A naturally occurring C-terminal truncated isoform of the latent nuclear antigen of Kaposi’s sarcoma-associated herpesvirus does not associate with viral episomal DNA

Maurice Canham and Simon J. Talbot

The latency-associated nuclear antigen (LANA) encoded by orf73 of Kaposi’s sarcoma-associated herpesvirus (KSHV) binds to viral episomal DNA and nuclear heterochromatin in infected cells. A 3-2 kb transcript in KSHV-positive primary effusion lymphoma (PEL) cells (BCP-1 and BC-3) encoding a C-terminal truncated form of LANA (LANA-Δ76) has been identified. This transcript has the addition of a poly(A) tail at nt 3264 of orf73 resulting in an in-frame stop codon (TAA) effectively truncating LANA by 76 aa (~8 kDa). Examination of the coding region revealed the presence of a non-canonical polyadenylation signal (AGTAAA) 17 nt upstream of the poly(A) tail. The protein expressed from this transcript is representative of the faster migration of the LANA doublet bands observed by SDS-PAGE and Western blot. Mutation of the poly(A) signal from AGTAAA to TGTACA produced a protein that co-migrated with the larger LANA isoform. A C-terminal LANA-Δ76 EGFP fusion protein localized to the nucleus but did not co-localize with endogenous LANA in BCP-1 cells, or heterochromatin in HEK293 cells. Using an electrophoretic mobility shift assay (EMSA), the authors were able to show that LANA-Δ76 does not bind to the KSHV terminal repeat motif known to interact with LANA. These data provide evidence for the presence of an isoform of LANA that may perform alternative functions in KSHV-infected cells.

INTRODUCTION

Kaposi’s sarcoma-associated herpesvirus (KSHV) (Chang et al., 1994) is implicated in the aetiology of all epidemiological forms of Kaposi’s sarcoma (KS), i.e. Mediterranean classic, African endemic, post-transplant or iatrogenic and the most commonly occurring AIDS-associated (Boshoff & Weiss, 1998). KSHV sequences have also been identified in several rare lymphomas such as multicentric Castleman’s disease and primary effusion lymphoma (PEL) (Cesarman et al., 1995a, 1996; Soulier et al., 1995). KSHV-positive cell-lines have been established from PEL, only some of which are co-infected with Epstein–Barr virus (EBV) (Boshoff et al., 1998; Cesarman et al., 1995b; Renne et al., 1996). Several of these KSHV-positive cell lines have been used in serological testing for antibodies against KSHV; in an indirect immunofluorescence assay (IFA) the sera of KSHV-infected individuals detects discrete nuclear antigen(s) in PEL cell-lines, analogous to the EBNAs of EBV (Gao et al., 1996a, b; Kedes et al., 1996; Lennette et al., 1996). The immunogenic protein detected in these assays – latency associated nuclear antigen (LANA) – is encoded by orf73 (Kedes et al., 1997; Kellam et al., 1997; Rainbow et al., 1997).

LANA, identified as a 226–234 kDa doublet by SDS-PAGE (Gao et al., 1996a, 1999), is a versatile protein with multiple functions. It tethers viral episomes to host chromatin during mitosis, allowing delivery of viral progeny to all daughter cells (Ballestas et al., 1999). LANA binds to two short motifs within the terminal repeat of the KSHV genome through a region in its C-terminal domain (Ballestas & Kaye, 2001; Garber et al., 2001, 2002). Interaction with host cell mitotic chromosomes is mediated through a 32 aa domain at the N terminus (Piolot et al., 2001). It is thought that LANA acts as a bridge between chromosomal and viral episomal DNA.

Similar to the oncogenes of other DNA tumour viruses, LANA binds to and interferes with the functions of the tumour suppressors p53 and retinoblastoma protein (Friborg et al., 1999; Radkov et al., 2000), and transforms primary rat embryo cells with Hras (Radkov et al., 2000). Recently, it was reported that LANA also exploits the Wnt-β-catenin pathway to activate genes that can promote cell growth (Fujimuro et al., 2003).

