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RFHVMn ORF73 is structurally related to the KSHV ORF73 latency-associated nuclear antigen (LANA) and is expressed in retroperitoneal fibromatosis (RF) tumor cells

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Abstract

Retroperitoneal fibromatosis herpesvirus (RFHV), the macaque homolog of the human rhadinovirus, Kaposi's sarcoma-associated herpesvirus (KSHV), was first identified in retroperitoneal fibromatosis (RF) tumor lesions of macaques with simian AIDS. We cloned and sequenced the ORF73 latency-associated nuclear antigen (LANA) of RFHVMn from the pig-tailed macaque. RFHVMn LANA is structurally analogous to KSHV ORF73 LANA and contains an N-terminal serine–proline-rich region, a large internal glutamic acidic-rich repeat region and a conserved C-terminal domain. RFHVMn LANA reacts with monoclonal antibodies specific for a glutamic acid–proline dipeptide motif and a glutamic acid–glutamine-rich motif in the KSHV LANA repeat region. Immunohistochemical and immunofluorescence analysis revealed that RFHVMn LANA is a nuclear antigen which is highly expressed in RF spindloid tumor cells. These data suggest that RFHV LANA is an ortholog of KSHV LANA and will function similarly to maintain viral latency and play a role in tumorigenicity in macaques.

Keywords: LANA; Latency associated; Retroperitoneal fibromatosis; Rhadinovirus; ORF73; KSHV; RFHV; Kaposi's sarcoma

Introduction

Kaposi's sarcoma-associated herpesvirus (KSHV)/human herpesvirus 8 was first discovered in AIDS patients in association with Kaposi's sarcoma (KS) (Chang et al., 1994) and has been linked with other AIDS-related malignancies, such as primary effusion lymphomas (PEL) and multicentric Castleman's disease (Cesarman et al., 1995; Soulier et al., 1995). Analysis of the KSHV genome revealed a strong similarity in sequence and gene organization with herpesvirus saimiri (HVS), the prototype of the *Rhadinovirus* genus of gammaherpesviruses found in the New World squirrel monkey (Russo et al., 1996). KSHV is detected in all epidemiologic forms of KS, including classical KS, endemic KS, and AIDS-associated KS, strongly implicating KSHV as the causative agent of the disease (Verma and Robertson, 2003). Essentially all of the characteristic spindle-shaped tumor cells in KS lesions are latently infected with KSHV (Boshoff et al., 1995; Staskus et al., 1997). KSHV latency is characterized by the expression of a restricted set of viral proteins that are believed to play important roles in the maintenance of the viral genome and in the tumorigenesis process leading to the development of KS (Sarid et al., 1998).

The most prominent protein expressed in cells latently infected with KSHV is the *orf73* gene product, the latency-associated nuclear antigen (LANA) (Kedes et al., 1997; Kellam et al., 1997; Rainbow et al., 1997). KSHV LANA is expressed from a polycistronic transcript with two other latent genes, K13,

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encoding the viral FLICE inhibitory protein (v-FLIP), and *orf72*, encoding the viral cyclin D homolog (v-cycD) (Dittmer et al., 1998; Glenn et al., 1999). LANA is a nuclear protein that functions to ensure the maintenance of the viral genome within the host cell by tethering the viral episomal DNA to host cell chromosomes (Ballestas et al., 1999). LANA also regulates the cellular transcription program in KSHV-infected cells through interaction with a number of cellular proteins, including transcriptional regulators, such as the mSin3-associated corepressor SAP30, Sp1, and ATF4/CREB2, as well as known tumor suppressors, p53 and pRB (Friborg et al., 1999; Krithivas et al., 2000; Radkov et al., 2000). Furthermore, KSHV LANA directly influences the viral transcription program and helps to maintain the latent state of the virus by inhibiting viral replication (Lan et al., 2004).

KSHV LANA is a large polypeptide with three structural domains (Russo et al., 1996). The serine- and proline-rich N-terminal domain contains a nuclear localization signal (NLS) (Piolot et al., 2001), a chromatin-binding motif (CBM) (Wong et al., 2004) and domains responsible for interaction with the transcription regulators, mSin3 complex and Sp1 (Krithivas et al., 2000; Verma et al., 2004). The central domain contains several highly repetitive acidic regions that vary in length and are responsible for the size variation of LANA proteins from different KSHV isolates that can range from 1003 to 1162 amino acids (Gao et al., 1999). The proline-rich C-terminal third domain contains another NLS and is responsible for dimerization of LANA and binding to the terminal repeats (TR) of the viral genomic DNA (Ballestas et al., 1999; Cotter and Robertson, 1999; Komatsu et al., 2004). The C-terminal domain is responsible for interaction with tumor suppressors pRB and p53 (Friborg et al., 1999; Radkov et al., 2000).

Two distinct lineages of rhadinoviruses related to KSHV have been identified in Old World primates. The RV1 rhadinovirus lineage consists of KSHV and its homologs in different non-human primate species, including African green monkeys, drills, gorillas, chimpanzees, and macaques (Greensill et al., 2000; Lacoste et al., 2000a; Lacoste et al., 2000b; Schultz et al., 2000). The macaque RV1 rhadinovirus, retroperitoneal fibromatosis-associated herpesvirus (RFHV), is the most fully characterized of the non-human RV1 rhadinoviruses to date. The complete sequences for a region of the viral genome containing the DNA polymerase and four other adjacent genes have been obtained for two strains of RFHV from different macaque species, *Macaca mulatta* (rhesus) (RFHVMm) and *Macaca nemestrina* (pig-tailed) (RFHVMn) (Rose et al., 2003). The RV2 rhadinovirus lineage consists of a group of more distantly related viruses which co-infect the same Old World primate species (Greensill et al., 2000; Lacoste et al., 2000b; Schultz et al., 2000; Lacoste et al., 2001; Whitby et al., 2003). Of this group, the macaque RV2 rhadinoviruses are the most completely characterized. The complete sequence of the genomes of two strains of the rhesus macaque RV2 rhadinovirus (RRV) have been determined (Alexander et al., 2000; Searles et al., 1999), and the glycoprotein B sequence and a partial sequence of the DNA polymerase gene are available from the virus found in pig-tailed macaques (MneRV2/PMRV) (Auerbach et al., 2000; Schultz et al., 2000). The presence of RV1 and RV2 rhadinoviruses in both Old World monkeys and apes suggests that the two viral lineages have evolved from an ancient non-speciative divergence within an ancestral primate host.

