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A short sequence immediately upstream of the internal repeat elements is critical for KSHV LANA mediated DNA replication and impacts episome persistence

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ABSTRACT

Kaposi's sarcoma-associated herpesvirus LANA (1162 residues) mediates episomal persistence of viral genomes during latency. LANA mediates viral DNA replication and segregates episomes to daughter nuclei. A 59 residue deletion immediately upstream of the internal repeat elements rendered LANA highly deficient for DNA replication and modestly deficient for the ability to segregate episomes, while smaller deletions did not. The 59 amino acid deletion reduced LANA episome persistence by ~14-fold, while sequentially smaller deletions resulted in ~3-fold, or no deficiency. Three distinct LANA regions reorganized heterochromatin, one of which contains the deleted sequence, but the deletion did not abolish LANA's ability to alter chromatin. Therefore, this work identifies a short internal LANA sequence that is critical for DNA replication, has modest effects on episome segregation, and substantially impacts episome persistence; this region may exert its effects through an interacting host cell protein(s).

Introduction

Kaposi's sarcoma-associated herpesvirus (KSHV or human herpesvirus 8 (HHV-8)) is the only gamma-2 herpesvirus that infects humans. KSHV has a causative role in Kaposi's sarcoma, primary effusion lymphoma, and multicentric Castleman's disease (Cesarman et al., 1995; Chang et al., 1994; Moore and Chang, 1995; Soulier et al., 1995). KSHV latently infects tumor cells and during latent infection only expresses a small subset of genes. Cells latently infected with KSHV maintain multiple copies of the viral genome as circular, covalently closed, extrachromosomal forms (episomes) (Cesarman et al., 1995; Decker et al., 1996). Latency-associated nuclear antigen (LANA), encoded by open reading frame 73 (ORF73) (Kedes et al., 1997; Kellam et al., 1997; Rainbow et al., 1997), is necessary and sufficient for episome persistence (Ballestas et al., 1999; Ballestas and Kaye, 2001).

There are two key components to episome persistence, DNA replication and segregation of episomes to daughter nuclei, and LANA fulfills both these functions. Both N-and C-terminal LANA are essential for episome maintenance. LANA associates with mitotic chromosomes and has two independent chromosome binding regions located in N- and C-terminal LANA (Fig. 1) (Ballestas et al., 1999; Barbera et al., 2004; Kelley-Clarke et al., 2007a, 2007b; Krithivas et al., 2002; Lim et al., 2004; Piolot et al., 2001; Szekely et al., 1999; Wong et al., 2004). N-terminal LANA is the dominant chromosome attachment region and binds mitotic chromosomes by directly interacting with histones H2A/H2B on the nucleosome surface. This interaction is essential for episome maintenance and efficient DNA replication (Barbera et al., 2004; Barbera et al., 2006; Hu et al., 2002). C-terminal LANA self-associates to bind two adjacent sites in each KSHV terminal repeat (TR) element to mediate DNA replication (Ballestas and Kaye, 2001; Cotter et al., 2001; Fejer et al., 2003; Garber et al., 2002; Garber et al., 2001; Grundhoff and Ganem, 2003; Hu et al., 2002; Komatsu et al., 2004; Lagunoff and Ganem, 1997; Lim et al., 2002). LANA mediates segregation of DNA to progeny nuclei by simultaneously binding TR DNA and mitotic chromosomes.

Expression of LANA in uninfected cells causes nuclear reorganization with release of DNA from heterochromatic areas, which can be observed in human and mouse cells (Mattsson et al., 2002; Stuber et al., 2007). These changes are more easily detected in murine cells due to the presence of pericentromeric alpha-satellite repeats that are organized into well defined heterochromatic chromocenters (Stuber et al., 2007). The LANA region exerting this effect was mapped to residues 275-331(Stuber et al., 2007).

We previously showed that, in addition to N- and C-terminal LANA, internal LANA sequence is also critical for episome persistence since fusion of N- and C-terminal LANA resulted in highly deficient episome maintenance (De Leon and Kaye, 2011a). Further, a panel of large internal deletion mutants suggested that a small region, located immediately upstream of the internal

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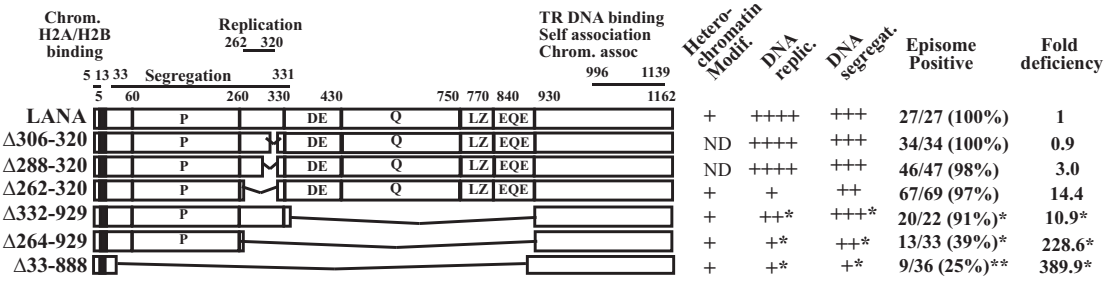

