Identification of a virus trans-acting regulatory element on the latent DNA replication of Kaposi’s sarcoma-associated herpesvirus

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Latency-associated nuclear antigen 1 (LANA1) of Kaposi’s sarcoma-associated herpesvirus (KSHV) plays a pivotal role in the maintenance of the virus genome in latently infected cells. LANA1 links virus genomes to host chromosomes via a C-terminal DNA-binding domain which interacts with the sequences located in terminal repeats (TRs) of the virus genome and via an N-terminal chromosome-binding sequence which associates with the host chromosomes, respectively. Recent data suggest that LANA1 also actively participates in the replication of KSHV TR-containing plasmid in the transient DNA replication assay. In this report, it was found that C33A and COS-1, but not NIH/3T3, cell lines are permissive for the transient replication of KSHV TR-containing plasmid. Using several LANA1-deletion mutants, the minimum domain of LANA1 required for replication activity was also determined. In addition, the N terminus of LANA1 inhibited the transient replication systems of KSHV and Epstein–Barr virus (EBV) in transiently transfected 293 and 293T cells, but the C terminus of LANA1 specifically inhibited the transient replication system of KSHV in other cell lines. Consistent with previous reports, these data further emphasize the functional importance of the N terminus of LANA1 on replication from the KSHV latent origin of DNA replication.

INTRODUCTION

Kaposi’s sarcoma-associated herpesvirus (KSHV) was first identified as a herpesvirus-like DNA sequence from Kaposi’s sarcoma tissues of a patient with acquired immuno-deficiency syndrome by representational display method (Chang et al., 1994). KSHV has been implicated in body cavity-based lymphoma, also known as primary effusion lymphoma, and some cases of multicentric Castleman’s disease (Cesarman et al., 1995a; Soulier et al., 1995). The virus genome consists of an ~140-5 kb long unique region flanked by a multiple GC-rich 801 bp terminal repeat (TR) sequence and encodes more than 80 open reading frames (Lagunoff & Ganem, 1997; Russo et al., 1996). Based on sequence analysis, it has been classified as a member of the gammaherpesvirus 2 subfamily, and is closely related to Herpesvirus saimiri and Epstein–Barr virus (EBV). The infection of endothelial and spindle cells of Kaposi’s sarcoma as well as lymphocytes by KSHV is predominantly latent, and the restricted set of virus-latent genes is expressed from circularized virus genome, which exists as a multicopy episome (Cesarman et al., 1995b; Decker et al., 1996; Sarid et al., 1998). Chemical treatment such as phorbol 12-myristate 13-acetate (PMA) and n-butyrate, or overexpression of the KSHV open reading frame 50 can switch the virus from a latent to a lytic cycle during which infectious virus particles are productively assembled in infected cells (Lukac et al., 1998, 1999; Miller et al., 1996; Moore et al., 1996; Renne et al., 1996; Sun et al., 1998).

Latency-associated nuclear antigen 1 (LANA1), encoded by open reading frame 73 of KSHV genome, is one of the virus genes expressed during latent infection (Russo et al., 1996; Rainbow et al., 1997). Based on the primary amino acid sequence, it was suggested that this 222–234 kDa nuclear protein was a transcription factor. LANA1 interacts with several cellular transcription factors such as p53, pRb, ATF4/CREB2 and CREB-binding protein, and modulates their transcriptional activities (Friborg et al., 1999; Lim et al., 2000, 2001; Radkov et al., 2000). When tethered to promoters via the heterologous DNA-binding domain, both the N terminus and the C terminus of LANA1 exhibit cell-type and promoter-specific transcriptional repressor activity, possibly through interactions with the components of the mSin3 corepressor complex and heterochromatin protein 1, respectively (Krithivas et al., 2000; Lim et al., 2003; Schwam et al., 2000). In addition, LANA1 has also been shown to participate in the transcriptional regulation of several cellular and virus promoters (An et al., 2002; Friborg et al., 1999; Groves et al., 2001; Hyun et al., 2001; Knight et al., 2001; Krithivas et al., 2000; Radkov et al., 2000; Renne et al., 2001).