We first identified the macaque RV1 rhadinovirus, RFHV, in retroperitoneal fibromatosis and related subcutaneous fibromatosis lesions of macaques, herein referred to as RF (Rose et al., 1997). RF is a fibroproliferative neoplasm associated with simian AIDS (SAIDS) which has strong similarities to AIDS-KS (Tsai et al., 1990). Like KS, RF lesions are multifocal with increased vascularity, and contain tumor cells exhibiting a characteristic spindle-shaped cell morphology (London et al., 1983; Tsai et al., 1985). We previously detected the presence of both macaque RV1 and RV2 rhadinoviruses in RF tumor lesions by PCR (Schultz et al., 2000; Bielefeldt-Ohmann et al., 2005). In order to study the biology of these viruses and determine their potential roles in the development of RF in macaques, we cloned and sequenced the ORF73 LANA homologs of the RV1 and RV2 rhadinoviruses of the pig-tailed macaque in which the majority of RF cases within the Washington National Primate Research Center (WaNPRC) occurred. We show here that RFHVMn LANA has strong sequence homology to KSHV LANA and exhibits the same structural domains and conserved motifs. We prepared recombinant RFHVMn, MneRV2 and RRV LANA proteins and identified monoclonal antibodies which specifically react with RFHVMn LANA. Using these antibodies, we determined that the spindle-shaped tumor cells within RF lesions are infected with RFHV and express RFHV LANA as a prominent nuclear antigen suggesting that it plays a role in the development and/or maintenance of RF tumors.

Results

Cloning and characterization of ORF 73 LANA of the macaque RV1 rhadinovirus, RFHVMn

A lambda library was prepared from high molecular weight DNA obtained from an archived RF tumor sample of a pig-tailed macaque from the WaNPRC which contained ~1 viral copy/cell of the macaque rhadinovirus, RFHVMn. A lambda clone was identified which contained 10kb of the RFHVMn genome, spanning the *orf73* gene. Sequence analysis revealed a large open reading frame of 3213 bp encoding a 1071-amino-acid protein which showed the highest similarity with the KSHV LANA in a BLAST search. This open reading frame was flanked by sequences upstream and downstream which showed close similarities to ORF72, K13 and K14 of KSHV indicating that the region of the RFHVMn genome containing the LANA locus was identical in structure and gene content to KSHV. Sequence alignment of the LANA homologs of the RFHVMn and KSHV RV1 rhadinoviruses revealed a close similarity in length and structure. Like KSHV LANA, RFHVMn LANA contained an N-terminal region of ~300 aa and a C-terminal region of ~225 aa which were separated by an acidic repeat region of

513 aa, comparable in size to the acidic repeat region within KSHV LANA (Fig. 1).

Alignment of the N-terminal regions of RFHVMn and KSHV LANAs revealed two distinguishable subdomains. The first was a basic amino acid-rich domain just downstream of the initiating methionine (aa 1–48, RFHVMn) which showed significant amino acid conservation between the RFHVMn and KSHV sequences (39% amino acid identity) (Fig. 2). Flanking that was a larger region of 279 aa with low sequence similarity (25% identity), primarily between serine and proline residues (aa 49–335, RFHVMn). Alignment of the C-terminal regions of the RFHVMn and KSHV LANA sequences revealed a conserved C-terminal domain of 223 amino acids (aa 849–1071, RFHVMn) which showed 54% sequence identity between the RFHVMn and KSHV sequences.

A comparison of the internal acidic repeat regions of the RFHVMn and KSHV LANA homologs revealed little sequence similarity except for the abundant presence of glutamic acid residues in both sequences. While the 513-amino-acid acidic repeat region of the RFHVMn LANA homolog contained ~57 copies of perfect and imperfect copies of a single consensus motif, "EEPEPEPE" (Fig. 3B), KSHV LANA contained four separate repeat domains with consensus motifs of EEDD/EEED (~25 repeats), QQQEP/QQREP (~31 repeats), QQQDE/QEQQDE (~32 repeats), and QEQELEE/EEQEEQELEEV (~21 repeats) (based on the 1162-amino-acid variant; Fig. 3A). KSHV LANA variants with sizes ranging from 1003 to 1162 amino acids have been detected with different numbers of repeats within these domains (Gao et al., 1999). Analysis of the underlying DNA sequence encoding the four different KSHV repeats revealed a single consensus DNA sequence element for each repeat that was present a variable number of times (Fig. 3A). Similarly, a single consensus DNA sequence encoded the "EEPEPEPE" motif of RFHVMn LANA (Fig. 3B). No sequence similarity was detected between the DNA consensus elements of the RFHVMn and KSHV LANA homologs.

Dot matrix analysis of the DNA and encoded amino acid sequences of the RFHVMn acidic repeat region revealed the presence of two larger motifs, each present twice in the domain. One repeated motif contained three exact consensus elements and five variant elements (single underline in Fig. 3B), while the other contained six exact consensus elements (double underline, Fig. 3B). These results suggest that the RFHVMn repeat motif has evolved through both short range duplication of the single "EEPEPEPE" sequence and a long range duplication of multiple repeats.

Cloning and characterization of the MneRV2 LANA homolog

In order to compare the LANA homologs of RV1 and RV2 rhadinoviruses from the same macaque host species, we cloned the *orf73* gene from the macaque RV2 rhadinovirus, MneRV2, which like RFHVMn infects the pig-tailed macaque. Sequence analysis revealed an open reading frame of 1314 nucleotides which encoded a protein of 438 amino acids. As shown in Figs. 1 and 2, MneRV2 LANA was similar in structure and length to the 448-amino-acid LANA homolog of RRV, the closely related RV2 rhadinovirus infecting rhesus macaques (*M. mulatta*) (Alexander et al., 2000; Piolot et al., 2001; Searles et al., 1999).

Overall, the LANA homologs of the macaque RV2 rhadinoviruses, MneRV2 and RRV, were 62% identical. The

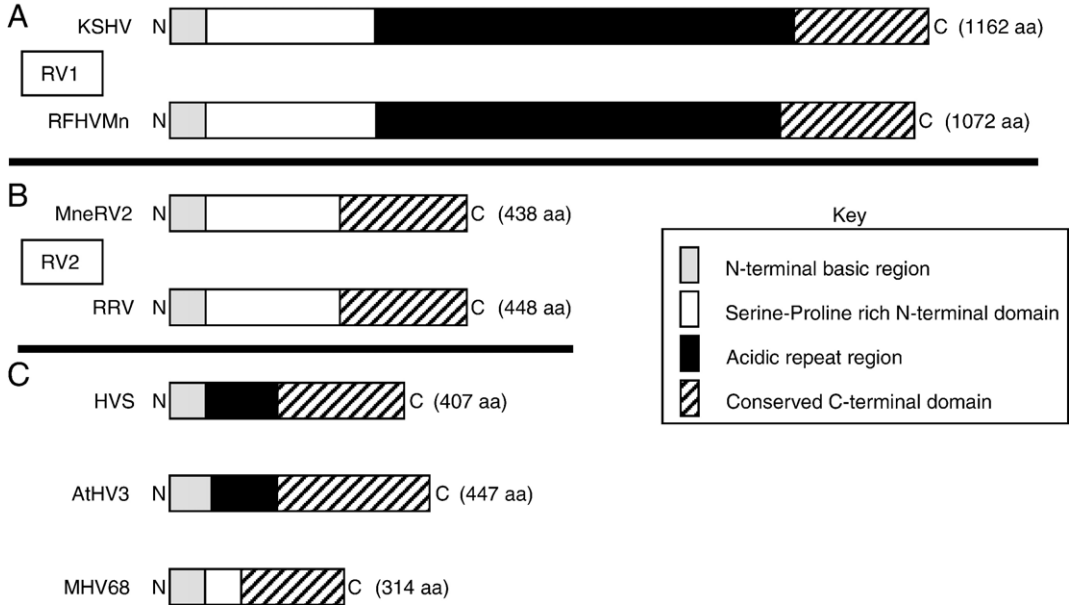

Fig. 1. Schematic structural comparison of the RFHVMn and MneRV2 ORF73 LANAs with homologs from other primate and murine rhadinoviruses. (A) RV1 Old World primate rhadinovirus lineage: KSHV (human; *NP_572129*) and RFHVMn (pig-tailed macaque; this study) (B) RV2 Old World primate rhadinovirus lineage: MneRV2 (pig-tailed macaque; this study), and RRV (rhesus macaque; *AAD21406*) (C) New World primate rhadinoviruses: HVS (squirrel monkey; *NP_040275*), AtHV3 (spider monkey; *NP_048045*), and the murine rhadinovirus: MHV68 (mouse; *NP_044913*).

