The herpesvirus saimiri ORF73 gene product interacts with host-cell mitotic chromosomes and self-associates via its C terminus

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The herpesvirus saimiri (HVS) ORF73 gene product shares limited homology with the ORF73 protein of Kaposi’s sarcoma-associated herpesvirus (KSHV). ORF73 is expressed in an in vitro model of HVS latency, where the genome persists as a non-integrated circular episome. This suggests it may have a similar role to KSHV ORF73 in episomal maintenance, by tethering viral genomes to host-cell chromosomes. Here, the association of ORF73 with host mitotic chromosomes is described. Deletion analysis demonstrates that the distal 123 aa of the ORF73 protein are required for mitotic chromosomal localization and for self-association. Moreover, deletion of the extreme C terminus disrupts both self-association and host mitotic chromosome colocalization. This suggests that HVS ORF73 has a similar role to KSHV ORF73 in episomal maintenance and that association of ORF73 to host mitotic chromosomes is dependent on its ability to form multimers.

Herpesvirus saimiri (HVS) is the prototype gamma-2 herpesvirus and has significant homology with other gamma herpesviruses including Kaposi’s sarcoma-associated herpesvirus (KSHV), Epstein–Barr virus (EBV) and murine gammaherpesvirus-68 (MHV-68) (Albrecht et al., 1992; Russo et al., 1996; Virgin et al., 1997). During latent infection the HVS genome, like that of other herpesviruses, persists as a non-integrated circular episome. Analysis has shown that a cluster of genes, namely open reading frames (ORFs) 71, 72 and 73, are transcribed as a polycistronic mRNA from a common latency-associated promoter in an in vitro model of HVS latency (Hall et al., 2000a). Similar profiles have been observed in various KSHV latently infected cell lines (Dittmer et al., 1998; Talbot et al., 1999). In both HVS and KSHV, ORF71 and ORF72 encode an anti-apoptotic FLICE inhibitory protein (Thome et al., 1997) and a cyclin D homologue (Chang et al., 1996; Jung et al., 1994), respectively. However, ORF73 encodes a protein with no known cellular homologue.

KSHV ORF73 encodes the latency-associated nuclear antigen (LANA) (Kedes et al., 1997; Kellam et al., 1997; Rainbow et al., 1997). HVS ORF73 has only limited sequence homology with KSHV ORF73; however, both proteins share a number of common features. Both consist of a large central acidic repeat domain, flanked by a small N-terminal region and a larger C terminus (Hall et al., 2000b). A major function of LANA is to maintain the extrachromosomal viral genome during KSHV latent infection (Ballestas et al., 1999; Cotter & Robertson, 1999). LANA has been shown to associate with host-cell mitotic chromosomes via a region at its N terminus encompassing aa 5–22 (Piolot et al., 2001) and to specifically bind KSHV terminal repeat DNA. Therefore, it acts as a tether attaching the KSHV latent viral genome to the host-cell chromosomes (Ballestas & Kaye, 2001). This suggests that KSHV ORF73 is functionally analogous to the EBV nuclear antigen 1 (EBNA1) (Kedes et al., 1997; Leight & Sugden, 2000). It has been well established that EBNA1 is the only viral protein required for EBV viral maintenance (Lee et al., 1999; Leight & Sugden, 2000; Lupton & Levine, 1985). EBNA1 binds to the family repeat element within the EBV genome (Yates et al., 1984, 1985) and interacts with metaphase chromosomes via human EBNA1 binding protein 2 (EBP2) (Shire et al., 1999; Kapoor & Frappier, 2003; Kapoor et al., 2001; Wu et al., 2002).

To date it is unknown if HVS ORF73 colocalizes with host mitotic chromosomes and if so which domains are required. Therefore, due to the lack of sequence homology between HVS and KSHV ORF73, we aimed to identify and characterize the HVS ORF73 chromosomal binding mechanism. To determine whether HVS ORF73 associates with host-cell mitotic chromosomes, the complete ORF73 coding region was PCR-amplified and inserted into the eukaryotic expression vector pEGFP-C2 (Invitrogen) to yield pEGFP-73, which contains an N-terminal enhanced green fluorescent protein (EGFP) tag (Fig. 1a). To determine the subcellular localization of ORF73, a transient transfection was...
performed and the resulting pattern of expression observed. Cos-7 cells were transfected using Lipofectamine 2000 (Invitrogen) and 2 µg of construct DNA and incubated for 36 h. Cells were then fixed and permeabilized before mounting in Vectashield containing DAPI (Vector). The slides were then examined using a laser confocal...

