Mapping the minimal regions within the ORF73 protein required for herpesvirus saimiri episomal persistence

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Herpesvirus saimiri (HVS) establishes a persistent infection in which the viral genome persists as a circular non-integrated episome. ORF73 tethers HVS episomes to host mitotic chromosomes, allowing episomal persistence via an interaction with the chromosome-associated protein, MeCP2. Here we demonstrate that ORF73 also interacts with the linker histone H1 via its C terminus, suggesting it associates with multiple chromosome-associated proteins. In addition, we show that the C terminus is also required for the ability of ORF73 to bind the terminal repeat region of the HVS genome. These results suggest that the ORF73 C terminus contains all the necessary elements required for HVS episomal persistence. Using a range of ORF73 C terminus deletions to rescue the episomal maintenance properties of a HVSΔ73 recombinant virus, we show that a C terminus region comprising residues 285–407 is sufficient to maintain the HVS episome in a dividing cell population.
285–407 is the minimal domain sufficient to maintain the HVS episome in a dividing cell population.

To further investigate the interactions between HVS ORF73 and chromosome-associated proteins, co-immunoprecipitation assays were performed. 293T cells were cotransfected with pEGFP-HistoneH1 in the presence of pMyc or p73-myc (Calderwood et al., 2004a). To ensure that the interaction between ORF73 and HistoneH1 was not due to a common affinity for DNA, co-immunoprecipitation assays were performed with cell extracts which remained untreated or treated with DNase I, as described previously (Griffiths & Whitehouse, 2007). To confirm that DNase I treatment had been successful, untreated and treated cell extracts were used as templates within a PCR utilizing primers directed against glyceraldehyde-3-phosphate dehydrogenase (GAPDH). This analysis demonstrated that complete digestion of cellular DNA had
occurred following addition of DNase I (Fig. 1a). Subsequently, the cell extracts were incubated with an ORF73-specific antibody and the immunocomplex captured using protein A agarose. Histone H1 was then detected by Western blotting using a GFP-specific antibody (Clontech). Results show that ORF73 interacted with Histone H1 and that this interaction was DNA-independent (Fig. 1a).

To confirm this interaction GST pull-down assays were performed using a recombinant ORF73 protein, pET21b-73C, which expresses the ORF73 C terminus (aa 241–407) as a histidine-tagged fusion protein (Griffiths & Whitehouse, 2007). pET21b-73C was transformed and grown in BL21 Escherichia coli and subsequently expressed and bound to nickel-conjugated agarose beads. Histone H1–GFP or GFP proteins were expressed in 293T cells and

Fig. 1. The HVS ORF73 C terminus interacts with histone H1. (a) (i) 293T cells were transfected with pORF73-myc in the presence of pEGFP or pEGFP-HistoneH1. Cell lysates were either left untreated or incubated with DNase I prior to incubation with an ORF73-specific antibody and immunocomplexes captured using protein A agarose. Analysis by Western blotting using a GFP-specific antibody indicates that ORF73-myc is immunoprecipitated in association with histone H1. Total cell extracts were run as positive controls (input). (ii) PCR analysis performed using primers directed against GAPDH indicates that DNase I treatment has successfully digested cellular DNA within the treated cell extracts. (b) pEGFP or pEGFP-HistoneH1-transfected cell extracts were incubated with Ni-NTA agarose beads immobilizing the histidine-tagged 73C protein (spanning positions 106,013–106,513 of the genomic coordinates). Following incubation, each ORF73C-bead preparation was washed, then analysed by Western blotting using a GFP-specific antibody. Total extract of pEGFP-Histone H1-transfected cells was run as a positive control (input). (c) Schematic representation of the pET21-ORF73C His-tagged deletion series. Right-hand side panel summarizes the interaction between Histone H1 and each recombinant deletion or indicates which constructs could not be used in the assay. (d) Recombinant ORF73C proteins bound to Ni-NTA beads (left panels) were incubated with cell extracts expressing either EGFP or pEGFP-HistoneH1. Proteins associated with ORF73-conjugated beads were analysed by Western blotting using a GFP-specific antibody (right panels).
the cell extracts incubated with the ORF73C-beads. After washing, proteins bound to the ORF73C-beads were separated by SDS-PAGE and detected by Western blotting using a GFP-specific antibody. The results confirm that ORF73C specifically bound the chromosome-associated protein HistoneH1 (Fig. 1b). Moreover, as we have previously shown that ORF73 also interacts with MeCP2, it suggests that ORF73 can interact with multiple chromosome-associated proteins.