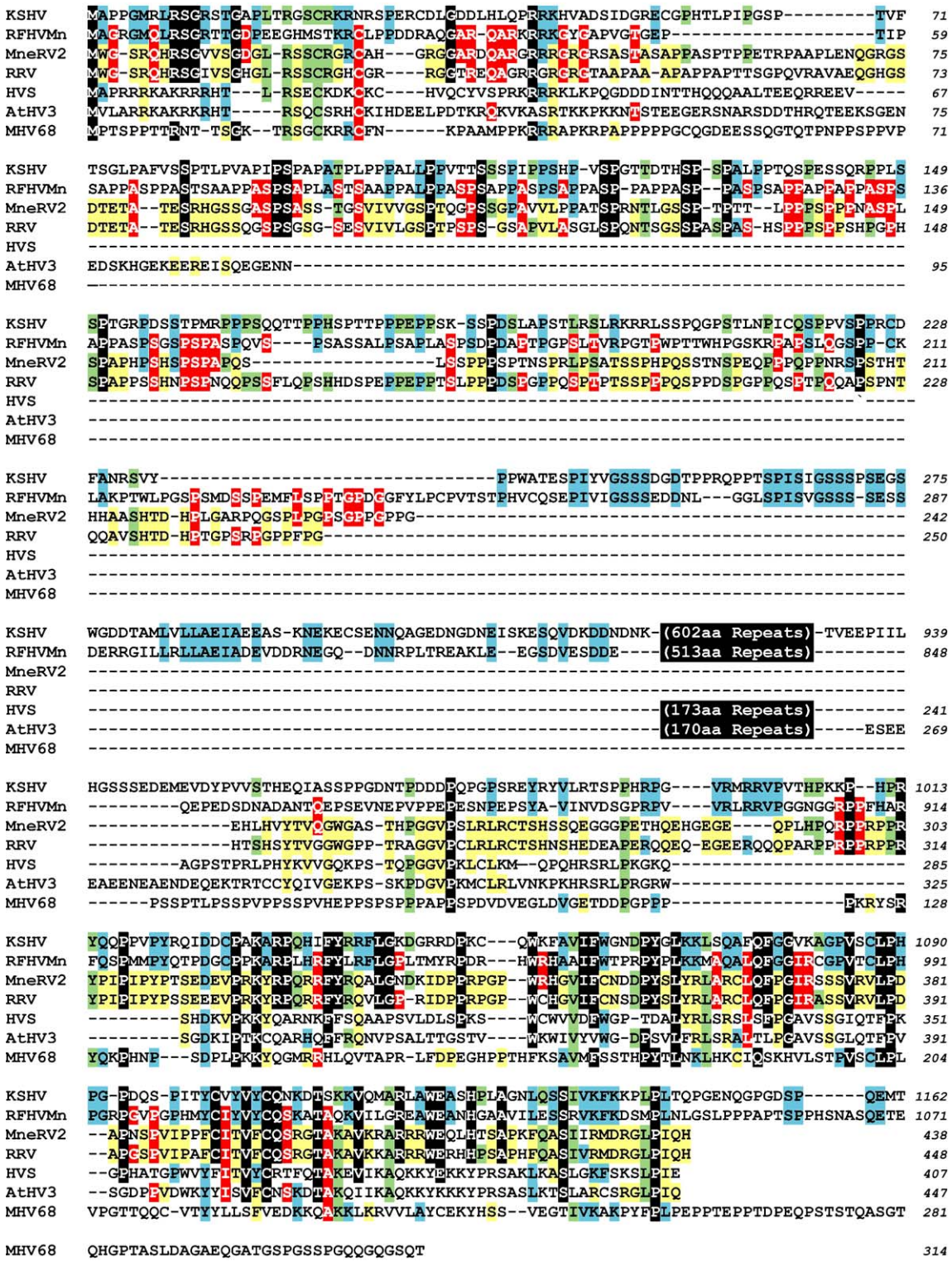

Fig. 2. Amino acid sequence comparison of the RFHVMn and MneRV2 ORF73 LANAs with homologs from other primate and murine rhadinoviruses. Protein alignment of the rhadinovirus ORF73 homologs listed in the legend to Fig. 1. Shaded residues indicate amino acid sequence conservation: (Black)—conserved in both RV1 and RV2 Old World primate rhadinoviruses, KSHV, RFHVMm, RRV and MneRV2; (Blue)—conserved in both human and macaque RV1 rhadinoviruses, KSHV and RFHVMn; (Yellow)—conserved in both macaque RV2 rhadinoviruses, RRV and MneRV2; (Green)—conserved in both the human RV1 rhadinovirus, KSHV, and either macaque RV2 rhadinovirus, MneRV2 or RRV; (Red)–conserved in the macaque RV1 rhadinovirus, RFHVMn, and either macaque RV2 rhadinovirus, MneRV2 or RRV. Amino acid sequences of the internal acidic repeat domains indicated as black boxes are provided in Fig. 3.

Fig. 3. Comparison of the internal acidic repeat domains of RFHVMn LANA with repeat domains of other rhadinoviruses. The amino acid sequences are given for the internal acidic repeat domains of the human KSHV LANA (A) (1162-aa isolate) and macaque RFHVMn LANA (B) (1071 aa, this study) RV1 rhadinoviruses and the squirrel monkey HVS (C) and spider monkey AtHV3 (D) New World primate rhadinoviruses (for accession numbers, see legend to Fig. 2). The four major repeat subregions within the KSHV LANA repeat domain (1162-aa variant) are shown. The consensus amino acid and nucleotide repeat elements are indicated. Duplications of identical sequences containing multiple consensus repeat elements in the RFHVMn sequence are indicated by single and double underlining.

first N-terminal 46 amino acids were highly conserved (75%) and like the analogous regions in the RFHVMn and KSHV sequences contained a number of basic amino acids (Fig. 2). The MneRV2 and RRV LANAs also contained a serine- and proline-rich region (aa 47–242, MneRV2) which was 51% conserved and analogous to those seen in RFHVMn and KSHV (Fig. 2). While the serine–proline-rich regions of the RRV and MneRV2 LANAs were 25–26% identical to the corresponding region in RFHVMn LANA, these regions were less than 20% conserved with KSHV LANA. The C-terminal region of MneRV2 LANA (aa 295–438) was strongly conserved with the corresponding region in RRV LANA (88% sequence identity), but only moderately conserved with RFHVMn and KSHV LANAs (33–35% sequence identity).

