Latency-associated nuclear antigen of Kaposi’s sarcoma-associated herpesvirus (human herpesvirus-8) binds ATF4/CREB2 and inhibits its transcriptional activation activity

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Latency-associated nuclear antigen (LANA), encoded by ORF 73 of Kaposi’s sarcoma-associated herpesvirus (KSHV; human herpesvirus-8), may play an important role in the persistence of the viral episome by tethering it to host chromosomes during mitosis. It also has been suggested from its amino acid sequence features that LANA may have transcription-regulatory activity. Here, it is reported that LANA interacts with activating transcription factor (ATF) 4/cAMP response element-binding protein (CREB) 2, a member of the ATF/CREB family of transcription factors, and represses the transcriptional activation activity of ATF4/CREB2. Repression by LANA is independent of the DNA-binding ability of ATF4/CREB2, since LANA also represses transactivation of ATF4/CREB2 fused to the GAL4 DNA-binding domain and does not affect the DNA-binding ability of ATF4/CREB2 in an electrophoretic mobility shift assay. The putative leucine zipper domain of LANA is required for binding to the relatively conserved basic region/leucine zipper domain (bZIP) of ATF4/CREB2, suggesting that the interaction may involve leucine zipper dimerization.

Introduction

Kaposi’s sarcoma-associated herpesvirus (KSHV), also called human herpesvirus-8, is the likely aetiological agent of Kaposi’s sarcoma (KS), the most common AIDS-related malignancy (Chang et al., 1994; Moore & Chang, 1995). KSHV also has been implicated in several B-cell lymphoproliferative diseases, including primary effusion lymphoma (PEL) (Cesarman et al., 1995), formerly designated body cavity-based lymphoma (BCBL), and some cases of multicentric Castleman’s disease (Soulier et al., 1995). KSHV establishes a latent infection in KS spindle cells, as well as PEL/BCBL cell lines. Among the viral genes expressed in the latent state, latency-associated nuclear antigen (LANA), encoded by ORF 73 of the KSHV genome, is a 222–234 kDa nuclear protein consisting of 1162 amino acids (Russo et al., 1996; Ganem, 1997; Cotter & Robertson, 1999). Recently, several cellular proteins have been shown to interact with LANA. These cellular proteins include histone H1 (Cotter & Robertson, 1999), RING3 (Platt et al., 1999) and p53 (Friborg et al., 1999).

We performed a yeast two-hybrid assay with full-length LANA as bait to identify cellular proteins that interact with LANA. Among several positive clones was a partial cDNA of activating transcription factor (ATF) 4 (Hai et al., 1989), also called cAMP response element (CRE)-binding protein (CREB) 2 (Karpinski et al., 1991) or TAXREB67 (Tsujimoto et al., 1991), which is a member of the ATF/CREB family of transcription factors. ATF4/CREB2 was initially characterized as a negative regulatory transcription factor of CRE (Karpinski et al., 1992). It was later shown to interact with CREB-binding protein (CBP)/p300 co-activators as well as several general transcription factors, including TATA-binding protein, TFIIB and the RAP30 subunit of TFIIF, and it functions as a transcriptional activator (Liang & Hai, 1997). Members of the ATF/CREB family of transcription factors share a relatively conserved basic region/leucine zipper (bZIP) domain required for direct contact with DNA and homo- and heterodimerization (Sassone-Corsi, 1995). They also dimerize selectively with the Fos/Jun family of transcription factors (Hai & Curran, 1991).
Through such cross-family dimerization, in addition to homo- 
heterodimerization, they extend their DNA-binding specific-
ity to allow differential regulation of transcription.