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infection is accomplished by two distinct events. These include replication of the virus genome, which is synchronized with the DNA synthesis of host cells, and the equal segregation of replicated virus genomes to daughter cells after mitosis. A long-term replication assay, in which these two activities can be collectively analysed by selecting drug-resistant cells containing origin of replication (oriP)-plasmids, revealed that KSHV LANA1 and TR sequences located at both ends of the virus genome act as virus trans- and cis-acting elements, respectively, in the latent replication of KSHV (Ballestas et al., 1999; Ballestas & Kaye, 2001). LANA1 binds to sequences within TR via the C-terminal DNA-binding domain (Ballestas & Kaye, 2001; Cotter et al., 2001; Garber et al., 2001, 2002; Lim et al., 2002) and associates with the chromosomes of host cells via the N-terminal chromosome-binding sequence (CBS) (Ballestas et al., 1999; Cotter & Robertson, 1999; Pirot et al., 2001), suggesting a model in which LANA1 tethers virus genome to host chromosome for its persistence during latent infection. Recently, we and others showed that LANA1 actively participates in replication of the virus genome using a short-term replication assay whereby a methylation-sensitive restriction enzyme removes unreplicated oriP-containing plasmid from transiently transfected cells (Garber et al., 2002; Hu et al., 2002; Lim et al., 2002). It was also shown that LANA1 might recruit the cellular replication machinery to KSHV TR, possibly via interaction with origin recognition complexes (Lim et al., 2002) as in the case of EBNA-1 (Epstein–Barr virus nuclear antigen-1), a functional analogue of KSHV LANA1 (Chaudhuri et al., 2001; Dhar et al., 2001; Schepers et al., 2001).

In this report, we verified the transient replication of KSHV TR-containing plasmid in cell lines derived from different species, and determined the minimum domain of LANA1 required for replication of the TR-containing plasmid, using a transient replication assay. We also identified dominant-negative mutants of LANA1 for the replication activity and speculated on their replication-inhibitory mechanisms using other cell lines and virus replication systems.

METHODS

Plasmids. Plasmids pCDNA3 LANA1, CMV VP16 LANA1 C and p4TR-luc have been described previously (Lim et al., 2000, 2002). pFLAG-CMV2 LANA1 and LANA1-deletion mutants have been described previously and constructed by similar procedures (Lim et al., 2001, 2002). pOLP containing EBV oriP, FE and FC, mammalian expression vectors encoding FLAG-tagged EBNA-1 and EBNA-1C, respectively, were generous gifts from Dr Kieff (Harvard Medical School) (Hung et al., 2001). EapCG, a mammalian expression vector encoding both E1 and E2 of human papillomavirus (HPV) type 18, was a generous gift from Dr Thierry (Pasteur Institute, France). p105core-luc containing HPV oriP was a generous gift from Dr Lee (Pennsylvania State University). Mammalian expression vectors for E2F1 and p53 have been described previously (Gwack et al., 2001; Hwang et al., 2002).

Cell culture, transfection and reporter assay. Human embryonic kidney cell lines, 293 and 293T, and human cervical carcinoma cell line, C33A, were maintained and transfected as described previously (Lim et al., 2000, 2003). Simian virus 40 (SV40)-transformed African green monkey kidney cell line, COS-1, and mouse embryo cell line, NIH/3T3, were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, and were transfected using LIPOFECTAMINE plus reagent according to the manufacturer’s instructions (Invitrogen). The quantity of total DNA used in the transfections was kept constant by including an appropriate blank vector. The transient reporter assay was performed as described previously (Lim et al., 2000).

Western blotting. 293T cells in 60 mm dishes were transfected with the indicated mammalian expression vectors. At 36 h after transfection, cells were harvested and cell pellets were resuspended in 100 μl of PBS. After addition of 100 μl of 2 × SDS buffer, lysates were briefly sonicated, and then boiled for 5 min. Total proteins were separated by SDS-PAGE, transferred to Hybond-C membrane (Amersham Biosciences), immunoblotted with an anti-FLAG M2 monoclonal antibody (Sigma) and rabbit polyclonal anti-LANA1 serum (a generous gift from Dr Jung, Harvard Medical School), and detected by ECL™ (Amersham Biosciences).

Immunofluorescence assay. 293T cells grown on coverslips were transfected with the indicated expression vectors. At 24 h after transfection, cells were fixed in 3% formaldehyde at room temperature for 30 min, and permeabilized in PBS containing 0.2% Triton X-100 at 4°C for 25 min. FLAG-tagged proteins were observed using an anti-FLAG M2 monoclonal antibody (Sigma) and a rhodamine-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories). Coverslips were mounted with Vectashield (Vector Laboratories) and examined by confocal laser scanning microscopy (Pascal, Carl Zeiss, Jena, Germany).