Fig. 1. Schematic diagram of KSHV LANA and deletion mutants. The proline rich region (P), aspartate and glutamate region (DE), glutamine and glutamate region (Q), glutamine and glutamate region (EQE), and putative leucine zipper (LZ) are indicated. The DE, Q, EQE and LZ regions are all comprised of repeat elements. Shaded region indicates the N-terminal nuclear localization (NLS) signal. C-terminal LANA can also localize to nuclei but an NLS has not been precisely mapped. Amino acids 5–13 mediate chromosome association through binding histones H2A/H2B. Amino acids 996–1139 have DNA binding, self-association and chromosome association functions. Capabilities for heterochromatin modification, DNA replication, DNA segregation, episome maintenance and fold deficiency for episome maintenance are summarized at the right for LANA and each mutant. Fractions are the number of G418 resistant cell lines containing episomes over the total number of G418 resistant cell lines assayed by Gardella analysis and percentages are shown in parenthesis. ND, not done. Asterisks indicate results of previous work (De Leon Vazquez et al., 2013).

repeat elements, may have a key role in episome persistence (De Leon Vazquez et al., 2013). Notably, this region overlaps with residues 275-332 that were reported to reorganize heterochromatin. Here we directly investigate the role of this region in episome maintenance. We find that this sequence is critical for DNA replication, modestly affects segregation and substantially impacts episome persistence. Although we confirmed that LANA reorganizes heterochromatic regions, LANA deleted for this small region was still capable of reorganizing heterochromatin.

Results

LANAΔ262-320 maintains the ability to release DNA from heterochromatic chromocenters

We recently showed that internal sequence exerts critical effects on LANA's ability to mediate episome persistence. N- and C-terminal LANA are essential for episome persistence. N-terminal LANA mediates mitotic chromosome attachment and C-terminal LANA binds KSHV TR DNA and also has a role in chromosome attachment. These components are essential for LANA mediated DNA replication and tethering of KSHV episomes to mitotic chromosomes, which provides a mechanism to segregate DNA to daughter nuclei. Deletion of all internal regions revealed that this sequence is also critical for episome persistence (De Leon and Kaye, 2011a). A panel of mutants deleted for large portions of the internal sequence suggested that a small internal LANA region immediately upstream of the internal repeat elements may exert an important role. LANA deleted for amino acids 332-929 (LANAΔ332-929) was 10.9 fold reduced for episome persistence efficiency, while LANA deleted for residues 264-929 (LANAΔ264-929) was reduced 228.6 fold (Fig. 1) (De Leon Vazquez et al., 2013). Here, we perform a targeted investigation of the region immediately upstream of the LANA internal repeat elements.

Previous work (Stuber et al., 2007) demonstrated that LANA reorganizes DNA staining patterns by releasing DNA from heterochromatic regions. These effects were particularly evident in murine cells, such as fibrosarcoma L cells, which contain pericentromeric DNA organized in well defined heterochromatic chromocenters. This function was mapped to LANA residues 275-332. Since these residues overlap with the LANA 264–331 sequence that were implicated as important for episome persistence, we asked whether the effect on episome persistence might be related to LANA's ability to reorganize heterochromatin.

We generated LANAΔ262-320 (Fig. 1), which is deleted for the indicated residues, and assessed its ability to reorganize heterochromatin. GFP, LANA or LANAΔ262-320 were expressed in murine L cells. GFP (green) (Fig. 2A panels a–c) did not cause rearrangement or modification of DNA (blue, or black and white) chromocenters (bright DNA foci, arrow indicates example in Fig. 2B). Consistent with previous results (Stuber et al., 2007), cells expressing LANA released DNA from chromocenters (Fig. 2A, panels d–f), causing disappearance of DNA foci, in contrast to adjacent, untransfected cells that lacked LANA expression. LANAΔ262-320 also released DNA from chromocenters (Fig. 2A, panels g–i). Fig. 2B shows a cell expressing a moderate level of LANAΔ262-320 and an adjacent cell expressing a very low level of LANAΔ262-320. Different optical slices of the cell are shown from a stack of images, which permits better definition of the bright chromocenters. The cell expressing a moderate level of LANAΔ262-320 shows release of DNA from chromocenters while the adjacent cell expressing a very low level of LANAΔ262-320 does not. These results suggest that LANA residues 262-320 are dispensable for the release of DNA from chromocenters.

It remained possible that LANA residues 321–332 exert a role in chromatin modification, since these amino acids were also implicated in the earlier work but were not deleted in LANAΔ262-320. Therefore, we transfected L cells with LANAΔ264-929, which lacks these residues, or LANAΔ332-929 which contain all but amino acid 332. Both LANAΔ332-929 (Fig. 2C) and LANAΔ264-929 (Fig. 2D) released DNA from chromocenters, indicating that residues 264-929 are dispensable for this effect. We also expressed LANAΔ33-888, which contains only N- and C-terminal LANA, in L cells, and this mutant also released DNA from chromocenters (Fig. 2E).

To further localize the LANA region(s) responsible for heterochromatin reorganization, we assessed a panel of LANA mutants for the ability to release DNA from chromocenters (Fig. 2F). GFP, LANA with an N-terminal GFP fusion (GFP LANA), or GFP LANA mutants were expressed in L cells, and the percent of GFP or GFP LANA expressing cells with reorganized heterochromatin was determined. As expected, GFP did not result in heterochromatin reorganization, while GFP LANA altered chromatin in 87.5% of cells. GFP LANAΔ33-888, comprised of N- and C-terminal LANA, released DNA from chromocenters in 67.2% of cells, modestly less than that of LANA. To investigate the role of N-terminal LANA as well as LANA 33-331, the sequence located just downstream of N-terminal LANA, we assessed GFP LANA 1-331, GFP LANA 1-32, GFP LANA 33-331, and GFP LANA 1-331 GMR (mutated at residues $_5$GMR$_7$, resulting in loss of histone H2A/H2B binding and mitotic chromosome association (Barbera et al., 2004)), which released DNA from chromocenters in 60.4%, 10.9%, 23.0%, and 32.8% of cells, respectively. These results indicated that LANA 1-32 and LANA ;33-331 appear to contain distinct functional regions capable of reorganizing heterochromatin.