Through a domain in its C terminus, LANA activates and/or represses certain cellular and viral promoters, probably

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Received 12 November 2003
Accepted 18 February 2004
through interaction with cellular transcription factors such as CREB, CBP and Sp1 (An et al., 2002; Krithivas et al., 2000; Lim et al., 2000; Radkov et al., 2000). A family of nuclear factors typified by RING3 has also been shown to interact with the C terminus of LANA resulting in the phosphorylation of serine and threonine residues between aa 951 and 1107 of LANA (Mattsson et al., 2002; Platt et al., 1999). The functional significance of this phosphorylation is not yet understood.

This report describes the identification of an isoform of LANA truncated by 76 aa at the C terminus. We have investigated the DNA binding and subcellular localization of this protein in PEL cells.

### METHODS

#### Cells

The KSHV-positive primary effusion lymphoma (PEL) B-cell lines BCP-1 (Boshoff et al., 1998) and BC-3 were grown in RPMI (Invitrogen) supplemented with 20% (v/v) fetal calf serum (FCS), 2 mM glutamine, 60 µg penicillin ml⁻¹ and 100 µg streptomycin ml⁻¹. HEK293 cells (Graham et al., 1977) were grown in DMEM (Invitrogen) supplemented with 10% (v/v) FCS, 2 mM glutamine, 60 µg penicillin ml⁻¹ and 100 µg streptomycin ml⁻¹. Cells were incubated at 37 °C under 4% CO₂.

#### Plasmids

The C-terminal coding region of LANA (nt 2805–3488) and LANA-Δ76 (nucleotides 2805–3258) were PCR amplified with the primers GATCATGCTTCCCAATATGTTCGACGGGTC and GCATTCTGATATGTTCTTGGAGAGTC (LANA) or GATCATGCTTCCCAATATGTTCGACGGGTC and GCATTCTAGATTAGGACACGGGGCCTGCCT (LANA-Δ76). These PCR products were cloned into the BglII/XbaI restriction sites of the plasmid pEGFP-C1 (Clontech). All inserts were fully sequenced and found to contain no mutations.

The poly(A) site identified in the LANA coding sequence was mutated from AGTAAA to TGTACA using a Quick-change mutagenesis kit (Stratagene) and oligonucleotides CTTCCAGTTTGGAGGTGTACATA from AGTAAA to TGTACA using a Quick-change mutagenesis kit (Stratagene). The poly(A) site identified in the LANA coding sequence was mutated from AGTAAA to TGTACA using a Quick-change mutagenesis kit (Stratagene). All inserts were fully sequenced and found to be correctly expressed of the proteins was confirmed by Western blot analysis using an anti-GFP antibody (Clontech). The complementary oligonucleotides TR-1 (GATCTCCGCGGGCATGGGGCC) and TR-2 (GATCGGCCCATGCAGCGGGCGGA) were annealed and the ends filled using Klenow polymerase and dGTP, dATP, dTTP and [α-³²P]deoxy-ATP (Amersham), according to the manufacturer’s instructions (NEB). The use of dideoxy-ATP ensured that only one residue was added to the end of each oligonucleotide probe. The RNA-protection assay was performed using the Multi-NPA kit from Ambion.

#### RNA protection assay (RPA)

Poly(A)⁺ RNA was isolated (Micro-FastTrack mRNA isolation system) from BCP-1 and HEK293 cells and hybridized to an oligonucleotide complementary to the 3’ terminus of LANA-Δ76 mRNA with a 20 nt 5’ T sequence (T₃AGGACAGCGGGCCTGCCTTT). The oligonucleotide was labelled at the 3’ end using terminal transferase (NEB) and [α-³²P]deoxy-ATP (Amersham), according to the manufacturer’s instructions (NEB). The use of dideoxy-ATP ensured that only one residue was added to the end of each oligonucleotide probe. The RNA-protection assay was performed using the Multi-NPA kit from Ambion.