Here, we report that LANA interacts with ATF4/CREB2 in 
vivo and in vitro and that the bZIP domain of ATF4/CREB2 is 
required for the interaction. LANA represses the transcriptional 
activation activity of ATF4/CREB2 and the ATF4/CREB2-
binding domain of LANA is required for repression. Since the 
bZIP domains of members of the ATF/CREB family have been 
shown to be targeted by many viral proteins, including 
adenvirus E1a (Liu & Green, 1994), human T-cell leukaemia 
Yeast two-hybrid assay. Yeast two-hybrid screening was per-
in the yeast strain EGY048, harbouring the LacZ reporter plasmid p8op-lacZ 
Clontech. More than two independent β-galactosidase assays were 
produced from a liquid culture using ONPG (Sigma) as a substrate 
Lundblad, 1992).

Cell culture, transfection and reporter assays. HeLa and 
293T cells were maintained in Dulbecco’s modified Eagle's medium supple-
mented with 10% foetal bovine serum and transfected by the calcium 
phosphate method (Kingston, 1992). Cells of 60–70% confluence were 
plated in 60 mm dishes at 12 h before transfection. The total amount of 
transfected DNA was adjusted with the appropriate blank vector. Cells 
were harvested at 36 h post-transfection. Luciferase and β-galactosidase 
assays were performed according to manufacturer’s instructions (Pro-
mega) and the luciferase activity was normalized with co-transfected β-
galactosidase activity. The results are the means of four independent 
experiments.

In vivo binding assay. HA-tagged ATF4/CREB2 expression 
vector (pSRs ATf4/CREB2) was co-transfected with either pEBG blank 
vector or pEBG LANA expression vector into 293T cells. At 36 h post-
transfection, the cells were harvested and lysed in ice-cold PBS containing 
0.5% Nonidet P-40 and a protease inhibitor cocktail (Roche Molecular 
Biocahems) with brief sonication. After precipitating cell debris, the 
supernatant was incubated with glutathione–Sepharose 4B (Amersham 
Pharmacia Biotech) at 4 °C for 3 h. The beads were washed three times in 
PBS containing 0.1% Nonidet P-40 and a protease cocktail and then 
resuspended in a SDS-gel loading buffer. The eluted proteins were 
separated by 10% SDS–PAGE, transferred to nitrocellulose membrane 
and then immunoblotted and detected by ECL (Amersham 
Pharmacia Biotech).

GST pull-down assay. GST fusion proteins were incubated with 
[35S]methionine-labelled proteins synthesized by the TnT T7 coupled 
transcription-translation reticulocyte lystate system, according to the 
manufacturer’s instructions (Promega). After 3 h incubation at 4 °C in a 
binding buffer [20 mM Tris–HCl (pH 7.5), 50 mM NaCl, 2 mM MgCl2, 
1 mM DTT and 0.1% Nonidet P-40], glutathione–Sepharose 4B beads were 
added and incubated further for 1 h at 4 °C. The beads were washed 
four times in binding buffer and resuspended in SDS-gel loading buffer. 
The eluted proteins were analysed by SDS–PAGE and subjected to 
 autoradiography.

Localization of GFP-fused LANA deletion mutants. HeLa cells 
grown on coverslips were transfected with GFP or GFP-fused LANA 
deletion mutant expression plasmids by the calcium phosphate method, 
as described above. At 36 h post-transfection, the cells were washed 
three times and then fixed with formaldehyde (3.7%, v/v) in PBS. After 
two more washes, the coverslips were mounted and examined with a 
confocal laser scanning microscope (LSM510, Carl Zeiss).

Production of rabbit anti-LANA polyclonal antiserum. The 
C-terminal 212 amino acids of LANA fused to MBP were expressed in 
bacteria, purified and used in intradermal immunization. One month after 
the priming immunization with complete Freund’s adjuvant (Sigma), four 
additional boosting immunizations were administered with incomplete 
Freund’s adjuvant at intervals of 1 week. Rabbit anti-LANA polyclonal 
antisum diluted 1:500 was used in Western blots of total cell lysates.