Transient replication assay. A transient replication assay was performed as described previously (Lim et al., 2002), with minor modifications. For the KSHV replication assay, 293, 293T, C33A, COS-1 and NIH/3T3 cells grown in 100 mm dishes were cotransfected with 2 μg of p4TR-luc containing KSHV TR, 2 μg of pGL2-basic as an internal control, and the indicated amount of trans-element expression plasmid. For the EBV replication assay, 293, 293T and C33A cells grown in 100 mm dishes were cotransfected with 2 μg of pOLP containing EBV oriP, 2 μg of pGL2-basic as an internal control, and the indicated amount of trans-element expression plasmid. For the SV40 replication assay, 293T cells grown in 100 mm dishes were cotransfected with 2 μg of pCDNA3 containing SV40 oriP and 8 μg of the indicated trans-element expression plasmid. For the HPV replication assay, 293 and C33A cells grown in 100 mm dishes were cotransfected with 2 μg of p105core-luc containing HPV oriP, and the indicated amount of trans-element expression plasmid. Cells were split at ~24–36 h after transfection and harvested at 96 h after transfection, unless otherwise indicated. Low molecular mass DNA was extracted from transfected cells by the Hirt lysis method (Hirt, 1967), followed by phenol/chloroform/isomyl alcohol and chloroform extraction. Ethanol-precipitated DNA was dissolved in RNase-containing distilled water. Plasmids p4TR-luc, pOLP, pCDNA3 and p105core-luc were linearized by digestion with AlwI44I, SacI, EcoRI and BamHI, respectively. Unreplicated DNA was removed by digestion with DpnI, and the complete digestion of unreplicated oriP-containing plasmid was monitored by comparison with that of unreplicating internal control, pGL2-basic in the cases of KSHV and EBV replication assay. Digested DNA was separated by ethophoresis on a 0.8% agarose gel and analysed by Southern blot hybridization. Probes specific for the luciferase gene of pGL2-basic and neomycin open reading frame of pCDNA3 were synthesized with a NEBlot Phototope kit and detected with a Phototope-Star Detection kit, according to the manufacturer’s instructions (New England Biolabs). All transient replication assays were performed more than twice and the representative result was shown.
[\textsuperscript{3}H]\textsuperscript{-}Thymidine incorporation assay. 293T cells in six-well plates or in 60 mm dishes were transfected with the indicated mammalian expression vectors. Transfected cells in six-well plates were incubated with 0.6 \textmu Ci ml\textsuperscript{-1} (22-2 kBq ml\textsuperscript{-1}) of [methyl-\textsuperscript{3}H]thymidine (90 Ci mmol\textsuperscript{-1}, Amersham Biosciences) for the final 12 h before the assay that was performed at 36 h post-transfection. Transfected cells in 60 mm dishes were split into six-well plates 36 h after transfection, and incubated with radioactive thymidine as above for the final 24 h before the assay that was performed at 96 h post-transfection. At the indicated time, culture medium was removed and transfected cells were washed twice with PBS and extracted three times with 10% trichloracetic acid. Precipitated materials were solubilized in 1 M NaOH, 1% SDS at room temperature for 1 h and counted by liquid scintillation (LS6500 Liquid Scintillation Counters, Beckman Coulter). More than two independent experiments were performed in duplicate.

RESULTS

Transient replication assay of KSHV TR-containing plasmid in cell lines originated from different species

Recently, we and others have shown that KSHV TR-containing plasmid only replicates in the presence of LANA1 in a short-term replication assay (Garber et al., 2002; Lim et al., 2002; Hu et al., 2002). Human cell lines used in that assay included 293 and 293T (embryonic kidney cell lines), BJAB (a KSHV- and EBV-negative B cell lymphoma cell line derived from Burkitt lymphoma biopsy) and SLK (a KSHV-negative endothelial cell line derived from KS). To determine the host range permissive for the replication of KSHV TR-containing plasmid by LANA1, we tested additional cell lines derived from human and other species in a similar transient replication assay. Cell lines used included C33A (a human cervical carcinoma cell line), COS-1 (a SV40-transformed African green monkey kidney cell line) and NIH/3T3 (a mouse embryo cell line). The p4TR-luc plasmid, which contains four copies of KSHV TR, and pGL2-basic were cotransfected with a blank or a LANA1 expression vector into the indicated cell line. Transfected cells were split at 24–36 h after transfection to remove untransfected DNA and harvested at the indicated time points. Low molecular mass DNA purified from transfected cells was digested by Alw44I and DpnI to linearize transfected plasmid DNA and remove unreplicated DNA, respectively. Twenty per cent of the DNA was digested with Alw44I alone to show that an equivalent amount of each DNA sample was included in the DpnI digestion and the completion of DpnI digestion was routinely monitored by the complete digestion of an unreplicated internal control, pGL2-basic. As shown in Fig. 1, we could observe DpnI-resistant, replicated p4TR-luc extracted from C33A and COS-1 cells transfected with the LANA1 expression vector but not with the blank vector. However, the mouse cell line (NIH/3T3) was not permissive for transient replication of KSHV TR-containing plasmid, even though a comparable amount of DNA was transfected, extracted and included in the DpnI digestion. Under the same conditions, the transient replication of EBV oriP-containing plasmid was not observed in NIH/3T3 cells (data not shown), consistent with the previous data (Mizuguchi et al., 2000; Yates et al., 1985).

