs for early proteins [large T antigen (TAg) and small t antigen (tAg)] and late proteins (VP1, VP2 and VP3) (Fig. 1). Although the MasPyV genome contains an ORF 5′ of the VP2 gene that may encode an agnoprotein, this putative small protein (154 aa) has no significant sequence similarity to other primate agnoproteins. The regulatory region contained the consensus pentanucleotide TAg-binding site (GAGGC).

The deduced amino acid sequences of the viral proteins were compared with those of known PyVs. Phylogenetic trees of the PyV proteins constructed by using the neighbour-joining method suggested that all of the MasPyV viral proteins were more closely related to those of the myotis PyV, murine pneumotropic PyV and squirrel monkey PyV, than to other well-known rodent PyVs, such as murine PyV and hamster PyV (Fig. 2).

Expression of viral proteins after transfection of the MasPyV genome

After transfection, the linearized PyV genome is usually circularized by host cell repair mechanisms and the viral mRNA is transcribed (Dugan et al., 2007). Following transcription of the early viral genes, DNA replication and transcription of the late viral genes occurs. TAg and the major capsid protein, VP1, are generated by alternative splicing of the early and late mRNA transcripts, respectively. To test whether the MasPyV genome could produce viral proteins in cultured cells, the entire linearized viral genome was transfected into various cell lines. RT-PCR detected spliced-transcripts that were predicted to generate the MasPyV TAg and VP1 proteins in cells.
transfected with the viral genome (data not shown). Immunoblot analysis using antibodies against SV40 TAg (PAb416) and JCV VP1 showed that both the MasPyV TAg and VP1 proteins, which comprise 654 and 362 aa, respectively, were expressed in baby hamster kidney (BHK) cells 4 days after transfection (Fig. 3a). In addition, immunofluorescence analysis showed that TAg and VP1 were mainly present in the cell nuclei of BHK and Vero cells (Fig. 3b, c). We also detected transient expression of TAg and VP1 in several other cell lines, including NIH3T3 (mouse embryonic fibroblasts) and NRK (normal rat kidney cells) (data not shown). However, only a few cells

Fig. 2. Phylogenetic relationships between the amino acid sequences of large T antigen, small t antigen, VP1 and VP2 of polyomaviruses. MasPyV (Mastomys) is indicated by grey shading. The sequences for the other polyomaviruses were obtained from GenBank (see Supplementary Table S1). Phylogenetic analysis was performed using the neighbour-joining method with 1000 bootstrap replicates. Significant bootstrap values are shown. Bars indicate 0.2 or 0.5 amino acid residue replacements per site.
expressed the viral proteins 2 weeks after transfection, suggesting that MasPyV is unlikely to infect these cell lines productively.

**Characterization of MasPyV TAg**

Genetic and biochemical studies of TAggs encoded by polyomaviruses have shown that they are multifunctional proteins comprising several functional domains. The SV40 and JCV TAggs not only possess helicase and DNA-binding activities, which are required for replication of the viral genome, they also target tumour suppressor and cell cycle regulatory proteins, including retinoblastoma tumour suppressor protein (pRb) and p53. Alignment analysis showed that MasPyV TAg had 50% aa sequence identity and 82% similarity to the TAggs from SV40 and JCV. The deduced MasPyV TAg sequence contained features known to be conserved in TAggs, including a DnaJ domain with the highly conserved motif HPDKGGD, an LXCXE Rb-binding motif, a replication origin-binding domain and helicase/ATPase domains (Fig. 4a). To compare the binding of the MasPyV, SV40 TAg and JCV TAggs to the tumour suppressor proteins, pRb and p53, each of the TAggs was Flag-tagged, expressed in a human neuroblastoma cells (IMR-32), and immunoprecipitated. Immunoblot analysis of pRb and p53 in the precipitates revealed that MasPyV TAg, like SV40 and JCV TAggs, interacted with pRb. However, it did not interact with p53 (Fig. 4b). No overexpression of p53 (which results from the inhibition of protein degradation by TAg binding) was observed in cells expressing MasPyV TAg (Fig. 4b).

**DISCUSSION**

The genome arrangement of MasPyV is the same as that of other PyVs, including the putative ORFs for the late capsid proteins and the early non-structural proteins, and the relevant splice sites. We detected transient expression of VP1 and TAg mRNA and protein in some cell lines transfected with the entire MasPyV viral genome. This implies that the early and late genes were transcribed by bidirectional viral promoters and cellular transcription factors within those cells. However, a spread of MasPyV infection was not observed in any of the cell lines. It seems probable that the cells lack some factor(s), such as cellular replication machinery that facilitates replication of the viral genome, a receptor that allows efficient viral entry into the cells, and/or a factor that allows efficient virion assembly and release. Because the MasPyV TAg did not interact with p53, failure to suppress p53-induced apoptosis may have prevented efficient virus production during the late phase of infection.