To further investigate this interaction, deletion analysis was used to determine the ORF73 minimal domain required to bind to histone H1. The ORF73C histidine-tagged fusion deletion series (Fig. 1c) (Griffiths & Whitehouse, 2007) was expressed as recombinant histidine-tagged proteins, bound to Ni-NTA agarose beads and incubated with a pEGFP-HistoneH1 transfected cell extract. Although several of the proteins were insoluble and could not be used in the assay, analysis of these deletion proteins demonstrated that an overlapping fragment between deletion 4 and 10 encompassing ORF73 aa 324–379 is sufficient for histone H1 binding (Fig. 1d). Interestingly, this region is similar to the domain which interacts with MeCP2, namely aa 324–396. However, it has been shown previously that the minimal region of the ORF73 C terminus for chromosomal association is aa 285–407 (Calderwood et al., 2004a), which suggests that additional domains either side of the minimal MeCP2- and histone H1-binding regions, termed chromosome association sites (CAS) 1 and 2, are required for chromosome association.

In addition to binding host mitotic chromosomes, ORF73 must bind the TR region to maintain the HVS episome in a dividing cell population. Therefore, the specific ORF73 domain responsible for binding HVS TR DNA was investigated using chromatin immunoprecipitation (ChIP) experiments. The previously constructed ORF73 deletion series (Hall et al., 2000b), consisting of pEGFP-73NC, pEGFP-73N, pEGFP-73C (Fig. 2a) or empty pEGFP vector, were co-transfected into 293T cells together with a plasmid containing four copies of the HVS TR sequence (pHVS-TR + hyg). After 24 h the cells were harvested and ChIP assays performed using the ChIP assay kit (Upstate Biotechnology). Chromatin extracts, cross-linking, sonication, immunoprecipitation, agarose bead elution and protein removal were carried out based on the manufacturer's protocol. DNA recovered from immunoprecipitates with the GFP-specific polyclonal antibody was used as a template for PCR amplifications using primers specifically directed against unique HVS TR sequences. To eliminate the possibility of non-specific binding resulting in precipitation of TR DNA, reactions were also performed in the absence of immunoprecipitating antibody. Results shown in Fig. 2(b) demonstrate that, in the presence of the GFP-specific antibody, TR DNA was successfully immunoprecipitated in association with both EGFP–73NC and EGFP–73C protein, resulting in amplification of the expected 1444 bp DNA fragment. These results demonstrate that the ORF73 C terminus is sufficient for binding to HVS TR DNA.

To identify the minimal domain sufficient for TR binding, five deletion proteins spanning the ORF73 C terminus were cloned downstream and in-frame of EGFP. As illustrated in Fig. 2(c), each deletion protein includes various combinations of the previously defined ORF73C chromosome association sites (CAS1 or 2), or the histone H1-binding domain. To confirm that each vector expressed an EGFP-tagged protein of the expected molecular mass, the vectors were transfected into 293T cells and cell extracts analysed by SDS-PAGE and Western blotting. Analysis using a GFP-specific antibody indicates that each vector successfully expresses a GFP-tagged ORF73C deletion protein of the correct size (Fig. 2d). Each deletion was then assessed for its ability to bind HVS TR DNA using ChIP assays. As shown in Fig. 2(e) full-length ORF73C and EGFP–73CΔ5 successfully bound HVS TR DNA. These results indicate that ORF73 residues 324–407 are sufficient for binding to HVS TR DNA.