Electrophoretic mobility shift assay (EMSA). Synthetic olio-
ucleotides containing CRE (‘AAGATTGCCCTAGGTCAGAG-
AGCTAG’3) were labelled with [32P]ATP by 14 polynucleotide kinase 
and annealed with the complementary strand. Labelled probe (∼ 100 

Methods

■ Plasmids. Total genomic DNA of KSHV was purified from a BCBL-
1 cell line by the Hirt lysis method, as described previously (Wilson & 
Patien, 1991). The 34 kb ORF 73 DNA was amplified by PCR and 
inserted into EcoRI/XhoI sites of pLexA (Clontech) and pGEXAT-1 
(Amersham Pharmacia Biotech) to generate pLexA LANA and pGEXAT-
1 LANA, respectively. To construct pEBG-LANA, a partially digested 
BamHI–NotI fragment of pGEXAT-1 LANA was cloned into pEBG, a 
eukaryotic expression vector encoding glutathione S-transferase (GST) 
(Mayer et al., 1995). pSG5 derivatives were constructed by inserting 
appropriate PCR products into the EcoRI/BglII sites of pSG5 (Stratagene) 
and they were designated pSG5 LANA(1–1162), pSG5 LANAAC(1–950) 
and pSG LANA-N(1–340) according to the size of their insert DNA 
fragment. For in vitro transcription and translation, pcDNA3 derivatives 
were made by inserting PCR products into the EcoRI/XhoI sites of 
pcDNA3 (Invitrogen) and were designated pcDNA3 LANA(1–1162), 
pcDNA3 LANA ΔC(1–950), pcDNA3 LANA NALZI(–756), pcDNA3 
LANA N(1–340), pcDNA3 LANA ADEΔ(431–1162) and pcDNA3 
LANA LZ-C(751–1162), according to the size of their DNA fragment.

For green fluorescent protein (GFP)-fusion expression vectors, PCR 
products of LANA and deletion mutants were subcloned into the 
EcoRI/Sall sites of pEGFP-C1 (Clontech). For bacterial expression of 
the CREB family have been 

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Results

Yeast two-hybrid assay

Full-length LANA fused to the LexA DNA-binding domain, which showed no autonomous activation of the *lei2* gene under the control of LexA operators in yeast, was used as bait for a yeast-two-hybrid assay to identify cellular proteins that could interact with LANA. A B cell cDNA library fused to the B42 activation domain was screened. From 3.5 × 10⁵ transformants, 26 positive clones were classified into seven groups according to their restriction enzyme pattern and DNA sequence analysis. Sequence analysis revealed the following: six clones were partial cDNAs of ribosomal protein S28; one clone was RING3, previously identified as a LANA-interacting protein by using the GAL4 yeast two-hybrid system (Platt et al., 1999); and six clones were ATF4/CREB2. We focused on ATF4/CREB2 because LANA has been thought to have transcription-regulatory activity (Russo et al., 1996; Ganem, 1997; Cotter & Robertson, 1999). We identified a partial cDNA of ATF4/CREB2 from a yeast two-hybrid that encoded the bZIP domain of ATF4/CREB2, which showed no autonomous activation of CREB2. Since other members of the ATF/CREB family of transcription factors (Sassone-Corsi, 1995), in order to confirm the interaction between LANA and ATF4/CREB2, the isolated library plasmid was co-transformed with pLexA LANA into the yeast strain EGY048, harbouring the LacZ reporter plasmid p8op-lacZ. Next, β-galactosidase assays were performed on a liquid culture. When both plasmids were co-transformed, more than 50-fold activation of β-galactosidase activity was observed, compared with transformants harbouring pLexA LANA alone or pLexA lamin instead of pLexA LANA as a negative control (data not shown).