Comparison of the RFHVMn and MneRV2 LANAs with ORF73 homologs of more distantly related rhadinoviruses

ORF73 homologs have been identified in several primate and non-primate rhadinoviruses, including the New World monkey rhadinoviruses, HVS and AtHV3 (ateline herpesvirus 3), and the murine rhadinovirus, MHV68. The ORF73 homologs of these rhadinoviruses contain an N-terminal basic amino-acid-rich region and a conserved C-terminal domain, as seen in the Old World primate rhadinoviruses (Figs. 1 and 2). The C-terminal domains of the New World primate rhadinoviruses were more strongly conserved with those of the RV2 macaque rhadinoviruses (34–35% identity) than with those of the RV1 macaque or human rhadinoviruses (25–27%). Both New World primate ORF73 homologs lacked a domain homologous to the ~200 amino acid serine–proline-rich N-terminal domain present in all Old World primate LANA homologs, making these proteins significantly smaller than RFHVMn LANA (407/447 aa vs. 1071 aa) (Figs. 1 and 2). Both New World primate ORF73 homologs contained internal acidic repeat regions, which were, however, smaller than those found in RFHVMn and KSHV (~170 vs. 513 and 602 aa, respectively; Figs. 3C and D). No similarity was detected between the internal acidic repeats of HVS and AtHV3, nor was there similarity with the RFHVMn and KSHV repeats at the level of either the amino acid repeat motifs or the consensus DNA elements encoding the repeats (Fig. 3). The murine MHV68 ORF73 homolog was smaller than all of the other rhadinovirus homologs, with only 314 aa (Figs. 1 and 2). While the MHV68 ORF73 contained a conserved C-terminal domain and a small N-terminal serine–proline-rich domain, these domains were the least conserved with RFHVMn, MneRV2 and the other rhadinovirus ORF73 homologs. MHV68 ORF73, like the macaque RV2 rhadinovirus ORF73 homologs, lacked an internal acidic repeat region.

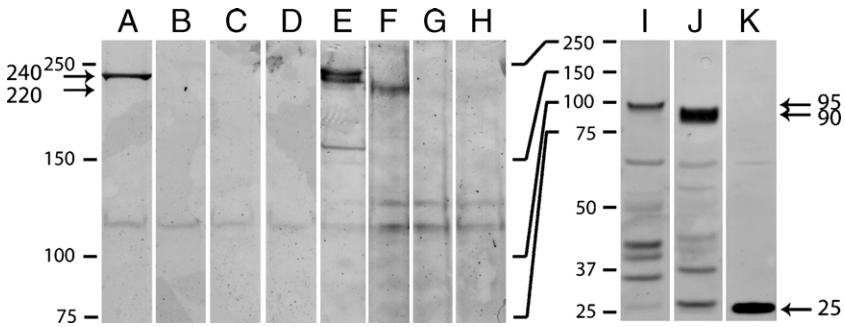

Fig. 4. Western blot analysis of COS-7 expressed recombinant RFHVMn LANA. Plasmid DNA was transfected into COS-7 cells and expressed proteins were harvested after 18 h, resolved by SDS-PAGE and immunoblotted with either the mAB247 anti-EP monoclonal antibody (lanes A–D), the LN53 anti-KSHV LANA monoclonal antibody (lanes E–H), or the Living Colors anti-EGFP antisera (lanes I–K). (pCDNA3/RFHV ORF73 (lanes A, E); pCDNA3/KSHV ORF73 (lanes B, F); pEGFP/RRV ORF73 (lanes C, G, I); pEGFP/MneRV2 ORF73 (lanes D, H, J); and pEGFP control plasmid (lane K)) Arrows indicate the positions of the positive LANA protein bands and their estimated molecular weights.

Identification of two monoclonal antibodies reactive with RFHVMn LANA

Sequence analysis of RFHVMn LANA revealed the presence of a large internal acidic repeat domain which contained multiple repetitions of the consensus sequence "EEPEPEPE" (Fig. 3B). A BLAST search of the NCBI protein database revealed a close similarity between the RFHVMn acidic repeat and an "EPEPEP" repeat found in the *Trypanosome brucei* procyclin against which a monoclonal antibody (mAB247) had been developed (Richardson et al., 1988). In order to determine whether the anti-EP antibody would react with the RFHVMn LANA, we cloned the RFHVMn *orf73* gene into the mammalian expression vector pCDNA3 to produce recombinant LANA. For controls, the MneRV2 and RRV *orf73* genes were cloned into the pEGFP vector to produce ORF73 fusions with the enhanced green fluorescent protein (EGFP) at the C-terminus to permit detection. In addition, a pCDNA3 expression vector containing the KSHV LANA gene was obtained that has been described previously (Renne et al., 2001). The LANA expression vectors were transfected into COS-7 cells. Western analysis of the expressed proteins revealed a strong reactivity between the anti-EP monoclonal antibody and a protein of approximately 240 kDa in the COS cells transfected with the pCDNA3/RFHV LANA construct (Fig. 4, lane A). Even though the predicted molecular weight of the RFHVMn LANA was approximately 118 kDa, the 240-kDa band was similar in size to the 220- to 234-kDa KSHV LANA which is known to migrate abnormally in SDS-PAGE (Rainbow et al., 1997). No reactivity was detected to proteins in the COS cells transfected with either the pCDNA3/KSHV LANA or the pEGFP/RRV or pEGFP/MneRV2 LANA fusion proteins (Fig. 4, lanes B–D). The presence of RRV and MneRV2 LANA in the transfected cells was demonstrated by reactivity with the anti-EGFP Living Colors antibody. The anti-EGFP antibody reacted with a 95-kDa protein in cells transfected with the RRV-EGFP construct (Fig. 4, lane I) and a 90-kDa doublet in cells transfected with the MneRV2-EGFP construct which was 10 amino acids smaller than the RRV-EGFP (Fig. 4, lane J). Several proteolytic degredation fragments were also observed, as has been noted for EGFP fusion proteins of KSHV LANA (Piolot et al., 2001). As a control, the anti-EGFP antibody also reacted with the 25-kDa EGFP fusion partner (Fig. 4, lane K). Previous studies have shown that Flag-tagged RRV LANA migrated as a 70-kDa doublet (DeWire and Damania, 2005) which is compatible with the size of RRV LANA within the 95-kDa LANA/EGFP fusion protein produced in our transfected cells.

We also tested the ability of recombinant RFHVMn LANA to react with the LN53 monoclonal antibody developed against KSHV LANA. This antibody reacts with a glutamic-acid-rich repeat "EQEQE" in the internal acidic repeat region of KSHV LANA (Kellam et al., 1999). Western analysis displayed a strong reactivity of the anti-KSHV LANA antibody with a 240-kDa doublet in the COS cells transfected with the pCDNA3/RFHV LANA construct (1071-aa protein) (Fig. 4, lane E). Similar reactivity was seen with a slightly smaller 220-kDa doublet in cells transfected with the KSHV ORF73 construct (1003-aa protein) (Fig. 4, lane F). No reactivity was detected with the MneRV2 and RRV LANA-EGFP fusion proteins (Fig. 4, lanes G and H).