#### Electrophoretic mobility shift assay (EMSA)

LANA-EGFP, LANA-Δ76-EGFP fusion proteins and EGFP were prepared using the TNT in vitro transcription–translation system (Promega). The correct expression of the proteins was confirmed by Western blot analysis using an anti-GFP antibody (Clontech). The complementary oligonucleotides TR-1 (GATCTCCGCGGGCATGGGGCC) and TR-2 (GATCGGCCCATGCAGCGGGCGGA) were annealed and the ends filled using Klenow polymerase and dGTP, dATP, dTTP and [α-³²P]deoxy-ATP (Amersham). Approximately 0.5–10 µl of the protein preparation was mixed with 50 000 c.p.m. ³²P-labelled double-stranded TR probe in 1× reaction buffer [20 mM Tris pH 7.5, 10% (v/v) glycerol, 50 mM KCl, 0.1 mM dithiothreitol, 10 mM MgCl₂, 1 mM EDTA, 20 µg poly(dI–dC) ml⁻¹] as described previously (Ballestas & Kaye, 2001). Reactions were resolved by electrophoresis through 5% (v/v) non-denaturing polyacrylamide gels in 1× TBE. The gels were fixed (acetic acid/methanol) and dried before exposure to X-ray film (X-OMAT-AR) at ~80 °C with an intensifying screen.

### RESULTS

#### Identification of an mRNA encoding a C-terminal truncated form of LANA

A BCP-1 cDNA expression library was screened with serum from an HIV-negative patient with active KS (Kellam et al., 1997). This serum contained high levels of antibodies recognizing LANA in BCP-1 cells by immunofluorescence and Western blot. Several reactive cDNA clones were identified during this screen, the majority of which encoded the C-terminal domain of LANA as well as the downstream orfs vCyclin and vFLIP, which are present on the same transcript (Dittmer, 2003; Kellam et al., 1997; Talbot et al., 1999). During this screen, one clone was identified (designated RD4) that was truncated in the C-terminal coding region of the LANA transcript due to the addition of a poly(A) tail at nt 3264 of orf73 (Fig. 1). The poly(A) tail creates an in-frame stop codon (TAA) effectively truncating LANA by 76 aa (~8 kDa). Examination of the coding region revealed the presence of a non-canonical polyadenylation signal (AGTAAA) 17 nt upstream of the poly(A) tail. Expression of this truncated form of LANA would result in a protein approximately 8 kDa smaller than full-length LANA. LANA is known to migrate as a high molecular mass doublet (226–234 kDa) (Gao et al., 1996a, 1999) when resolved by SDS-PAGE and Western blot. The molecular
mass difference between these two LANA isoforms is identical to the difference between full-length LANA and the putative RD4 protein, suggesting that the smaller LANA species may be encoded by the RD4 transcript identified here.

To confirm the presence of the RD4 transcript in KSHV-positive cells, we utilized both Northern blot and RPA (Fig. 2). Poly(A)$^+$ RNA was isolated from BCP-1, BC-3 (KSHV-positive) and BJAB (KSHV-negative) cell lines and probed with a double-stranded DNA probe encompassing nt 2939–3161 of orf73. This probe should hybridize to the LANA-vCyclin-vFLIP tri-cistronic transcript (5·8 kb) and the putative 3·2 kb RD4 transcript, but not to the 1·8 kb vCyclin-vFLIP bicistronic transcript (Talbot et al., 1999). The Northern blot reveals the presence of two mRNA species at approximately 6 kb and 3·2 kb in BCP-1 and BC-3 but not BJAB cells. To further confirm the existence of RD4 mRNA in KSHV cells, RPA was performed using a 40 nt DNA oligonucleotide complementary to 20 nt of the poly(A) tail and the last 20 coding nucleotides of the

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**Fig. 1.** DNA sequence and protein translation of (a) the C-terminal 91 aa of LANA and (b) the C-terminal 15 aa of the RD4-clone. The non-canonical polyadenylation signal (AGTAA) is shown in capital letters. The DNA sequence complementary to the oligonucleotide used in the RPA is underlined. The addition of the poly(A) tail to RD4 creates an in-frame stop codon truncating LANA at aa 1086. Three potential serine and one threonine phosphorylation sites are underlined in bold.