Fig. 1. Transient DNA replication assay of KSHV TR-containing plasmid in cell lines derived from different species. C33A, COS-1, and NIH/3T3 cells in 100 mm dishes were cotransfected with 2 \mu g of p4TR-luc containing KSHV TR, 2 \mu g of pGL2-basic as an internal control, and 8 \mu g (C33A) or 2 \mu g (COS-1 and NIH/3T3) of pcDNA3 or pcDNA3 LANA1. At \sim 36 h (C33A) or \sim 24 h (COS-1 and NIH/3T3) post-transfection, cells were split and harvested at the indicated times. Hirt-extracted DNA was digested with Alw44I alone (left) or Alw44I/DpnI (right), separated by electrophoresis on a 0.8% agarose gel, and analysed by Southern blot hybridization with luciferase gene-specific probe. Input, 20\% of DNA used in DpnI digestion.
Determination of the minimum domain of LANA1 required for the replication of KSHV TR-containing plasmid

Previously, we reported that the N-terminal 90 amino acids of LANA1, when fused to the C-terminal DNA-binding and dimerization domain of LANA1, could facilitate the replication of plasmids containing KSHV TR at a level comparable to wild-type LANA1 in a transient DNA replication assay (Lim et al., 2002). As depicted in Fig. 2(A), this region of the N-terminal LANA1 contains close but distinct CBS and nuclear localization signal (Piolot et al., 2001). To refine further the minimal requirement for replication activity within this region, a series of expression vectors encoding FLAG-tagged LANA1-deletion mutants were

Fig. 2. Determination of minimal domain of LANA1 required for the replication of TR-containing plasmid in a transient replication assay. (A) N-terminal amino acid sequence of LANA1 spanning chromosome-binding sequence (CBS) and nuclear localization signal (NLS) (upper) and a schematic diagram of LANA1-deletion mutants used in our study (lower). (B) Western blot of total lysates from transiently transfected 293T cells expressing FLAG-tagged LANA1 mutants. 293T cells in 60 mm dishes were transfected with 4 µg of the indicated mammalian expression vector, and FLAG-tagged proteins were detected with anti-FLAG M2 monoclonal antibody. Protein size marker is depicted on the left. (C) Immunofluorescence assay of transiently transfected 293T cells expressing FLAG-tagged LANA1 mutants. 293T cells grown on coverslips were transfected with 2 µg of the indicated mammalian expression vector, and FLAG-tagged proteins were detected using an anti-FLAG M2 monoclonal antibody and rhodamine-conjugated goat anti-mouse secondary antibody. (D) Transient replication assay of KSHV TR-containing plasmid by LANA1-deletion mutants. 293T cells in 100 mm dishes were cotransfected with 2 µg of p4TR-luc, 2 µg of pGL2-basic, and 8 µg of the mammalian expression vector encoding the indicated LANA1 mutants. Cells were split and harvested 36 h and 96 h after transfection, respectively. Hirt-extracted DNA was analysed as in Fig. 1.
constructed (Fig. 2A). Their expression and appropriate size was verified by Western blotting of total lysates from transiently transfected 293T cells with anti-FLAG monoclonal antibody (Fig. 2B). Immunofluorescence assay indicated that all of them exclusively localize to the nuclei of transiently transfected 293T cells, although ΔCBS NLS C and LANA1 C showed relatively weak speckled pattern compared to other LANA1 mutants (Fig. 2C). In the transient replication assay, expression vectors encoding these mutants were transiently cotransfected with p4TR-luc containing KSHV TR and pGL2-basic into 293T cells and transfected cells were harvested at 96 h after transfection. DpnI digestion of purified low molecular mass DNA and Southern blotting with luciferase gene–specific probe was similarly performed as in Fig. 1. As shown in Fig. 2(D), the N-terminal 22 amino acids of LANA1 containing CBS was necessary and sufficient for the mediation of the C-terminal LANA1 to facilitate the replication of p4TR-luc. It is noteworthy that much more replicated p4TR-luc was reproducibly detected in the presence of LANA1Δ31–950 and LANA1Δ23–950 than LANA1Δ91–950 and LANA1Δ53–950. It seems likely that deletion of the region encompassing amino acids 31 to 52 of LANA1 may alter its conformation in the context of internal deletion mutants, forming a more favourable structure for its replication activity, or may induce dissociation from cellular proteins which are targeted by this region and negatively regulate its replication activity.