RFHVMn and MneRV2 LANA proteins localize to the cell nucleus

To determine whether the RFHVMn and MneRV2 LANA proteins localize to the cell nucleus, as seen with KSHV LANA, the expression constructs for RFHVMn and KSHV LANA proteins, and the RRV and MneRV2 LANA-EGFP fusion proteins were transfected into COS-7 cells. After 12 h,

Fig. 5. Immunofluorescence localization of recombinant RFHVMn LANA in transfected COS-7 cells. Plasmid DNA was transfected into COS-7 cells. After 12 h, the cells were fixed, permeabilized, and incubated with Topro-3 DNA stain to detect nuclei (Blue) and either (A) the anti-EP antibody or (B) the LN53 anti-KSHV LANA antibody to detect the LANA proteins. Antibody-bound proteins were visualized using a red fluorescent secondary antibody while the GFP-tagged LANA fusions were visualized as green fluorescence. Inserts contain the merged Topro-3 and antibody immunofluorescence images of magnified cells. Transfected plasmids included, pCDNA3/RFHV ORF73, pCDNA3/KSHV ORF73, pEGFP/RRV ORF73, pEGFP/MneRV2 ORF73 and the pEGFP control. Arrows indicate positive cell nuclei.

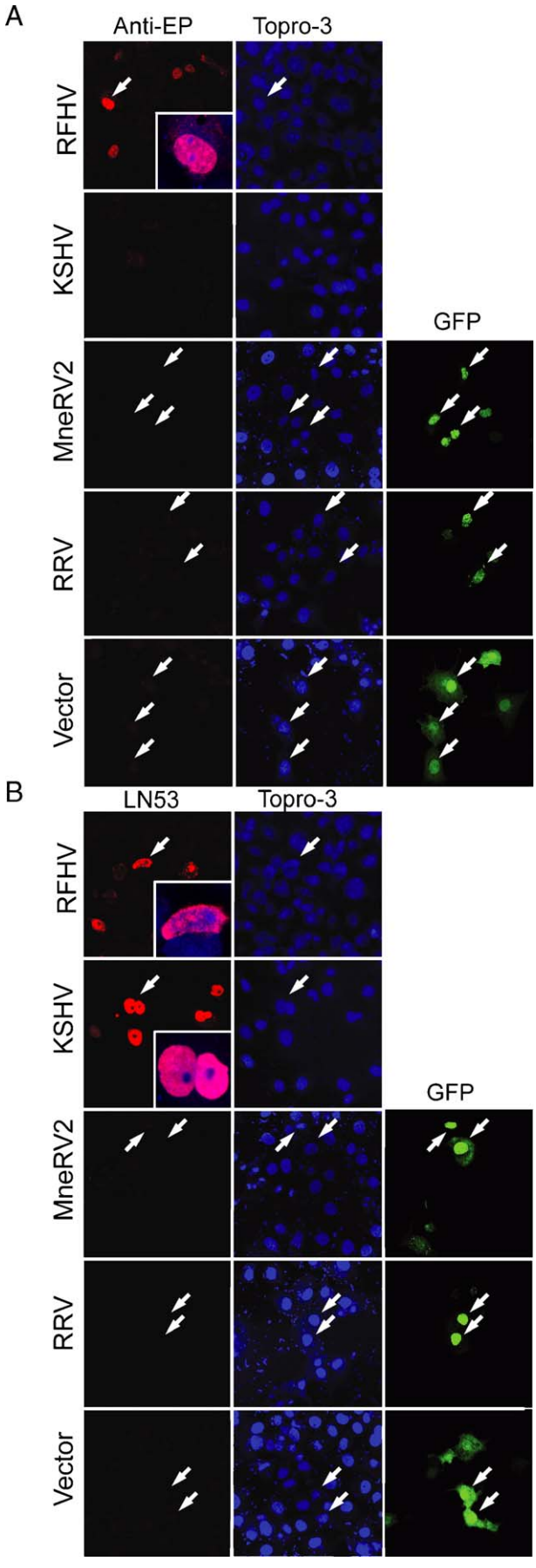

Fig. 5.

the cells were fixed with 8% paraformaldehyde. In the cells transfected with pCDNA3/RFHV ORF73, a strong immunofluorescence signal was detected with the anti-EP antibody in approximately 40% of the cells (Fig. 5A). The anti-EP immunofluorescence signal was confined to the nucleus and co-localized with the Topro-3 DNA fluorescence (Fig. 5A,

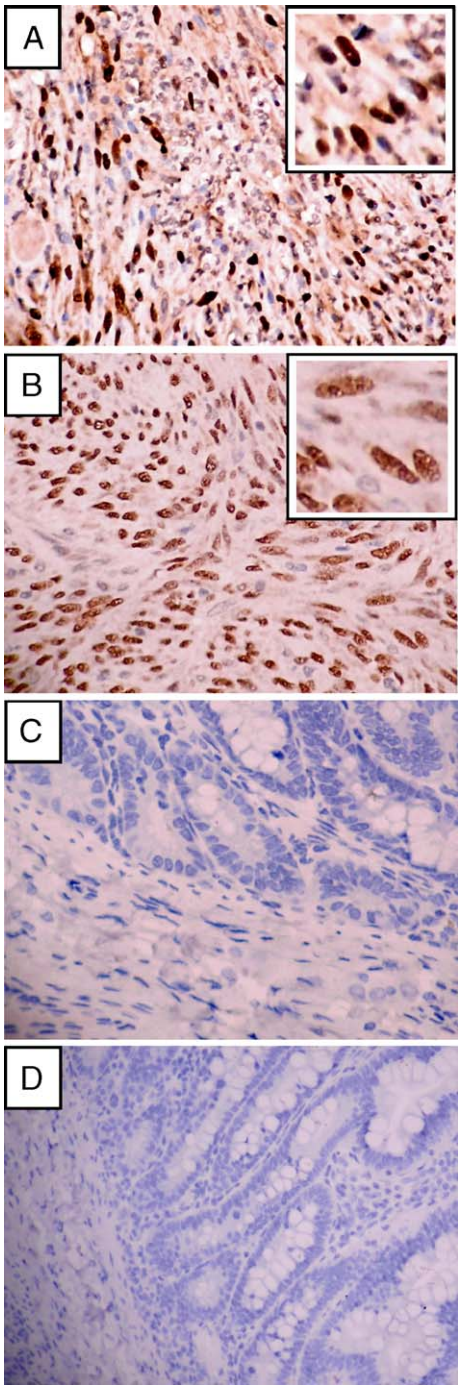

Fig. 6. Immunohistochemical localization of RFHVMn LANA in RF tumor biopsies. 5-μm sections of paraffin-embedded RF tumor (panels A, B) and normal jejunum (panels C, D) tissues were deparaffinized, subjected to antigen-retrieval, incubated with the LN53 anti-KSHV LANA monoclonal antibody (panels A and C) or the anti-EP monoclonal antibody (panels B and D), and finally counterstained with hematoxylin. Inserts in panels A and B show magnification of positive nuclei recognized by the monoclonal antibodies.