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**Fig. 2.** (a) Northern blot analysis of poly(A)$^+$ RNA from BCP-1, BC-3 or BJAB cells. The poly(A)$^+$ RNA extracted from $5 \times 10^6$ cells was loaded in each lane and the blot probed with $^{32}$P-labelled double-stranded DNA probes corresponding to nt 2939–3161 (NruI–XcmI) of the LANA sequence (Russo et al., 1996). This probe should hybridize to the LANA-vCyclin-vFLIP tri-cistronic transcript (5·8 kb) and the putative 3·2 kb RD4 transcript, but not to the 1·8 kb vCyclin-vFLIP bicistronic transcript (Talbot et al., 1999). The Northern blot reveals the presence of two mRNA species at approximately 6 kb and 3·2 kb in BCP-1 and BC-3 but not BJAB cells. To further confirm the existence of RD4 mRNA in KSHV cells, RPA was performed using a 40 nt DNA oligonucleotide complementary to 20 nt of the poly(A) tail and the last 20 coding nucleotides of the (KSHV-positive) and BJAB (KSHV-negative) cell lines and probed with a double-stranded DNA probe encompassing nt 2939–3161 of orf73. This probe should hybridize to the LANA-vCyclin-vFLIP tri-cistronic transcript (5·8 kb) and the putative 3·2 kb RD4 transcript, but not to the 1·8 kb vCyclin-vFLIP bicistronic transcript (Talbot et al., 1999). The Northern blot reveals the presence of two mRNA species at approximately 6 kb and 3·2 kb in BCP-1 and BC-3 but not BJAB cells. To further confirm the existence of RD4 mRNA in KSHV cells, RPA was performed using a 40 nt DNA oligonucleotide complementary to 20 nt of the poly(A) tail and the last 20 coding nucleotides of the

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RD4 transcript. This probe was labelled at the 3' end with terminal transferase and [γ-32P]dideoxy ATP, ensuring that only one nucleotide was added to the end of the probe. The data in Fig. 2(b) show that mRNA from BCP-1 but not from the KSHV-negative cell line HEK293 cells protects the 40 nt probe and a 20 nt fragment. The 20 nt fragment probably arises from hybridization of the probe to the full-length LANA transcript and subsequent digestion of the 20 nt oligo(dT) tail, which will not hybridize to this message. These data confirm the presence of the RD4 transcript in BCP-1 cells.

The RD4-encoded protein co-migrates with the smaller LANA isoform

The full-length RD4 transcript was cloned into the eukaryotic expression vector pBK-CMV (Stratagene). A full-length clone of LANA in which the alternative poly(A) signal was mutated from AGTAAA to TGTACA was also cloned into pBK-CMV. These constructs were transfected into HEK293 cells, and the proteins immune-precipitated using a mouse monoclonal antibody recognizing LANA. The Western blot of the immune-precipitated proteins shown in Fig. 3 was probed with the LANA monoclonal antibody LN53, which recognizes an epitope in the central repeat domain of LANA (Kellam et al., 1999). The figure shows that the RD4-encoded protein co-migrates with the smaller LANA isoform (~225 kDa). The RD4-encoded protein is therefore designated LANA-Δ76 (deletion of the terminal 76 aa of LANA). Mutation of the poly(A) signal resulted in the expression of a single protein co-migrating with the larger LANA isoform (~235 kDa) observed in BCP-1 cells.