(a)

(b)

(c)
microscope (Leica TCS SP) and a PlanApo 100 × UV oil-immersion lens.

As expected pEGFP displayed a fluorescent signal throughout the cell nucleus and cytoplasm. In contrast, pEGFP-73 resulted in a distinct nuclear speckling pattern as previously reported (Hall et al., 2000b). More interesting was the observation that EGFP-73 fluorescence was coincident with the DAPI-stained mitotic chromosomes (Fig. 1b). This result suggests that HVS ORF73 encodes a chromosome-binding domain (CBD) and is capable of association with host-cell chromosomes during mitosis.

In order to further characterize the ORF73 CBD, a range of
ORF73 deletion mutants (Fig. 1a) was produced by a similar PCR method as described above and assessed for the ability to colocalize with host mitotic chromosomes. Results demonstrated that a deletion containing the ORF73 N terminus (pEGFP-73N) localized to the nucleus of cells but failed to associate with mitotic chromosomes. This confirms previous data identifying two nuclear-localization signals (NLSs) in the N terminus (Hall et al., 2000b) but suggests that neither of them is capable of acting as a CBD (data not shown). Previous studies have also demonstrated that deletion of the central acidic domain resulted in a wild-type distribution of fluorescence (Hall et al., 2000b). Analysis of the clone pEGFP-73N-C in mitotic cells also demonstrated a wild-type association with host-cell chromosomes (Fig. 1b). These two mutants therefore suggest that the CBD is located in the C terminus. Although we have previously suggested that an additional NLS may reside within the C terminus (Hall et al., 2000b), the C-terminal deletions used were all produced in a second vector, pEGFP-NLS, which contains the well-characterized NLS from SV40 (Kalderon et al., 1984) (Fig. 1c). Transfection of clone pEGFP-NLS-73C resulted in a strong nuclear distribution of fluorescence which co-localized with the host-cell chromosomes (Fig. 1d). This indicates that the CBD is located in the ORF73 C terminus. This was further supported using pEGFP-NLS-73CA1, which lacks the first 44 aa of the C terminus: fluorescence was observed in the nucleus which co-localized with the host-cell chromosomes during mitosis. However, pEGFP-NLS-73CA2 and pEGFP-NLS-73CA3, which contain small deletions from the C or N terminus of pEGFP-NLS-73CA1 respectively, both localized to the cell nucleus but failed to associate with mitotic chromosomes. Overall, these results show that the C terminus is required for chromosome association of HVS ORF73 and this interaction requires the distal 123 aa. However, removal of 18 aa from the start or 12 aa from the end of this domain abolishes chromosomal association and we have described these domains as chromosome association sites (CAS) 1 and 2, respectively. This suggests that the chromosomal association of ORF73 may require multiple distinct elements, hence explaining the relatively large domain required for chromosomal association.

In order to further investigate the role of the essential regions at each end of pEGFP-NLS-73CA1, sequence analysis was performed and identified a motif at aa 291–293 consisting of a proline followed by two lysines in CAS1. This motif is similar to a SPKK motif identified in histone H1 and H2 proteins which is involved in histone binding to the minor groove of the DNA (Churchill & Suzuki, 1989; Suzuki, 1989). This PKK motif is also found in a number of herpesvirus ORF73 homologues and in EB2. To analyse the possible significance of this motif in chromosomal association, a series of constructs was produced in which the amino acids in the PKK motif were replaced with alanines (Fig. 2a). Substitution of all three amino acids resulted in a complete loss of chromosome association (Fig. 2b). This suggests that this motif is involved in the association of ORF73 with host-cell mitotic chromosomes. However, single amino acid substitutions across this region failed to inhibit chromosomal association, indicating a degree of redundancy in this domain. In addition, deletion analysis suggests that the extreme C terminus is also required for chromosomal association; however, sequence analysis of this region, CAS2, failed to identify any known motifs. Therefore, single point mutations of selected amino acids across the last 12 residues were generated and assessed for chromosomal association. However, these point mutations failed to disrupt the ability of ORF73 to associate with mitotic chromosomes.