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**Fig. 3.** Expression of viral proteins after transfection of the MasPyV genome. BHK or Vero cells were transfected with the MasPyV genome 4 days before immunoblot analysis (a) or 3 days before immunofluorescence staining (b, c) with a mAb to SV40 TAg (pAb416) and a polyclonal antibody to JCV VP1. (a) The TAg (654 aa residues) and VP1 (362 aa residues) proteins from MasPyV were detected in BHK cells transfected with the MasPyV genome (MasPyV+). (b) MasPyV TAg was detected in the cell nuclei (green). (c) MasPyV VP1 was detected mainly in the cell nuclei, although some was detected in the cytoplasm (green). Cell nuclei were stained with DAPI (blue). Bars, 50 μm.
We detected the novel polyomavirus, MasPyV, in a single sample isolated from Mastomys spp. Mastomys natalensis is widespread in sub-Saharan Africa, except for the southwestern portion of the continent. The detection rate for the MasPyV genome in DNA samples from mastomys spleen tissues was low (one of 45 mastomys) in this study. Although we found the MasPyV genome not only in the spleen but also in kidney and liver tissues from the mastomys using the broad-spectrum PCR (data not shown), the mastomys showed no noticeable gross pathological findings. Like other mammalian polyomaviruses in their natural hosts, infection with MasPyV is likely to be asymptomatic in a healthy individual.

Phylogenetic analysis showed that MasPyV is closely related to murine pneumotropic PyV (77% identity to VP1 at the amino acid level). Natural murine pneumotropic PyV infection of immunocompetent adult mice does not cause disease, but induces fatal interstitial pneumonia in newborn mice (Greenlee, 1979). It is, therefore, possible that MasPyV also causes disease in newborn or immunodeficient mastomys.

Most mammalian polyomaviruses exhibit transforming properties in cell culture and are able to induce malignant tumours after inoculation into non-permissive rodents. The early proteins encoded by the early regions of these polyomaviruses are responsible for this transformation. SV40 TAg is responsible for most of the transforming
activity through interfering with the functions of pRb and p53 (Ali & DeCaprio, 2001). However, murine PyV TAg does not interact with p53, but an additional middle t antigen is responsible for the transforming activity via other mechanisms (Treisman et al., 1981). Although MasPyV TAg did not interact with p53, further investigation of the role of the early proteins is required to assess the oncogenic potential of MasPyV.

METHODS

Animals and DNA extraction from tissues. A total of 100 rodents was collected from the Namwala, Lusaka and Mfuwe areas of Zambia using Sherman traps. All the tissues from captured rodents were kept at −80 °C. Total DNA was extracted from rodent spleens using a QIAamp DNA mini kit (Qiagen) according to the manufacturer's instructions. The study of rodents in Zambia was authorized by the Zambia Wildlife Authority.

PCR. The VP1 and VP3–VP1 regions of polyomavirus were amplified using a nested broad-spectrum PCR method as described by John et al. (2005). The first round of amplification was performed using 100 ng extracted DNA and high fidelity Taq DNA polymerase (Roche Diagnostics) in 20 μl reaction mixtures. The cycling protocol comprised 2 min of incubation at 95 °C, followed by 45 cycles each of 94 °C for 30 s, 46 °C for 1 min and 72 °C for 1 min, and 72 °C for 5 min. For nested PCR, 0.5 μl of the first PCR product was used as the template in a similar reaction: 95 °C for 2 min, followed by 45 cycles each of 94 °C for 30 s, 56 °C for 1 min and 72 °C for 1 min, and 72 °C for 5 min. PCR products were visualized after electrophoresis in ethidium bromide stained 1.5% agarose gels. The entire MasPyV genome was obtained by inverse PCR using PrimerSTAR GXL DNA polymerase (Takara) and primers facing outwards from the initial VP1 gene product. The cycling protocol used was: 2 min incubation at 94 °C, followed by 40 cycles each of 98 °C for 10 s, 60 °C for 15 s and 68 °C for 5 min, and 68 °C for 5 min. The PCR product was then cloned into the pcR4-TOPO vector (Invitrogen) and sequenced using a BigDye Terminator v3.0 Cycle Sequencing kit on an ABI Prism 3130 Genetic Analyzer (Applied Biosystems).

Species identifications were verified by sequencing of cytochrome b, acid phosphatase type V (APS) and recombination activating gene 1 (RAG1) (Steppan et al., 2005) using DNA obtained from spleen tissue.