Assay of binding in vivo between LANA and ATF4/CREB2

In order to confirm that LANA interacts with ATF4/CREB2 in mammalian cells, an in vivo binding assay was performed. Plasmids expressing GST or GST–LANA were co-transfected with HA-tagged ATF4/CREB2 expression plasmid into 293 T cells. After cells were harvested, proteins were precipitated with glutathione–Sepharose beads and immunoblotted by using a monoclonal antibody against GST (Fig. 1a). In Fig. 1(b), total cell lysates (lane T) or glutathione-bound proteins (lane P) were immunoblotted by using a monoclonal antibody against HA. Although HA-tagged ATF4/CREB2 protein was expressed at comparable levels (lanes 3 and 5), it was only co-precipitated with GST–LANA protein (lane 6). When the GST–LANA expression plasmid (lane 2) or GST- and HA–ATF4/CREB2-expressing plasmids (lane 4) were transfected, no such band was observed, confirming that the pulled-down protein was not a non-specific protein of similar size. This result shows that LANA associates with ATF4/CREB2 in vivo.

In vitro binding assay and mapping of the binding domains in LANA and ATF4/CREB2

In order to define the binding domains of LANA and ATF4/CREB2, we carried out an in vitro GST pull-down assay. Various deletion mutants of ATF4/CREB2 and LANA used in this assay are shown in Fig. 2(a, c). GST-fused ATF4/CREB2 and deletion mutant proteins were expressed in bacteria and purified by using glutathione–Sepharose beads. The amounts of GST-fused proteins used in the GST pull-down assay were comparable in a Coomassie blue-stained gel (Fig. 2b). Fig. 2(c) shows that the bZIP domain of ATF4/CREB2 is necessary and sufficient for binding to in vitro-translated, radiolabelled LANA. LANA deletion mutants containing a putative leucine zipper domain were precipitated with GST–LANA/CREB2 fusion protein immobilized on glutathione–Sepharose beads (Fig. 2c). GST–ATF4/CREB2 bZIP fusion proteins showed a higher affinity for LANA than did the wild-type ATF4/CREB2. The above results show that LANA binds ATF4/CREB2 in vitro.

Effect of LANA on transcriptional activation by ATF4/CREB2

Next, we examined whether LANA could modulate the transcriptional activation activity of ATF4/CREB2. When co-transfected into HeLa cells with a luciferase reporter plasmid containing three tandem ATF/CRE sites (pATFx3-luc), ATF4/CREB2 activated transcription of the reporter plasmid (Liang & Hai, 1997). Fig. 3(a) shows that increasing amounts of LANA repressed the transactivation activity of ATF4/CREB2 on the ATF/CRE promoter in a dose-dependent manner. However, pSG5 LANA-N, which does not contain the binding domain for ATF4/CREB2, could not repress the transactivation activity of ATF4/CREB2. This finding indicates that repression by LANA requires an ATF4/CREB2-binding domain. Since LANA may repress the transactivation activity of ATF4/CREB2 by binding to the bZIP domain, which is required for dimerization/DNA binding, and affecting the DNA-binding ability of ATF4/CREB2, we adopted the GAL4 fusion system to exclude this possibility. The use of the GAL4 system simplifies the investigation of the functional interaction between LANA and ATF4/CREB2, since other members of the ATF/CREB family of transcription factors can also bind the same ATF/CRE sites (Hai et al., 1989). GAL4-fused ATF4/CREB2 was constructed and a luciferase reporter containing
Fig. 1. Association of LANA and ATF4/CREB2 in vivo. 293T cells were co-transfected with pEBG or pEBG LANA along with pSRx ATF4/CREB2, expressing HA-tagged ATF4/CREB2. After cell extracts were pulled down with glutathione–Sepharose 4B beads, bound proteins were immunoblotted with anti-GST (a) or anti-HA (b) antibody. T, total lysates; P, pull-down.