insert). An obvious speckled pattern of immunofluorescence was detected with an absence of staining in the nucleoli. The anti-KSHV LANA monoclonal antibody gave a strong nuclear immunofluorescence signal in the pCDNA3/RFHV ORF73 transfected cells (Fig. 5B), confirming the reactivity of this antibody with RFHVMn LANA in Western analysis. This fluorescence also exhibited a speckled pattern which was absent from the nucleoli (Fig. 5B, insert). In the cells transfected with the pCDNA3/KSHV ORF73, no staining was detected with the anti-EP antibody (Fig. 5A), while a strong nuclear staining was detected with the LN53 anti-KSHV LANA antibody, as expected (Fig. 5B). These cells also showed a speckled pattern of immunofluorescence and a lack of nucleolar staining. No staining was apparent with either the anti-EP or LN53 anti-KSHV LANA antibodies in the cells transfected with the pEGFP/MneRV2 or pEGFP/RRV LANA constructs. The presence of the RRV and MneRV2 LANA-EGFP fusion proteins in these transfected cells was demonstrated by the presence of EGFP fluorescence confined to the nucleus (Figs. 5A and B). Cells transfected with the pEGFP vector alone showed staining throughout the cell demonstrating that the LANA portion of the LANA/EGFP fusion protein was responsible for the nuclear localization (Figs. 5A and B). These studies confirmed the antibody reactivity seen in our Western blot analysis.

Expression of RFHVMn LANA in the nucleus of RF tumor cells

We have previously detected RFHV DNA in RF and SF tumor lesions (Rose et al., 1997; Schultz et al., 2000). In order to determine whether RFHVMn LANA was expressed in the tumor cells of RF lesions, the anti-EP and anti-KSHV LANA monoclonal antibodies were reacted with formalin-fixed paraffin-embedded sections of RF tumor lesions. The LN53 anti-KSHV LANA antibody showed strong staining of the spindeloid tumor cells present in the RF lesion (Fig. 6A). This staining was confined to the nuclei of the tumor cells (see inset). No staining was detected in normal cells adjacent to the RF tumor nor was staining observed in normal cells in the jejunem of a non-affected macaque (Fig. 6C). The anti-EP monoclonal antibody also showed intense staining of the nuclei of the spindloid RF tumor cells (Fig. 6B and inset), replicating that seen with the LN53 antibody. No reactivity was seen with normal cells adjacent to the RF tumor nor with cells present in jejunem of a non-affected macaque with the anti-EP antibody (Fig. 6D). Histological and morphological similarities have previously been noted between the spindle-shaped tumor cells in RF lesions and those detected in KS lesions (London et al., 1983; Tsai et al., 1985; Bielefeldt-Ohmann et al., 2005). Our results demonstrate a strong similarity between the anti-LANA antibody staining of the nuclei of the spindloid tumor cells of RF lesions and that seen in KS lesions (Rainbow et al., 1997).

Discussion

In the current study, we cloned and sequenced the ORF73 LANA homologs of the RV1 and RV2 rhadinoviruses infecting the pig-tailed macaque, RFHVMn and MneRV2, respectively. Analysis of RFHVMn LANA revealed a close similarity in size and structure to KSHV LANA. RFHVMn LANA contained the three distinct domains that have previously been identified in KSHV LANA, including a serine–proline-rich N-terminal domain initiated by a region of basic amino acids, a large highly acidic internal repeat region, and a conserved C-terminal domain (Russo et al., 1996). The 1071-amino-acid RFHVMn LANA, with a predicted molecular weight of approximately 118 kDa, resolved on SDS-PAGE as a 240-kDa protein that variably migrated as a doublet (Fig. 4). This is similar to the anomalous migration observed with KSHV LANA which has a predicted molecular weight of 116–135 kDa but migrates in SDS-PAGE as a doublet of 220–234 kDa. The migration of RFHVMn LANA may be affected by the presence of the internal acidic repeat region, as has been suggested for KSHV LANA (Rainbow et al., 1997).

Analysis of MneRV2 LANA revealed a close similarity in structure and sequence to the LANA of the closely related rhesus macaque RV2 rhadinovirus, RRV. Both proteins contained a serine–proline-rich N-terminal domain of approximately 250 amino acids, initiated with a basic amino-acid-rich region, and a C-terminal domain of approximately 200 amino acids which was highly conserved between the two sequences (88% identical amino acids). Although the N- and C-terminal domains were similar to those seen in RFHVMn and KSHV, neither of the macaque RV2 rhadinovirus LANA homologs contained the large internal acidic repeat region characteristic of the RV1 rhadinovirus LANA homologs.

The serine–proline-rich N-terminal domains of RFHVMn and KSHV LANA showed little sequence similarity except for the presence of serine and proline residues. Only the corresponding domains in the closely related RV2 rhadinoviruses, RRV and MneRV2, had a significant sequence similarity beyond the proline and serine residues, with 51% conserved amino acids. Interestingly, in this region, the RFHVMn sequence was as closely related to the two macaque RV2 rhadinoviruses (25–26% identity) as it was to the KSHV homolog (25% identity) (see residues shaded in red, Fig. 2). Since the N-terminal domain of KSHV LANA is known to interact with the host chromatin, the sequences conserved in the macaque RV1 and RV2 rhadinoviruses may be important for interacting with macaque chromatin.

The C-terminal domains of all the rhadinovirus LANA homologs showed the strongest cross-sequence homology. The LANA sequences of the two macaque RV2 rhadinoviruses, MneRV2 and RRV, were 88% identical in this region, while the macaque and human RV1 rhadinoviruses, RFHVMn and KSHV, were 51% identical. The RV2 C-terminal sequences were as similar to the New World primate rhadinovirus ORF73 sequences as they were to the RV1 sequences (~35% in both cases). A number of functionalities have been assigned to the C-terminal domain of KSHV LANA, including dimerization, transcriptional activation and repression, inactivation of cell cycle regulatory proteins, binding to nuclear heterochromatin (An et al., 2005; Lan et al., 2005; Mattsson et al., 2002; Platt et al., 1999; Radkov et al., 2000; Schwam et al., 2000; Viejo-

Borbolla and Schulz, 2003) and binding to the terminal repeat of the KSHV genome (Cotter et al., 2001). Some of these functionalities have also been identified in the LANA homologs of RRV (DeWire and Damania, 2005) and HVS (Borah et al., 2004; Verma and Robertson, 2003) supporting a conservation of structure and function, even with the more distantly related proteins. Further studies will reveal the extent of the functional similarities between the RFHVMn and KSHV LANA homologs.