**Dominant-negative mutants of LANA1 on the replication of KSHV TR-containing plasmid**

Previous reports showed that the C terminus of LANA1 alone can bind to KSHV TR in an electrophoretic mobility shift assay and repress the transcription from KSHV TR in the transient reporter assay (Garber et al., 2001, 2002; Lim et al., 2002). However, the C-terminal LANA1 alone could not support the replication of TR-containing plasmids in the transient replication assay (Lim et al., 2002; see also Fig. 2D). Therefore, it is possible that the C-terminal LANA1 competes with the wild-type LANA1 for the binding sites within TR when coexpressed in transiently transfected cells, resulting in a dominant-negative inhibition of the replication of TR-containing plasmids by the wild-type LANA1. First, we designed the transient reporter assay that can test this competitive binding to TR between the wild-type and the C terminus of LANA1 in transfected 293T cells. Although both wild-type and C-terminal LANA1 repress the transcriptional activity from TR, the fusion of VP16 activation domain to the C-terminal LANA1 can switch itself from a repressor to a specific activator of TR-dependent transcription (Lim et al., 2002). We thereby assumed that if this activator competes with the wild-type LANA1 for binding to TR, the transcriptional repression of TR by LANA1 would be relieved in the presence of C-terminal LANA1 fused to the VP16 activation domain. As shown in Fig. 3(A), the increasing amount of VP16 LANA1 C relieved the transcriptional repression of TR by wild-type LANA1 in a dose-dependent manner. The expression level of the wild-type LANA1 was not influenced by the cotransfection of VP16 LANA1 C expression vector, further supporting the hypothesis that the competitive binding to TR between them occurs in the transfected cells (data not shown). Next, we examined the effect of overexpressed C-terminal LANA1 on the replication of TR-containing plasmid by the wild-type LANA1 in the transient replication assay. As shown in Fig. 3(B), the C-terminal LANA1 significantly repressed the replication activity of the wild-type LANA1, compared with the effect of overexpressed hHP1α on it. Unexpectedly, however, the N terminus of LANA1 also showed inhibitory effect on the replication activity of wild-type LANA1. These inhibitory effects of the N- and C-terminal LANA1 were not due to the reduced expression level of the wild-type LANA1 by the coexpression of them, which was verified by Western blotting (Fig. 3C).

**Effect of LANA1 and its derivatives on the other virus replication systems and DNA synthesis of host cells**

To address the specificity of dominant-negative deletion mutants of LANA1 on the replication-inhibitory effect of KSHV TR-containing plasmid, we examined their effects on the other virus replication systems in 293T cells. These include EBV, another member of the gamma herpesvirus family, and SV40, a representative small DNA tumour virus of *Papovaviridae*. The virus replication of EBV during latent infection is accomplished by virus cis- and trans-acting elements as KSHV. The replication of the plasmid containing a family of repeats and a dyad symmetry sequence, which are separated by ∼1 kb within the EBV genome, requires the presence of EBNA-1, a functional analogue of KSHV LANA1 (Hung et al., 2001; Lupton & Levine, 1985; Middleton & Sugden, 1994; Reisman et al., 1985). Most of the mammalian expression vectors used in our study also contain SV40 oriP that efficiently replicates in cells expressing SV40 large T antigen such as 293T cells. Therefore, the replication of SV40 can be easily estimated in 293T cells without the exogenous expression of the virus trans-element.

As described previously (Hung et al., 2001), EBV oriP-containing plasmid, pPOLP, replicated only in the presence of EBNA-1, but not LANA1 (Fig. 4A, left panel). Overexpression of the wild-type and the N terminus of LANA1 dramatically reduced the replication of pPOLP by EBNA-1, while the C-terminal LANA1 did not show any effect on it. Western blot of total lysates from transfected cells indicated that this inhibitory effect of the wild-type and the N terminus of LANA1 on the transient replication of EBV oriP-containing plasmid is not due to the change in the expression level of EBNA-1 (Fig. 4A, right panel). Reciprocal transient replication assay was also performed to investigate the effect of EBNA-1 upexpression on the replication of KSHV TR-containing plasmid, p4TR-luc, which only replicates in the presence of LANA1. As shown...
in Fig. 4(B), overexpression of EBNA-1 neither inhibited the replication of KSHV TR-containing plasmid nor affected LANA1 expression. Since the N-terminal LANA1 seemed to possess the general inhibitory activity on the herpesvirus replication systems under the condition of our transient replication assay in 293T cells, we questioned whether this domain of LANA1 could also suppress other virus replication other than herpesviruses. When these herpesvirus replication proteins were overexpressed in the transient replication assay of pcDNA3 containing SV40 oriP in 293T cells, only the wild-type and the N terminus of LANA1 showed significant inhibitory effect on the SV40 replication (Fig. 4C). To determine which region of the N-terminal LANA1 is responsible for the inhibitory activity of the virus replication, a series of LANA1-deletion mutants depicted in Fig. 2(A) was included in the transient replication assay of EBV. As shown in Fig. 4(D), LANA1-deletion mutants supporting the replication of KSHV TR-containing plasmid were different from those restraining the replication of EBV oriP-containing plasmid, and only the
LANA1Δ91–950 showed the inhibitory activity comparable to N-terminal LANA1.