Fig. 2. Mapping of binding domains in LANA and ATF4/CREB2. (a) Schematic representation of ATF4/CREB2 deletion mutants. bZIP, Basic region/leucine zipper domain; ZIPII, second leucine zipper domain. (b) Coomassie blue stain of GST fusion proteins used in GST pull-down assay before autoradiography. (c) Schematic representation of LANA deletion mutants (left panel) and autoradiogram of in vitro-translated LANA and deletion mutants bound to indicated GST-fusion proteins (right panel). P-rich, Proline-rich region; DED, aspartate/glutamate-rich repeat region; Q-rich, glutamine-rich region; ZIP, putative leucine zipper domain. Lane 1 shows 10% of radiolabelled LANA and deletion mutants used in GST pull-down assay.

five GAL4-binding sites (pFR-luc) was used as a reporter plasmid. As shown in Fig. 3(b), pFR-luc was activated by GAL4–ATF4/CREB2 and its transactivation was repressed by LANA in a dose-dependent manner. Repression of ATF4/CREB2 transactivation activity by LANA is also dependent on the presence of a binding domain for ATF4/CREB2. Re-
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Fig. 3. Repression by LANA of ATF4/CREB2-dependent transcriptional activation. HeLa cells were co-transfected with 1–5 µg pATFx3-luc, 2 µg pCG or pCG ATF4/CREB2 and indicated amounts of pSG5 derivatives expressing LANA deletion mutants (a) or 1–8 µg pFR-luc, 1 µg pM or pM ATF4/CREB2 and indicated amounts of pSG5 derivatives expressing LANA deletion mutants (b). Relative luciferase activity (RLU) was normalized to co-transfected β-galactosidase activity (β-gal). Each result is the mean of four independent experiments and standard deviations are indicated by error bars.

Pression by LANA was not cell type specific, as a similar result was obtained with 293T cells (data not shown).

The cellular localization of LANA and C-terminal deletion mutants was examined in order to confirm that the inability of the LANA deletion mutant to repress the transactivation activity of ATF4/CREB2 was not due to the cellular localization of LANA but was due to the absence of an ATF4/CREB2-binding domain. As shown in Fig. 4, LANA and all C-terminal deletion mutants fused to GFP were localized in the nucleus, which is consistent with the results of a previous report (Friborg et al., 1999). Interestingly, an N-terminal deletion mutant of LANA (amino acids 324–1162) designated as LANA ΔN was localized exclusively in the cytoplasm (Fig. 4f). This exclusively cytoplasmic localization of LANA ΔN was not due to aberrant expression or a frameshift between GFP and LANA. Expression of LANA ΔN with the correct size was demonstrated by a Western blot of a total cell lysate transfected with the indicated expression vectors by using a rabbit polyclonal antiserum against the C terminus of LANA (Fig. 4g). These findings imply that the N terminus of LANA (1–324) may contain the nuclear localization signal (NLS). Taken together, these results suggest (i) that LANA inhibits the transcriptional activation activity of ATF4/CREB2 specifically by binding to the bZIP domain and (ii) that the ATF4/CREB2-binding domain of LANA is necessary for repression of the transactivation activity of ATF4/CREB2.

Effect of LANA on the DNA-binding ability of ATF4/CREB2

Although LANA repressed the transcriptional activation activity of GAL4–ATF4/CREB2, we cannot exclude the possibility that LANA affects the DNA-binding ability of ATF4/CREB2 and thereby represses the transcriptional activation activity of ATF4/CREB2 on a reporter promoter containing ATF/CRE. Therefore, we examined the effect of...
LANA in EMSA with GST–ATF4/CREB2. As shown in Fig. 5, when labelled probe containing the CRE sequence was incubated with GST–ATF4/CREB2, two major shifted bands were observed (lane 3, C1 and C2). The addition of a 10-fold excess of unlabelled oligonucleotides containing a CRE- or Oct-1-binding site was used in competition reactions. IVT mock, Control reticulocyte lysate; IVT LANA, in vitro-translated LANA.

Fig. 5. EMSA of ATF4. The indicated components (top) were included in each binding reaction and GST–ATF4/CREB2–DNA complex-specific and non-specific bands are indicated by C1 and C2, respectively. A 10-fold molar excess of unlabelled oligonucleotides containing a CRE- or Oct-1-binding site was used in competition reactions. IVT mock, Control reticulocyte lysate; IVT LANA, in vitro-translated LANA.