The deletion analysis of ORF73 suggests that the minimal domain for both chromosome association and TR DNA binding resides in the C terminus. Therefore, we next aimed to determine which residues within the C terminus were sufficient to support HVS episomal maintenance. We have demonstrated previously that replacement of the complete ORF73 gene into a recombinant HVS lacking ORFs 71–73 is sufficient to rescue HVS episomal persistence (Calderwood et al., 2005). We therefore assessed whether any C-terminal deletion constructs could rescue the episomal persistence ability of HVS-BACΔ71-73. To this end, SW480 cells were transfected with each N- and C-terminal ORF73 deletion construct (Fig. 2), after 24 h the transfected cells were superinfected with HVS-BACΔ71-73 (m.o.i. of 1) and maintained in 600 µg G418 ml\(^{-1}\) and 200 µg hygromycin ml\(^{-1}\), which selected for the 73 deletion constructs and HVS-BAC, respectively, for a further 24 h. SW480 cells were used in this analysis as we have previously shown that HVS establishes a latent infection in these cells where the genome persists as a non-integrated episome (Smith et al., 2001). The cells were then trypsinized and diluted to a cell density of 1 × 10^7 cells ml\(^{-1}\). The cells were then seeded at approximately 10 cells per single well in 96-well microtitre plates and grown under G418 and hygromycin selection for 2 weeks. The plates were then analysed for colony formation and scored as a percentage of wells positive for colony outgrowth. Results demonstrated that only cells pre-transfected with pEGFP-73NC, pEGFP-73C or pEGFP-73C-CAS1+2 enabled HVS-BACΔ71-73 to be maintained in a dividing cell population (Fig. 3). Moreover, transfected cells were also seeded in duplicate at 1 × 10^6 cells and grown under G418 and hygromycin selection for 2 weeks. Similar results were observed as above, where cell growth was only present in cells pre-transfected with pEGFP-73NC, pEGFP-73C or pEGFP-73C-CAS1+2. RNA was then isolated from each cell line and used in RT-PCR analysis to confirm the expression of each ORF73 expression construct in SW480 cells, following 2 weeks growth in selection. Results
demonstrate that expression of each ORF73 C-terminal deletion was observed in each selected cell line (Fig. 3b). To confirm the presence of the HVS-BACΔ71-73 episome in each selected cell line, DNA was isolated from cells after 14 days selection using the low molecular mass DNA isolation method (White et al., 2003). The DNA (1 μl) was then electroporated into E. coli ElectroMAX DH10B (Invitrogen) and plated on LB agar supplemented with 12.5 μg chloramphenicol ml⁻¹. Results demonstrated that episomal DNA was recovered from cells pre-transfected with pEGFP-73NC, pEGFP-73C or pEGFP-73C-CAS1+2. No DNA was isolated from the few small colonies pre-transfected with the other ORF73 constructs. To confirm the bacterial colonies were due to transformation

Fig. 2. The HVS ORF73 C terminus binds TR DNA. (a) Schematic representation of the pEGFP-73 deletion series. (b) PCR amplification of HVS TR DNA from GFP antibody or no antibody immunoprecipitates using cell extracts transfected with the pEGFP-73 deletion construct indicated. (c) Schematic representation of the pEGFP-73C deletion series. (d) Vectors encoding the pEGFP-73C deletion series were transfected into 293T cells and protein expression analysed by Western blotting using a GFP-specific antibody. (e) PCR amplification of HVS TR DNA from GFP antibody or no antibody immunoprecipitates using cell extracts transfected with the pEGFP-73C deletion construct indicated.
by HVS episomes, DNA isolated from these bacterial colonies was analysed by restriction digest and pulsed-field gel electrophoresis. Restriction digests demonstrate that the episomes isolated were consistent with those expected for HVS-BACΔ71-73. These results therefore indicate that the ORF73 C terminus can rescue the episomal persistence ability of HVS-BACΔ71-73. Moreover, residues 285–407 within the ORF73 C terminus, which encompass both CAS1 and CAS2, is the minimal region required for episomal persistence and for efficient establishment of a latent infection.

