Development of stealth transgenes for gene therapy : evaluation of cis-acting inhibitors of antigen presentation

Raamsman-Ossevoort, M.

Chapter 5

In-Cis Inhibition of Antigen Processing by the Latency-Associated Nuclear Antigen I of Kaposi Sarcoma Herpes Virus

Zaldumbide, A., Ossevoort, M., Toes, R.E.M., Hoeben, R.C.

In Cis Inhibition of Antigen Processing by the Latency-Associated Nuclear Antigen I of Kaposi Sarcoma Herpes Virus.

Arnaud Zaldumbide[1], Martine Ossevoort[1], Rene E.M. Toes[2,3], Rob C. Hoeben[1,*]

[1]Depts of Molecular Cell Biology, [2]Immunohematology and Blood Bank, [3]Rheumatology, Leiden University Medical Center, P.O. Box 9503 2300 RA, Leiden, The Netherlands.

*Corresponding author: Prof. Dr. Rob C.Hoeben
Mailing address:
Department of Molecular Cell Biology
Leiden University Medical Center
Wassenaarseweg 72
2333 AL Leiden
The Netherlands.
Phone: +31 71 5276119.
Fax: +31 71 527 6284
E-mail: R.C.Hoeben@lumc.nl.

Abstract

Kaposi Sarcoma Herpes Virus (KSHV), also known as Human Herpes Virus 8 (HHV8), can persist as an episome in target cells. The Latency-Associated Nuclear Antigen 1 (LANA-1) is a key component of the latency process, and may be a functional equivalent of the EBNA-1 protein of Epstein-Barr Virus. EBNA-1 can subdue immune recognition by virtue of a long glycine and alanine-rich repeat, which interferes with the proteasomal degradation of EBNA-1 and in this way averts the presentation of antigenic peptides. LANA-1 contains a strongly acidic repeat region of approximately 580 amino acids, which consists almost exclusively of aspartic-acid, glutamine, and glutamic-acid residues. Despite the LANA-1 repeat is not homologous to the EBNA-1 Gly-Ala-rich repeat, we demonstrate that this acidic region interferes with antigen processing *in-cis*. Upon transfection of expression vectors containing LANA-1-eGFP fusion genes the cells did not present an ovalbumin-derived $H2K^b$-restricted CTL epitope inserted at the C-terminus of the fusion protein. Deletion of the central acidic-repeat region of LANA-1 abolished the capacity of LANA-1 to block antigen presentation. Similar to the EBNA-1-derived Gly-Ala-rich repeat, the LANA-1 repeat does not inhibit presentation *in trans:* co-transfection of LANA-1 expression vectors does not inhibit presentation of the OVA epitope from the GFP_{ova} fusion protein. These data demonstrate that the acidic repeat region of LANA-1 can function as *in-cis* acting inhibitor of antigen presentation. This may contribute to the immune evasion of cells latently infected by KSHV.

Introduction

Kaposi Sarcoma Herpes Virus (KSHV) also called Human herpes virus 8 (HHV8), is a gamma herpes virus associated with AIDS-related lymphoproliferative disorders [20,25]. Like other herpes viruses KSHV can persist for life in a latent form in infected cells [5,29,33].

One of the most studied proteins implicated in herpes virus latency is the Epstein-Barr Virus (EBV) Nuclear Antigen 1 (EBNA-1). EBNA-1 binds to the viral origin of replication and to metaphase chromosomes [14 17,30,37], thus allowing EBV episomal maintenance within the infected cell and equal partitioning to the daughter cells. EBNA-1 is the only viral protein present in all EBV-associated tumours. By a mechanism not totally understood yet, the Gly-Ala repeat can interfere with the proteasomal degradation and prevent cytotoxic T lymphocyte epitope generation [15,16,21]. The current model explaining this phenomenon dictates that a long stretch of alanines interspaced by 1, 2 or 3 glycines would give to EBNA-1 the appropriate conformation to interact with a proteasomal component that contain a hydrophobic pocket [27]. Although this 30 to 200 GA repeat (depending on virus isolates) confers a benefit mechanism

for the virus, it appears that this sequence has not been evolutionary conserved, since none of the other herpes virus proteins contain a homologous repeat.

Like EBV, KSHV infects human B cells, macrophages, endothelial cells, and epithelial cells. The infected cells are not eradicated from host cells by the immune system, suggesting that KSHV developed a strategy to evade the immune system [5,12,13,24]. During latency, KSHV persists as a multicopy circular episomal DNA in the nucleus, and here it expresses a small subset of viral genes. One of the proteins encoded by these genes is the Latency-Associated Nuclear Antigen 1, LANA-1 (also called LNA or LNA-1), the EBNA-1 homologue in KSHV [1]. LANA-1 is a large multi-functional nuclear protein of 1162 amino acids, expressed from ORF73, and is involved in numerous cellular processes [9]. It has been reported to improve dissemination of KSHV by modulating expression of oncosuppressor proteins [6,8,23]. The central region of LANA-1 is occupied by a long acidic sequence that can be arbitrary divided in 3 parts: aspartic acid/glutamic acid (DE), glutamine/glutamic acid (QE), and aspartic acid/glutamin (DQ) [2] repeats. The function of the central domain is not well described yet, but seems to be required for the activation of the latent EBV promoter Cp[34]. LANA-1 contains two nuclear localisation signals (nls), one located in the N-terminal part (aa 24 to aa 30) [22] and one in the C-terminal part [26]. LANA-1 is associated with heterochromatin during interphase and with chromosomes during mitosis [19,31].