Phylogenetic analysis. The amino acid sequences associated with the following reference virus genomes were obtained from GenBank: JC virus, BK virus, WU virus, KI virus, Merkel cell PyV, SV40, SA12, B-lymphotropic PyV, squirrel monkey PyV, murine PyV, hamster PyV, murine pneumotropic PyV, myotis PyV, bovine PyV, avian PyV, goose hemorrhagic PyV, finch PyV and crow PyV (see Supplementary Table S1 for accession numbers, available in JGV Online). Multiple sequence alignments of the predicted large T antigen, small t antigen, VP1 and VP2 ORFs were made using CLUSTAL_X 2.0 (Larkin et al., 2007). Phylogenetic analysis was performed using the neighbour-joining method with 1000 bootstrap replicates (Saitou & Nei, 1987; Felsenstein, 1985). Phylogenetic trees were generated using the NJPlot drawing software (Perriere & Gouy, 1996).

Cells and plasmids. African green monkey Vero and IMR-32 cells (Health Science Research Resources Bank) were maintained under an atmosphere of 5% CO2 at 37 °C in Dulbecco’s minimum essential medium supplemented with 10% FBS, penicillin (100 U ml−1), streptomycin (0.1 mg ml−1) and 2 mM L-glutamine. For IMR-32 cells, 0.1 mM non-essential amino acids were added to the medium. BHK cells were maintained in minimum essential medium supplemented with 5% FBS. The full MasPyV genome was cloned into the BamHI sites of the pUC19 vector to yield pUC19-MasPyV. The BamHI site within pUC19-MasPyV separates the TAg coding region. The TAg expression vectors, pCMV-Flag-JCV TAg, pCMV-Flag-SV40 TAg and pCMV-Flag-MasPyV TAg, were constructed using the early region of each viral genome.

Transfection. pUC19-MasPyV was digested with BamHI to release the MasPyV genome. The DNA was purified using a MonoFas column (GL Science) and transfected into Vero cells (or various other cell lines) using FuGENE HD (Roche) or TransIT 2020 (Mirus Bio) according to the manufacturer’s instructions.

Immunofluorescence. Cells were seeded into 12-well dishes and transfected with the BamHI-digested pUC19-MasPyV 3 days before fixation. Cells were fixed for 3 min in 100% methanol at −20 °C. Non-specific binding sites were blocked with 1% BSA and the cells were incubated with a mAb to SV40 Tag (PA:416; Calbiochem), which recognizes the epitope corresponding to aa 83–128, and a polyclonal antibody to JCV VP1 (Suzuki et al., 2001) overnight at 4 °C. Immune complexes were visualized by incubation with AlexaFluor 488-conjugated secondary antibodies (In vitrogen) for 1 h at room temperature. Cell nuclei were counterstained with DAPI (Invitrogen). Fluorescence signals were analysed using an inverted fluorescence and phase-contrast microscope (IX70, Olympus), and images were collected with a charge-coupled device camera with the use of DP controller software (Olympus).

Immunoblot and immunoprecipitation analysis. BHK cells were seeded into 12-well dishes and transfected with BamHI-digested pUC19-MasPyV 4 days before collection. Cells were suspended in RIPA buffer [1% Triton X-100, 150 mM NaCl, 0.1% SDS, 1% deoxycholic acid, 10 mM Tris/HCl (pH 7.5), 5 mM EDTA, 10% glycerol (v/v), 50 mM NaF, 1 mM PMSF and complete protease inhibitor cocktail (Roche)]. Lysates proteins (30 μg) were fractionated by SDS-PAGE and subjected to immunoblot analysis with antibodies to the SV40 TAg and JCV VP1. For immunoprecipitation, IMR-32 cells seeded onto 6 cm dishes were transfected with the indicated constructs 3 days before collection. Cells were then lysed in IP lysis buffer [1% NP-40, 150 mM NaCl, 10 mM Tris/HCl (pH 7.5), 1 mM EDTA, 50 mM sodium fluoride, 1 mM PMSF and complete protease inhibitor cocktail]. Lysed proteins were immunoprecipitated with Dynabeads Protein G (Invitrogen) for 2 h at 4 °C after coating with Flag M2 antibody (Sigma). Protein complexes were eluted with SDS sample buffer [125 mM Tris/HCl (pH 6.8), 10% 2-mercaptoethanol, 4% SDS, 10% sucrose and 0.04% bromophenol blue] after washing with IP lysis buffer. Eluted proteins and 20 μg of the original input proteins were fractionated by SDS-PAGE and subjected to immunoblot analysis with antibodies to p53 (DO-7; Dako), pRB (Calbiochem) and HRP-conjugated M2 antibody (Sigma). Immune complexes were detected with HRP-conjugated secondary antibodies (Biosource International), Immobilon Western HRP substrate (Millipore) and a LAS-1000 Plus system (Fujifilm).

ACKNOWLEDGEMENTS

This study was supported in part by grants from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) and the Ministry of Health, Labour, and Welfare of Japan; the Japan Health Science Foundation, and the Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases, MEXT.
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