The most striking structural resemblance between KSHV and RFHVMn LANA was the presence of the large internal acidic repeat domains (see Figs. 1 and 3). In the RFHVMn isolate sequenced, this repeat domain was approximately 513 amino acids in length. This is similar to the size of the repeat domain in KSHV LANA which varies from 416–602 amino acids in different KSHV isolates (Gao et al., 1999). The KSHV repeat region consists of at least four separate repeat elements with consensus sequences of "EEDD/EEED", "QQQEP/QQREP", "QQQDE/QEQQDE", and "QEQELEE/EEQEEQELEEV" where the number and exact make-up of these domains varied between isolates (see Fig. 3A). The RFHVMn repeat, however, consisted almost entirely of a single consensus element "EEPEPEPE". This consensus sequence and some minor variants were repeated approximately 57 times (Fig. 3B). This suggests that the 513-amino-acid repeat domain evolved through both short range and long range duplication events. No obvious sequence similarity was observed between the KSHV and RFHVMn LANA repeat domains, other than the presence of a large number of glutamic acid residues. Although the origin and evolution of the internal repeat domains within the KSHV and RFHVMn LANA homologs appear to be distinct, it is of interest that the size of the domains are quite similar. Internal acidic repeat domains have been identified in the LANA homologs of the New World primate rhadinoviruses, however, these domains were much smaller (<200 amino acids) (Fig. 3), and showed no homology to the repeats of RFHVMn or KSHV, except for the presence of acidic amino acids.

The presence of similarly sized large internal acidic repeat regions within the LANA homologs of RFHVMn and KSHV is suggestive of a shared structure and function. While no function has yet been associated with the repeat region within KSHV LANA, it has been suggested that it could participate in the control of euchromatin/heterochromatin transition through facilitating the opening of closed heterochromatic areas via its large acidic structure (Szekely et al., 1999). Repeating patterns of glutamic acid and proline residues, as seen within the RFHVMn LANA internal repeat, have been identified in other proteins. The trypanosome procyclin, for example, has a small domain containing approximately 30 "EP" repeats which is reactive with the mAB247 anti-EP monoclonal antibody (Richardson et al., 1988). Molecular modeling and nuclear magnetic resonance studies have indicated that glutamic acid–proline dipeptide repeats have an extended rod-like structure (Evans et al., 1986), and it was hypothesized that the "EP" repeats in the procyclin provide it with a highly extended structure important for its function as a major surface glycoprotein (Roditi et al., 1989). Although the consensus glutamic acid–proline repeat "EEPEPEPE" in RFHVMn LANA is a variant of the dipeptide repeat, a similar extended structure could be important for tethering the RFHVMn episome to host cell chromatin and participating in chromatin remodeling.

We found that the mAB247 monoclonal antibody which recognizes the dipeptide "EP" repeats of the trypanosome procyclin (Richardson et al., 1988) also reacts with "EP" repeats within the recombinant RFHVMn LANA in both Western and immunofluorescence analysis (see Figs. 4A and 5A). This reactivity is specific given that neither MneRV2, RRV nor KSHV LANA sequences are recognized in similar assays. We also found that the LN53 anti-KSHV LANA monoclonal antibody which recognizes the "EQEQE" epitope in the KSHV LANA repeat region (Kellam et al., 1999) also reacts with RFHVMn LANA (see Figs. 4E and 5B). This reactivity is specific for RFHVMn LANA, as the antibody does not recognize either of the MneRV2 and RRV LANA homologs in both Western and immunofluorescence assays. Sequence analysis revealed that RFHVMn LANA lacked the repeated "EQEQE" epitopes recognized by the LN53 monoclonal antibody. Given the presence of multiple iterations of the sequence "EPEPE" within the RFHVMn internal repeat, this monoclonal antibody may recognize a structural motif based on the repetitive glutamic acid residues. Since the anti-EP monoclonal does not react with the KSHV LANA repeats, the reactive epitopes of the anti-EP and LN53 anti-KSHV LANA antibodies appear to be different.

Our studies demonstrated that KSHV, RFHVMn, MneRV2 and RRV LANA recombinant proteins associated with the cell nucleus when expressed from plasmids transfected into COS cells. This was true for RFHVMn LANA, and for MneRV2 and RRV LANA which were expressed as N-terminal fusions with EGFP (see Fig. 5). We have also shown that RFHVMn LANA localized to the nucleus of spindloid tumor cells in macaque RF lesions using the LN53 anti-KSHV LANA and mAB247 anti-EP monoclonal antibodies (Figs. 6A and B). The nuclear localization signal for KSHV LANA has been localized to a region of basic amino acids "RKRNRSP" (aa 24–30) (Piolot et al., 2001). This sequence has similarity with nuclear localization signals of other proteins, such as EBNA1 of EBV (eKRpRSP) (Ambinder et al., 1991). A comparison of the aligned RFHVMn LANA sequence at this position, i.e., aa 24–30, revealed minimal similarities, with conservation of only three of the seven amino acids (tKRclpP). Even less conservation was evident with the aligned sequences of RRV and MneRV2 (aa 22–28-"Rghcahg" and "RgRcahg", respectively). A previous study demonstrated that the N-terminal domain of RRV (aa 1–25), which is highly conserved with the MneRV2 sequence (22/25 identical residues) induced nuclear localization of red fluorescent protein (DeWire and Damania, 2005), suggesting that this region contained a functional NLS. Using a software program to predict putative NLSs we identified a putative NLS sequence within RFHVMn that spanned aa 41–47 and encoded the highly basic "ARKRRKG" motif. Further studies to determine the sequences necessary to mediate nuclear localization of RFHVMn, as well as MneRV2 and RRV LANA are ongoing.

Using both the anti-EP and anti-KSHV LANA antibodies, we have shown that RFHVMn LANA is a highly expressed nuclear antigen in the spindle-shaped tumor cells in RF lesions of macaques with SAIDS-RF (Figs. 6A and B). RF and the closely related subcutaneous fibromatosis (SF) are progressive fibrovascular proliferations with morphological and histological similarities to KS lesions (London et al., 1983; Tsai et al., 1990). The neoplastic lesions of RF/SF and KS are multifocal and contain characteristic spindle-shaped tumor cells. While the KS spindle cells are of endothelial origin and carry lymphatic markers, the exact nature and origin of the RF/SF spindle cells are still in question (Bielefeldt-Ohmann et al., 2005). However, the expression and localization of the RFHVMn and KSHV LANA in the nuclei of the spindle tumor cells are a common feature of these two related diseases. Functional analysis of KSHV LANA has demonstrated that it plays a role in tethering the KSHV genome to host chromatin in the infected cells and functions to maintain viral latency by suppressing the lytic program of virus replication, stimulating host cell proliferation, interfering with cellular tumor suppressor functions and blocking proapoptotic pathways (Ballestas et al., 1999; Friborg et al., 1999; Krithivas et al., 2000; Lan et al., 2004; Radkov et al., 2000). The close structural similarity between RFHVMn and KSHV LANA and their common presence in the nuclei of infected spindle tumor cells suggests that RFHVMn LANA, like KSHV LANA, may play an important role in maintaining viral latency and inducing cellular proliferation.