Herein we show that HVS ORF73 can associate with the ubiquitous chromosome-associated protein, histone H1. Taken together with the previous observation that ORF73 interacts with MeCP2, it suggests that ORF73 can interact with multiple chromosome-associated proteins. Similar to the ORF73-MeCP2 association; the interaction between ORF73C and histone H1 occurs in the absence of the previously defined CAS1 and CAS2 chromosome association sites. However, these CAS regions are essential for chromosome association, suggesting they have additional roles, possibly in ORF73 protein folding or multimerization. We have previously shown that MeCP2 is essential for HVS episomal maintenance; however, the presence of up to eight histone H1 genetic variants precludes similar functional analysis of histone H1–ORF73C association by histone H1 knock-down, or chromosomal association inhibition. It is not surprising that gamma herpesviruses have evolved to bind host chromosomes via several alternative interactions. The eukaryotic chromosome is a dynamic structure, and although the basic organization of the nucleosome is known, the exact composition of heterochromatin remains enigmatic. However, it is clear

**Fig. 3.** HVS ORF73C residues 285–407 are sufficient to maintain the HVS episome in a dividing cell population. (a) Colony-forming assays performed on SW480 cells initially transfected with the pEGFP-ORF73 deletion construct and then superinfected with HVS-BACΔ71-73. The variation between two replication assays is indicated and is shown as so. (b) SW480 cells were initially transfected with the pEGFP-ORF73 deletion constructs and then superinfected with HVS-BACΔ71-73. RNA was then isolated from any colonies formed after 14 days growth, in the presence of G418 and hygromycin, and RT-PCR analysis performed to specifically amplify the ORF73 carboxy terminal (residues 324–407). (c) Low molecular mass DNA was extracted from any SW480 colonies formed previously transfected with the pEGFP-ORF73 deletion constructs and then superinfected with HVS-BACΔ71-73 after 14 days growth in selection. For each population, DNA was transformed into bacteria and two bacterial colonies were picked and DNA extracted. After digestion with AgeI, DNA was analysed by pulse field gel electrophoresis.
that chromosome-associated proteins play a vital role in the continual and essential modification of the chromosomal environment (Adkins et al., 2004). Therefore, even ubiquitous chromosome-associated proteins such as histone H1 may remain in a constant state of chromosome association and disassociation. Hence, the ability of HVS ORF73 to bind several tightly associated chromosomal proteins provides an effective mechanism by which it can secure long-term latent persistence.

Moreover, we demonstrate that the C terminus can also bind TR DNA. Interestingly, Cotter et al. demonstrated that a 200 amino acid domain within the C terminus of KSHV LANA is sufficient for binding to KSHV TR DNA (Cotter et al., 2001). Deletion analysis of ORF73C demonstrated that only the terminal 83 aa of the protein are required for interaction with the TRs. The ORF73C TR-binding domain includes the MeCP2- and histone H1-binding region and also contains the previously identified CAS2. Analysis of ORF73C multimerization has shown that CAS2 is essential for the formation of ORF73C homo-multimers (Calderwood et al., 2004a). Therefore, akin to many DNA-binding proteins, self-association of ORF73 is probably required to bind DNA.

In summary, we demonstrate the HVS ORF73 C terminus contains multiple functional domains required for chromosome association and DNA binding. Moreover, the minimal C terminus region, comprising residues 285–407, is sufficient to maintain the HVS episome in a dividing cell population. This analysis will help towards the development of safe replication-disabled HVS-based vectors for gene therapy applications, such as the HVS amplicon system (Macnab et al., 2008). The region identified herein, required for HVS episomal maintenance, will help minimize the viral sequence required in these HVS amplicon-based vectors.

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