Another common feature displayed by gammaherpesvirus ORF73 proteins is the ability to self-associate. To determine if HVS ORF73 formed multimers and which domains were involved, we performed a co-immunoprecipitation experiment using the EGFP-ORF73 deletion series and an ORF73 Myc fusion protein as bait. To produce the ORF73 Myc fusion protein, the ORF73 gene was PCR-amplified and inserted into the eukaryotic expression vector pcDNA3.1myc-His-B (Invitrogen) to yield pMYC-73. Transient transfection of 1 μg of pMYC-73 into Cos-7 cells resulted in the expression of full-length ORF73 fused to the Myc peptide, as detected by Western blotting using anti-c-Myc antibodies (data not shown). In order to perform the multimerization studies, Cos-7 cells were initially co-transfected with pMYC-73 and pEGFP-NLS-73C; 36 h post-transfection the cells were washed in ice-cold PBS, before being harvested in 1 ml of lysis buffer (50 mM Tris/HCl, pH 7–4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 μM AEBSF). The insoluble cellular debris was removed and the supernatant was incubated with 50 μl of anti-c-Myc agarose conjugate bead slurry (Sigma) for 2 h at room temperature. The beads were then washed, separated on a 10% SDS-PAGE gel and blotted onto nitrocellulose membranes. ORF73 was then detected using a 1:2000 dilution of anti-GFP antibody (Clonetech), followed by a 1:2000 dilution of anti-mouse immunoglobulin conjugated with horseradish peroxidase (Dako) and developed using enhanced chemiluminescence (Amersham). Following immunoprecipitation with the anti-Myc affinity resin, EGFP-NLS-73C was detected when co-expressed with pMYC73 (Fig. 3). This demonstrated that the ORF73 protein can self-associate and that the C terminus is sufficient for multimerization. To further analyse the domains responsible for self-association, further EGFP-ORF73 constructs covering the C terminus were utilized. Results showed that pEGFP-NLS-73CA1 was sufficient for self-association and that deletion of CAS1 did not disrupt self-association; however, no interaction was observed upon deletion of CAS2 (Fig. 3). Moreover, to determine whether the ORF73 co-immunoprecipitation is due to a common interaction with DNA, rather than a specific protein–protein interaction, we repeated the homodimerization assay before and after treatment of the ORF73 complexes with 10 μg of DNase I (Fig. 3c). Confirmation that the digestion conditions...
eliminated DNA was obtained by gel electrophoresis (data not shown). Results demonstrate that the DNase treatment had little effect on the self-association of the ORF73 protein.

Taken together these results suggest that chromosome association of HVS ORF73 may be dependent upon the ability to form multimers. Deletion of CAS2 is sufficient to disrupt self-association and host-cell mitotic chromosome association. However, chromosomal association also requires additional elements, as deletion of CAS1 does not disrupt self-association but is sufficient to inhibit the co-localization with host-cell chromosomes.

This C-terminal 123 aa region maps to the same region of LANA required for dimerization (Schwam et al., 2000). The last 205 aa of the LANA C terminus are required for self-association and this region is conserved in other LANA-like proteins. Schwam et al. (2000) also suggest that this C-terminal region is required for the accumulation of LANA as discrete nuclear speckles, suggesting an involvement in the nuclear distribution of LANA. This was further explained by the identification of a 15 aa domain located close to the LANA C terminus, deletion of which disrupted the interaction of LANA with nuclear heterochromatin and the characteristic nuclear speckling pattern (Viejo-Borbolla et al., 2003). However, on further analysis this region did not associate with heterochromatin following high-salt washes, suggesting that this was not sufficient for the interaction with heterochromatin. The current view remains that LANA interacts with nuclear heterochromatin via an N-terminal region, aa 5–22 (Piolot et al., 2001); however deletion of aa 1129–1143 from full-length LANA may result in conformation changes which disrupt the interaction of the N-terminal CBD (Viejo-Borbolla et al., 2003). Similarly, deletion of the extreme 12 aa of HVS ORF73 abolishes chromosomal binding and self-association; however, at present this analysis cannot determine whether these residues play a specific role in the conformation of the protein which may affect these functions.

In summary, we have demonstrated that HVS ORF73 co-localizes with mitotic chromosomes and this localization is dependent upon the distal 123 aa. We have identified

![Fig. 2. Point mutation analysis of CAS1 and 2. (a) Schematic representation of the series of point mutations within CAS1 and 2 indicating the amino acid substitutions produced. The effect of each mutation on chromosomal association is shown in the table on the right. (b) Cos-7 cells were transfected with pEGFP-NLS-73CΔPKK. Cells were fixed 36 h later and visualized by laser scanning confocal microscopy.](http://vir.sgmjournals.org)
a PKK motif, termed CAS1, with similarity to a motif found in other chromosome-associated proteins and shown that substitution of this motif abolishes chromosomal localization. A second essential domain for chromosome association, termed CAS2, has also been shown to be essential for the formation of ORF73 multimers, leading us to speculate that the association of ORF73 with host-cell chromosomes is dependent upon its ability to form homo-multimers.

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REFERENCES