In contrast to the lytic replication of herpesviruses, in which virus replication proteins including helicase, primase and DNA polymerase directly participate in the productive replication activity at the lytic origin of replication within the virus genomes, one virus protein, such as KSHV LANA1 or EBV EBNA-1, marks the latent origin of replication for the recruitment of cellular replication machinery during latent infection. In this way, the latent replication of virus genomes is dependent on the cellular replication proteins and synchronized with the DNA synthesis of host cells. Since the N-terminal LANA1 generally inhibited the transient replication of virus oriP-containing plasmid in transiently transfected 293T, it is possible that the observed activity may have arisen from the effect of N-terminal LANA1 on DNA synthesis of host cells. To check out this possibility, the DNA synthesis of 293T cells transfected with the indicated mammalian expression vectors was estimated by [3H]thymidine incorporation assay. As shown in Fig. 4(E), the magnitude of thymidine incorporation of transfected cells was negligibly affected by the expression of LANA1 or its derivatives in the condition that overexpression of E2F1 and p53, which is known to cause cell cycle arrest and apoptosis (Almasan et al., 1995; Qin et al., 1994; Shan & Lee, 1994; Wu & Levine, 1994), reduced it. Therefore, it seems unlikely that the general inhibitory activity of N-terminal LANA1 on the replication of virus oriP-containing plasmid is due to the reduced DNA synthesis of transiently transfected 293T cells.

Cell-type specificity of dominant-negative effect of LANA1 and its derivatives on the virus replication systems

Cell lines such as 293T and COS-1 endogenously express SV40 large T antigen, thereby permitting the replication of SV40 oriP-containing plasmid. The transient replication assay of virus replication systems in those cell lines is obligatorily coupled to SV40 replication system if any SV40 oriP-containing plasmid is used. Since all mammalian expression vectors used in our study contain SV40 oriP, it is possible that SV40 replication system affects the outcome of transient replication assay in 293T cells and the general replication-inhibitory activity of the N-terminal LANA1 results from the reduced replication of SV40 oriP-containing plasmid by the N-terminal LANA1. To exclude this possibility, we performed similar transient replication assays in other cell lines that do not express SV40 large T antigen. These include human embryonic kidney cell line 293 (Fig. 5A) and human cervical cancer cell line C33A (Fig. 5B). In transiently transfected 293 cells, LANA1 and its derivatives showed similar effects on the transient replication of KSHV TR- and EBV oriP-containing plasmid as in 293T cells. The N-terminal LANA1 inhibited both virus replication systems, but the replication-inhibitory activity of C-terminal LANA1 was KSHV-specific (Fig. 5A, top and middle). In contrast, only the C-terminal LANA1 inhibited the replication of KSHV TR-containing plasmid, albeit weakly but reproducibly, and we could not observe any comparable replication-inhibitory activity of wild-type and the N-terminal LANA1 in transiently transfected C33A cells (Fig. 5B, top and middle).

Additionally, we tested the effect of LANA1 and its derivatives on the transient replication system of HPV, another member of Papovaviridae in those cell lines. HPV replication system, as in the case of other virus replication systems described above, consists of virus trans- and cis-acting elements. These correspond to the two early virus proteins, E1 and E2, of HPV and the binding sites for them within the virus genome (Chiang et al., 1992; Del Vecchio et al., 1992; Demeret et al., 1995; Lee et al., 1997; Ustav & Stenlund, 1991). However, in both cell lines, LANA1 and its derivatives did not show any effect on the highly amplifying replication system of HPV, compared to those of KSHV and EBV (Fig. 5A and B, bottom).

DISCUSSION

Using a transient replication assay, it has been shown that KSHV TR-containing plasmid replicates in several human cell lines expressing LANA1 (Garber et al., 2002; Hu et al., 2002; Lim et al., 2002). In this report, we further verified additional cell lines originated from different species. C33A (a human cervical cancer cell line) and COS-1 (a monkey kidney cell line), but not NIH/3T3 (a mouse embryo cell line), were permissive for the replication of KSHV TR-containing plasmid in the presence of LANA1 (Fig. 1). Interestingly, Krithivas et al. (2002) recently reported that LANA1 does not associate with mouse chromosomes in transiently transfected NIH/3T3 but the overexpression of human MeCP-2 and DEK, which interact with the N-terminal CBS and the C terminus of LANA1, respectively, can target LANA1 to mouse chromosomes. Since the CBS of LANA1 was necessary for the replication activity in our transient replication assay (Fig. 2), these results suggest that the association of LANA1 with host chromosome may be critical not only for the equal segregation of replicated virus genome during mitosis of infected cells but also for its replication activity, thereby restricting species-specificity for DNA replication. Consistent with our data, Shinozaka et al. (2002) recently reported that the deletion of the N-terminal 22 amino acids of LANA1 abolished the maintenance of oriP-containing plasmid in the long-term replication assay and H1, but not H2B, can be functionally substituted for CBS of LANA1.