In transient co-transfection experiments, LANA repressed transcriptional activation of the reporter promoter by ATF4/CREB2. The ATF4/CREB2-binding domain in LANA was necessary for repression, implying that LANA may exert a transcription-inhibitory effect via direct binding to ATF4/CREB2. Since LANA inhibited transcriptional activation of the promoter by a GAL4–ATF4 fusion protein and did not affect the DNA-binding ability of ATF4/CREB2 under our experimental conditions, another mechanism may be involved in the process of repression by LANA. However, the possibility that LANA modulates DNA binding of ATF4/CREB2 in the context of a promoter cannot be excluded.

The PSORT II program (http://psort.nibb.ac.jp/) predicted three putative NLSs in the published amino acid sequence of LANA (GenBank accession no. AAC57158). These were PRRKH (amino acids 43–47), RKRR (amino acids 200–203) and PGVRMRR (amino acids 995–1001). The two N-terminal putative deletion mutants of LANA fused to GFP localized exclusively in the nucleus, but an N-terminal deletion mutant lacking these motifs (LANA ΔN) did not. Also, we found that the N-terminal 213 amino acids of LANA were necessary and sufficient for exclusively nuclear localization and that the C-terminal 212 amino acids of LANA were also necessary for nuclear localization in an N-terminal deletion mutant of LANA, which showed a similar localization pattern to that of a GFP control (data not shown).

Three models of active repression have been defined (Galvin & Shi, 1997). From our results, we deduced that LANA belongs by definition to the type II quenching repressors; that is, a protein that represses transcription by interacting with the activator itself. In order to elucidate the repression mechanism of LANA, we examined whether LANA acted as a direct repressor tethered to a promoter by using the GAL4–LANA fusion system. GAL4–LANA repressed a thymidine kinase promoter containing tandem GAL4-binding sites weakly in a dose- and GAL4 DBD-dependent manner (data not shown). Because the expression level of GAL4–LANA in our study was relatively low, we could not determine the reason for the observed weak repression. We also tested whether LANA recruited a co-repressor complex containing histone deacetylase activity (Knoepfler & Eisenman, 1999) to the promoter via ATF4/CREB2. LANA did not interact directly with GST-fused histone deacetylases 1, 2 and 3 in an in vitro GST pull-down assay. The repression of LANA upon transcriptional activation by GAL4–ATF4/CREB2 was not relieved by treatment with trichostatin A, a histone deacetylase inhibitor (Johnson & Turner, 1999), in a transient co-transfection experiment (data not shown). The above result indicated that the repression of LANA is not related to histone deacetylase activity.

Moderate repression of ATF4/CREB2-dependent transcription by LANA can be explained by multiple interactions of ATF4/CREB2 with general transcription factors and CBP (Liang & Hai, 1997). The C-terminal bZIP domain of
ATF4/CREB2, in addition to DNA binding/dimerization, has the ability to interact with general transcription factors/CFP and activates transcription weakly. However, LANA may be unable to inhibit the transactivation activity of ATF4/CREB2 completely because the N terminus of ATF4/CREB2 can also interact with general transcription factors and still activates transcription more efficiently than the C-terminal bZIP domain.

The physiological functions of ATF4/CREB2 have not been defined. It can repress protein kinase A-dependent transcriptional activation (Karpinski et al., 1992) or activate transcription when tethered to a promoter by a GAL DBD fusion or via ATF4/CREB2-binding sites (Liang & Hai, 1997). A previous study reported that LANA shows preferential binding to different regions of KSHV DNA in vitro (Cotter & Robertson, 1999). However, it is uncertain whether LANA binds directly to DNA with sequence preference, because in vitro-translated LANA used in a DNA-binding assay may be contaminated with cellular proteins in a rabbit reticulocyte lysate. LANA may use sequence-specific DNA-binding adator proteins, such as ATF4/CREB2, for sequence-preferential DNA binding or may target a viral or cellular promoter to regulate a transcriptional network during virus infection. It would be interesting to investigate a viral or cellular promoter targeted by LANA via ATF4/CREB2, in addition to the repression mechanism of LANA.

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