In conclusion, we have identified and characterized the ORF73 LANA homologs of the macaque RV1 and RV2 rhadinoviruses, RFHVMn and MneRV2, respectively. Our studies have shown that RFHVMn LANA is the closest known structural homolog of KSHV LANA, and like KSHV LANA, is a nuclear antigen which is highly expressed in virus-infected tumor cells. The functional comparison of RFHVMn and KSHV LANA and the exploitation of their structural similarities may provide an important approach for establishing the role these proteins play in viral latency, pathogenicity and pathogenesis of the virus-associated neoplastic lesions.

Materials and methods

Cells

The COS-7 African green monkey fibroblast cell line was obtained from the ATCC, while the rhesus primary fetal fibroblast (RPFF) cell line was a kind gift of M. Axthelm, Oregon National Primate Research Center (ONPRC). Cells were cultured in DMEM complete medium at 37 °C (DMEM with 10% Cosmic-Calf Serum (Hyclone), 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM HEPES).

Rhadinovirus isolates

An isolate of the rhesus macaque RV2 rhadinovirus, RRV strain 17577, was kindly provided by M. Axthelm and S. Wong (ONPRC). We have previously obtained an isolate of the pig-tailed macaque RV2 rhadinovirus, MneRV2 from a naturally infected pig-tailed macaque (*M. nemestrina*) (J97167) at the WaNPRC (Bruce et al., 2005).

Tissues

RF tissues for cloning studies were obtained from a simian retrovirus-2 (SRV-2) positive pig-tailed macaque (M78114) with SAIDS-RF (animal 2 in Tsai et al., 1985). RF tissues for immunohistochemistry were obtained from a 5.4-year-old SRV-2 negative male pig-tailed macaque (K99174) which was experimentally infected with SIV. This animal had two neoplastic masses in the distal jejunum, which on histology were diagnosed as RF.

Cloning and sequence analysis of RFHVMn LANA

High molecular weight DNA was isolated from an archived frozen RF tumor lesion and used to construct a MboI-partial genomic DNA library in lambda DASH-II vector (Stratagene). A lambda clone containing the RFHVMn *orf73* gene was identified using a PCR-based screening technique, essentially as described in (Bloem and Yu, 1990). This clone contained a region of the RFHVMn genome that included the open reading frames RF13, 72, 73, RF14 and 75. The sequence of the RFHVMn *orf73* gene was determined and deposited in Genbank with accession number 816131.

PCR amplification and sequence analysis of MneRV2 LANA

The *orf73* coding sequences for MneRV2 were identified using a consensus-degenerate hybrid oligonucleotide (CODEHOP) PCR approach, essentially as described in Rose et al. (2003). DNA isolated from RPFF cells infected with the MneRV2 rhadinovirus isolate (J97167) was used as template. The complete sequence of the *orf73* LANA, *N14* and *orf72* flanking sequences were obtained and the MneRV2 *orf73* sequence was deposited in Genbank with accession number 824200.

Sequence analysis

The sequences of the LANA homologs from KSHV, RRV, MneRV2 and RFHVMn were multiply aligned using ClustalW. In order to identify possible nuclear localization sequences, the LANA sequences were analyzed using the web-based "predictNLS" program (http://cubic.bioc.columbia.edu/predictNLS).

Construction of LANA expression vectors

The RFHVMn *orf73* coding sequences and 117 bp of 5′ non-coding sequence were cloned into pCDNA3 (Invitrogen) yielding the expression plasmid, pCDNA3/RFHV ORF73. The MneRV2 and RRV *orf73* coding sequences including 22 bp and 17 bp of 5′ non-coding sequences, respectively, were

cloned into the pEGFP vector (Invitrogen) yielding expression plasmids pEGFP/MneRV2 ORF73 and pEGFP/RRV ORF73 producing ORF73-EGFP fusion proteins. The KSHV pCDNA3.1 V5/HISA *orf73* (pCDNA3/KSHV ORF73) encoding the 1003-aa KSHV LANA was a kind gift from Rolf Renne, and has been described previously (Renne et al., 2001).

Transfections

COS-7 cells were transfected with either the pEGFP (vector control), pEGFP/MneRV2 ORF73, pEGFP/RRV ORF73, pCDNA3/RFHV ORF73, or pCDNA3/KSHV ORF73 vector using Transit LT-1 (Mirus). Cells were incubated at 37 °C and either processed for immunofluorescence after 11–24 h or harvested for SDS-PAGE/Western analysis. For immunofluorescence, the cells were first analyzed on an inverted fluorescence microscope for EGFP expression and then fixed with 8% paraformaldehyde for subsequent immunofluorescence processing and Topro-3 staining.

Antibody reagents

The rat monoclonal LN53 antibody, reactive with the "EQEQE" epitope of the KSHV ORF73 LANA protein (Kellam et al., 1999), was purchased from ABI Advanced Biotechnologies, Columbia, MD. The mouse monoclonal mAB247, reactive with the "EP" repeat of the T. brucei procyclin (Richardson et al., 1988), was a kind gift of Terry Pearson, University of Victoria, Canada. The rabbit anti-EGFP Living Colors antibody was purchased from Clontech. Secondary antibody reagents included alkaline phosphatase-labeled goat antibodies against rabbit (Promega), rat (Biosource), and mouse IgG (Promega), Alexa 594 (red) fluorescent-labeled goat antibodies against mouse(rat) IgG (Molecular Probes), and biotinylated goat antibodies against rat and mouse IgG (Vector Laboratories).

Western analysis

Transfected cells were solubilized and the proteins were resolved on SDS-PAGE (Invitrogen) and transferred to nitrocellulose membranes. The membranes were incubated with either the LN53 anti-KSHV LANA antibody (1:1000), the mAB 247 anti-EP antibody (1:100), or the Living colors anti-EGFP antibody (1:1000). The membranes were then incubated with alkaline phosphatase-labeled goat anti-immunoglobulin antibodies. The proteins were visualized with 5-Bromo-4-Chloro-3-Indolylphosphate/Nitro Blue Tetrazolium (BCIP/NBT) Liquid Substrate System (Sigma). The Precision Plus Protein Dual Color standard (Bio-Rad) was used for molecular weight estimation.

Immunofluorescence assays

The COS-7 cells transfected with the different LANA expression vectors were fixed, permeabilized and reacted with either the LN53 anti-KSHV LANA antibody (1:1000) or the mAB247 anti-EP antibody (1:40) diluted in Blocker. Bound immunoglobulins were detected with fluorescent labeled goat anti-mouse(rat) IgG. Nuclear DNA was visualized with Topro-3 (1:500) (Molecular Probes). The cells were analyzed using a confocal fluorescent microscope (Zeiss).

Immunohistochemistry

Tissue sections were deparaffinized and subjected to antigen-retrieval by boiling in 0.1mM EDTA, pH 8.0, for 15 min. The sections were incubated with the LN53 anti-KSHV LANA antibody or the mAB247 anti-EP antibody and bound antibody was visualized with biotinylated anti-immunoglobulin antibody and avidin–biotin–horseradish-peroxidase conjugates (Vector Laboratories) with diamino-benzidine or 3-amino-9-ethylcarbazole as the chromogen. Sections were counterstained with Harry's hematoxylin and examined in a Nikon Eclipse E600 microscope. Microphotographs were taken using a Nikon Coolpix 4500 camera and prepared for publication using Adobe Photoshop CS.

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