From the facts that the C-terminal LANA1 alone can bind sequences within TR and repress the transcription from TR (Garber et al., 2001, 2002; Lim et al., 2002), but not facilitate the replication of TR-containing plasmid in the transient replication assay under our experimental condition (Lim et al., 2002), we reasoned that it can compete with the wild-type LANA1 for binding sites within TR, and act as a dominant-negative inhibitor of the replication of
Fig. 4. General inhibitory effect of N-terminal LANA1 on the virus replication systems in transiently transfected 293T cells. (A) Left panel, the transient replication of EBV oriP-containing plasmid, which is mediated by EBNA-1, is inhibited in 293T cells expressing the wild-type and N terminus of LANA1. 293T cells in 100 mm dishes were cotransfected with 2 μg of pOLP containing EBV oriP, 2 μg of pGL2-basic, 0.25 μg of FE encoding FLAG-tagged EBNA-1, and 8 μg of the indicated expression vector for FLAG-tagged LANA1 or LANA1-deletion mutants. Cells were split and harvested 36 h and 96 h after transfection, respectively. Hirt-extracted DNA was digested with SacI alone (left) or SacI/DpnI (right) and digested DNA was analysed similarly as in Fig. 1. Right panel, effect of FLAG-tagged LANA1 and LANA1-deletion mutants on the expression of FLAG-tagged EBNA-1 from FE, pcDNA3 derivative. 293T cells in 60 mm dishes were cotransfected with 2 μg of FE and 4 μg of mammalian expression vector indicated at the top, and harvested 36 h after transfection. Total lysates were analysed by Western blotting with anti-FLAG M2 monoclonal antibody. The protein size marker is depicted on the left. (B) Left panel, the transient replication of KSHV TR-containing plasmid is not affected by the overexpression of EBV EBNA-1 in transiently transfected 293T cells. 293T cells in 100 mm dishes were cotransfected with 2 μg of p4TR-luc, 2 μg of pGL2-basic, 0.25 μg of pcDNA3 LANA1, and the increasing amount (2 or 4 μg) of FLAG-tagged EBNA-1 expression vector. Cells were split and harvested 36 h and 96 h after transfection, respectively. Hirt-extracted DNA was analysed similarly as in Fig. 1. Right panel, the effect of FLAG-tagged EBNA-1 on the expression of wild-type LANA1 from pcDNA3. 293T cells in 60 mm dishes were cotransfected with 2 μg of pcDNA3 LANA1 and the increasing amount (2 or 4 μg) of FLAG-tagged EBNA-1 expression vector, and harvested 36 h after transfection. Total lysates were analysed by Western blotting with rabbit polyclonal anti-LANA1 serum and anti-FLAG M2 monoclonal antibody, respectively. Protein size marker is depicted on the left. (C) The transient replication of SV40 oriP-containing plasmid, which is mediated by SV40 large T antigen endogenously expressed in 293T cell, is inhibited by the wild-type and N terminus of LANA1. 293T cells in 100 mm dishes were cotransfected with 2 μg of pcDNA3 containing SV40 oriP, and 8 μg of the indicated mammalian expression vector. Cells were split and harvested 36 h and 96 h after transfection, respectively. Hirt-extracted DNA was digested with EcoRI alone (left) or EcoRI/DpnI (right) and digested DNA was analysed similarly as in Fig. 1, except that neomycin-resistant gene-specific probe was used in Southern blotting to detect linearized pcDNA3. (D) Determination of N-terminal region of LANA1 that is responsible for the replication-inhibitory activity. Transient replication assay of EBV oriP-containing plasmid in 293T cells was similarly performed as in (A), using expression vectors of the indicated LANA1-deletion mutants. (E) Effect of the transient overexpression of LANA1 and LANA1-deletion mutants on the cellular DNA synthesis. Transfected 293T cells were incubated with radioactive thymidine for 24 h. [3H]Thymidine incorporation assay was performed 36 h (white bar) or 96 h (black bar) after transfection as described in materials and methods. The results represent the mean of more than two independent experiments in duplicate, and standard deviations are depicted with error bars.
oriP-containing plasmid, although Hu et al. (2002) reported the partial replication activity of C-terminal LANA1. As expected, the C terminus of LANA1 inhibited the replication of TR-containing plasmid by LANA1, but not other virus replication systems including EBV, SV40 and HPV in other cell lines. However, unexpectedly, the N terminus of LANA1 also showed the inhibitory activity of KSHV, EBV and SV40 replication systems in transiently transfected 293 and 293T cells, but not in C33A cells. The mode of replication-inhibitory activities of these two LANA1-deletion mutants may be distinct. In the case of the C-terminal LANA1, competitive binding with the wild-type LANA1 for specific sequences within KSHV TR could be the explanation for the observed KSHV-specific inhibition of replication. It seems unlikely that cellular replication factors including origin recognition complexes, which are commonly targeted by both the C terminus of LANA1 and EBNA-1 (Chaudhuri et al., 2001; Dhar et al., 2001; Lim et al., 2002; Schepers et al., 2001), were limiting for virus replication because the overexpression of C-terminal LANA1 did not influence the replication of EBV oriP-containing plasmid by EBNA-1 which recruits origin recognition complexes to the virus origin of replication. In contrast, the N terminus of LANA1, which is required for the association with host chromosome and the replication activity, may squelch cellular replication factors and/or replication sites on host chromosomes where cellular replication factors are assembled for virus replication,
resulting in the inhibition of virus replication systems in human embryonic kidney cell lines. Since most of mammalian expression vectors used in our experiments contain SV40 oriP, the transient replication assay of other virus replication systems in 293T cells is obligatorily coupled to SV40 replication system. Therefore, it is also possible that the observed suppression of virus replication by the N-terminal LANA1 involves the effect of SV40 replication by large T antigen and arises from the reduced replication of expression vectors for trans-acting element of virus replication, theoretically resulting in the reduced expression of them. However, we could not observe any significant effect of the N-terminal LANA1 on the expression of wildtype LANA1 and EBNA-1 from transiently transfected expression vectors. In addition, the population of multifunctional LANA1, which actually takes part in the replication activity of virus genome may be small. This is inferred from two observations: (i) that the titration experiment in the transient replication assay showed that even a small amount of LANA1 expression vector achieved the maximum efficiency in TR-containing plasmid replication (Hu et al., 2002; Lim et al., 2002); (ii) a relatively low copy number of virus genome is maintained in latently infected cells, suggesting that the expression level of virus