Subcellular localization of LANA-Δ76

The C termini of LANA and LANA-Δ76 were cloned into the plasmid pEGFP-C1 and transfected into BCP-1 or HEK293 cells. As shown in Fig. 4(a), the LANA-EGFP fusion protein co-localizes with the endogenous LANA in BCP-1 cells. The antibody used to visualize the endogenous LANA recognizes an epitope within the central repeat domain, not included in the LANA-EGFP fusion construct. In the KSHV-negative HEK293 cell line the LANA-EGFP fusion protein associated with the nuclear heterochromatin (Fig. 4d). Conversely, although the LANA-Δ76-EGFP fusion protein localized to the nucleus in both BCP-1 and HEK293 cells, it did not co-localize with endogenous LANA or nuclear heterochromatin (Fig. 4b, e).

Fig. 3. LANA was immune-precipitated from BCP-1 cells. LANA-Δ76 and LANA-Δpoly(A) were immune-precipitated from HEK293 cells. The position of the LANA doublet at approximately 225 and 235 kDa is indicated. The RD4-encoded protein (designated LANA-Δ76) co-migrates with the lower protein species of the doublet (~225 kDa). Mutation of the poly(A) signal (AGTAAA to TGTACA) in LANA produced a protein that co-migrates with the upper protein species of the doublet (~235 kDa).

Fig. 4. BCP-1 cells were transfected with (a) c-term LANA EGFP-, (b) c-term LANA-Δ76-EGFP-, or (c) EGFP-expressing plasmids. Cells were fixed with paraformaldehyde and stained with LN53 anti-LANA antibody (red) that recognizes an epitope in the repeat region of LANA (Kellam et al., 1999). This antibody does not recognize LANA C-terminal sequences. HEK293 cells were transfected with (d) c-term LANA EGFP, (e) c-term LANA-Δ76-EGFP, or (f) EGFP. Nuclei are counterstained with TOPRO-3 (blue).
LANA-Δ76 does not bind to the KSHV terminal repeats

LANA has been shown to bind to a 20 nt sequence (tccgcccgcgcagcgggcc) present in the terminal repeats (TR) of the KSHV genome (Ballestas & Kaye, 2001). As described previously (Ballestas & Kaye, 2001), an EMSA was used to determine whether LANA-Δ76 also interacted with this TR sequence. The data in Fig. 5(b) show that in vitro–translated C-terminal LANA but not LANA-Δ76 or EGFP bound specifically to the TR probe. The specificity of the LANA-TR interaction was confirmed by competing-out the specific shifted band with excess unlabelled TR probe (Fig. 5c). These data indicate that the terminal 76 aa of LANA are critical for binding to the KSHV TR.

DISCUSSION

Since LANA was first identified as an immunogenic protein expressed in cells latently infected with KSHV, it has been shown to migrate as a doublet of 226–234 kDa by SDS-PAGE (Gao et al., 1996a; Kellam et al., 1997; Rainbow et al., 1997). Gao et al. (1999) analysed the molecular polymorphism of LANA in several PEL cell lines and found that although the molecular mass of the protein varied (due to variation in the length of the central repeat domain), LANA always migrated as a doublet with an apparent molecular mass difference of approximately 8 kDa. We have identified a 3·2 kb transcript in KSHV-positive primary effusion lymphoma (PEL) cells (BCP-1 and BC-3) encoding a C-terminal truncated form of LANA (LANA-Δ76). This transcript has the addition of a poly(A) tail at nt 3264 of orf73 resulting in an in-frame stop codon (TAA) effectively truncating LANA by 76 aa (~8 kDa). Expression of LANA-Δ76 in HEK293 cells produced a protein co-migrating with the lower of the LANA isoforms when analysed by SDS-PAGE and Western blot. Examination of the coding region of LANA-Δ76 revealed the presence of a non-canonical polyadenylation signal (AGTAAA) 17 nt upstream of the poly(A) tail. Mutating this sequence from AGTAAA to TGTACA abrogated the function of the poly(A) signal, resulting in the expression of a protein co-migrating with the larger of the LANA isoforms. This poly(A) signal sequence has been shown to occur at a frequency of between 5 and 8% for human genes (Caron et al., 2001). Functional AGTAAA poly(A) signals have been identified in at least two herpesvirus genes (Husain et al., 1999; Wakeling et al., 2001). The introduction of an in-frame stop codon by the addition of a poly(A) tail to an mRNA represents a novel mechanism for the expression of a truncated isoform of a protein.