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**Fig. 5.** Effect of LANA1 and its derivatives on the virus replication systems in cell lines that do not express SV40 large T antigen. (A) Top and Middle, transient replication assay of KSHV and EBV oriP-containing plasmid in 293 cells was performed similarly as in 293T cells (Fig. 3B and 4A). Bottom, for transient replication assay of HPV oriP-containing plasmid, 293 cells in 100 mm dishes were co-transfected with 2 μg of p105core-luc containing HPV oriP, 1 μg of EapCG encoding both HPV E1 and E2, and 8 μg of expression vectors for FLAG-tagged LANA1 and LANA1-deletion mutants. Cells were split and harvested 36 h and 96 h after transfection, respectively. Hirt-extracted DNA was digested with BamHI/DpnI and digested DNA was analysed similarly as in Fig. 1. (B) Top, transient replication assay of KSHV in C33A with the increasing amount (2 or 4 μg) of FLAG-tagged N- or C-terminal LANA1 expression vector was also similarly performed. Middle and Bottom, transient replication assay of EBV and HPV oriP-containing plasmid in C33A was similarly performed as in 293 cells.
trans-acting element may not significantly affect the replication efficiency of herpesvirus replication systems under our experimental condition. Finally, we could observe the replication-inhibitory activity of N-terminal LANA1 in transiently transfected 293 cells, which does not express SV40 large T antigen and support the replication of SV40 oriP-containing plasmid, excluding that possibility. Interestingly, we could not detect any transcriptional repression activity of N-terminal LANA1 using transient reporter assay in C33A cells (Lim et al., 2003), where the N-terminal LANA1 did not also show the replication-inhibitory activity, suggesting the possible link between them.

While performing transient replication assays, we observed that the replication efficiency of virus oriP-containing plasmid showed a certain degree of variation in different experiments. There could be several reasons for the fluctuations in the replication efficiency. First, the different batch of serum and passage numbers of cells used in the transient replication assay may affect the cellular context such as the rate of cell cycle and the signal pathways up- or down-regulating the replication of virus oriP-containing plasmid. It is noteworthy that the overexpression of the catalytic subunit of protein kinase A or the treatment of phorbol 12-myristate 13-acetate during the transient replication assay negatively regulated the replication of KSHV TR-containing plasmid, supporting this possibility (data not shown). Secondly, the transiently transfected plasmid could be differentially chromatinized in separate transfections, which may lead to variations in the accessibility and process of cellular replication machinery on the virus oriP. Finally, since the copy number of herpesvirus genomes maintained by infected cells is limited by some unknown mechanism, it is possible that the initial amount of virus oriP-containing plasmid transfected into cells may determine its replication efficiency in the transient replication assay.

Because of its functional importance, we were interested in cellular proteins targeted by the CBS of LANA1. We found several candidate proteins that interact with CBS of LANA1 by proteomics approach. Currently, their functional roles on the chromosome association and replication activity of LANA1 are under investigation.

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REFERENCES