Fig. 5. (a) In vitro expression of LANA C-terminal EGFP fusion (lane 1; molecular mass 58 kDa), LANA-Δ76 C-terminal EGFP fusion (lane 2; molecular mass 50 kDa) and EGFP (lane 3; molecular mass 33 kDa). Proteins were resolved by SDS-PAGE, Western blotted and detected using an anti-GFP antibody (Clontech). (b) LANA but not LANA-Δ76 interacts with the KSHV terminal repeat sequence. Decreasing amounts (10, 2 and 0·5 µl) of LANA-EGFP, LANA-Δ76-EGFP or EGFP were mixed with radiolabelled TR probe and the complexes resolved on a non-denaturing polyacrylamide gel. An arrow indicates the shifted protein–DNA complex. The positions of a non-specific band (NSB) and unbound TR probe (*) are also indicated. (c) EMSA showing the specificity of the interaction of LANA and the TR. The shifted LANA–DNA complex (arrow) was successfully competed with excess cold competitor (unlabelled TR probe) but not a non-specific (NS) oligonucleotide probe.
The C terminus of LANA has been shown to interact with a number of cellular proteins (p53, Rb, RING3) (Friborg et al., 1999; Platt et al., 1999; Radkov et al., 2000) as well as being involved in the transcriptional regulation of cellular and viral genes mediated through transcription factors such as CREB, CBP, and Sp1 (An et al., 2002; Krithivas et al., 2000; Lim et al., 2000; Radkov et al., 2000). The C terminus of LANA also mediates dimerization (Schwam et al., 2000) and is responsible for binding to two short sequence motifs within the terminal repeats (TR) of the KSHV genome (Garber et al., 2002). Although localized in the nucleus, the LANA-Δ76 protein does not co-localize with LANA in KSHV-infected cells, suggesting that it has lost the domain responsible for dimerization and/or the domain required for TR binding. Using an EMSA, LANA-Δ76 failed to bind to the KSHV TR motif (Ballestas & Kaye, 2001). Viejo-Borbolla et al. (2003) have identified a short domain in the C terminus of LANA (aa 1129–1143) that plays a role in heterochromatin binding, probably by modulating the heterochromatin-binding domain identified at the N terminus (aa 5–22) (Piilot et al., 2001). These data are consistent with the nuclear localization of LANA-Δ76 (lacks aa 1086–1162), which also fails to associate with either heterochromatin or full-length LANA.

Although it does not bind to the KSHV TR or associate with full-length LANA, LANA-Δ76 still possesses the domains responsible for interacting with p53, Rb and RING3. The interaction of RING3 has been shown to result in the phosphorylation of serine and threonine residues in the C terminus of LANA (Platt et al., 1999). There are three predicted serine and one predicted threonine phosphorylation sites in the terminal 76 aa of LANA (Fig. 1a), but these fall outside the domain phosphorylated through interaction with RING3 (aa 951–1107) (Platt et al., 1999). The role of LANA-Δ76 and whether it interacts with these proteins in KSHV-infected cells still remains to be determined.

ACKNOWLEDGEMENTS

This work was supported by a Medical Research Council career establishment grant to S.J.T.

REFERENCES


C-terminal truncated form of KSHV LANA