Here we demonstrate that the Latency-Associated Nuclear Antigen-1 of KSHV can inhibit the presentation of antigenic peptides. In this respect it resembles latency-associated proteins in EBV and MHV68 [1]. The mechanism involved is not clear but it is evident that the long central acidic region of LANA-1 is required for this inhibition.

Results

To examine the effect of LANA-1 on antigen presentation in vitro, we generated a Green fluorescent protein gene that carries the codons for a heterologous CTL epitope (Fig.1A). The resulting protein GFP_{ova} can be used to monitor the antigen presentation with the well-characterized B3Z CTL hybridoma cell line [28]. Briefly, B3Z can recognize Ova peptide (SIINKFEL) when the peptide is presented in the context of $H2K^b$ MHC I. Recognition induces expression of the IL-2 gene, which can be monitored by the induction of an heterologous IL-2 promoter linked to the E.coli *lacZ* gene. Responsiveness of B3Z to GFP/GFP_{ova} transfected cells, evaluated by LacZ quantification, shows 8 fold increase in $H2K^b$ context, whereas no induction is observed without the appropriate MHC I molecules (Fig.1B), demonstrating the specificity of the assay.

To assess whether LANA-1 or EBNA-1 can affect this presentation, $H2K^b$ 293T cells were co-transfected with GFP_{ova} and increasing amounts (0, 0.05, 0.1, 0.2, 0.8 µg DNA) of LANA-

,

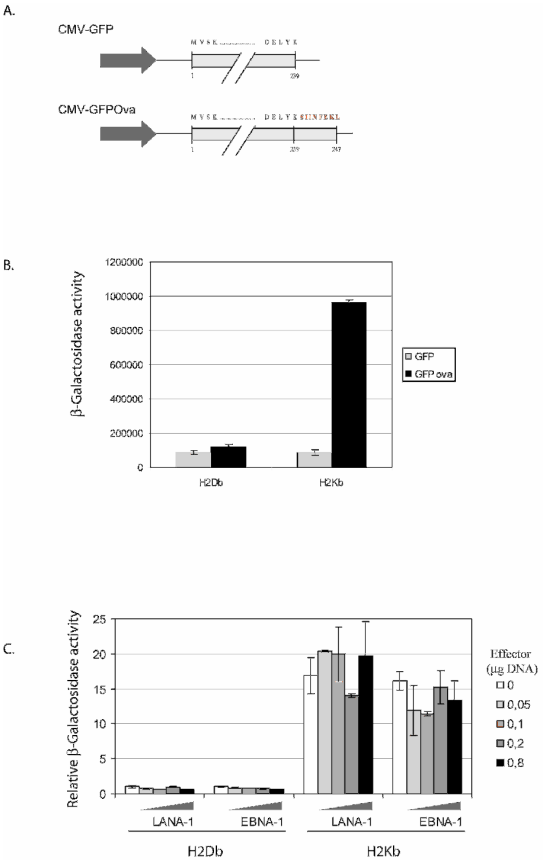

Figure 1
EBNA-1 and LANA-1 have no *in-trans* acting inhibitory effect on OVA presentation. (A) Schematic diagram of GFP and GFP_{ova} showing the insertion of the octameric antigenic peptide (OVA) SIINFEKL. (B) B3Z assay on OVA expressing cells. 293T cells were transfected with 0.8 µg of pRRL-CMV-GFP/GFP_{ova} and 0.2 µg MHC I encoding plasmid (pcDNA $H2K^b$/$H2D^b$). β-galactosidase activity was measured 24h after co-culture (48h post transfection). (C) Trans-inhibition B3Z assay. 293T cells were cotransfected like previously (1B) with increasing amounts of pCMV-EBNA-1 or pcDNA3.1-LANA-1. Point "0" $H2D^b$ is used to normalized *LacZ* activity and arbitrary set to 1.

1 or EBNA-1 expressing vector. In the negative control (GFP_{ova} only), we observed, like previously, a strong induction of β-galactosidase activity in the presence of $H2K^b$ indicating that the Ova epitope is efficiently recognized by the B3Z hybridomas (Fig.1C). As expected, EBNA-1 does not affect the antigen presentation *in-trans*, as is evident from the absence of significant differences in *LacZ* expression upon EBNA-1 co-transfection (p=0.0901 compared to mock-transfected cells at 0.8 µg DNA). Also LANA-1 did not affect antigen presentation *in-trans* in this assay (p=0.2702).

To evaluate whether LANA-1 could inhibit antigen presentation *in-cis* we generated new GFP_{ova} fusion proteins (Fig.2A). We and others have shown previously that the Gly-Ala repeat of EBNA-1 can interfere with the antigen processing [21]. We fused the N-terminal part of EBNA-1 to GFP_{ova}, generating the EBNA-GFP_{ova} to serve as a positive control in our experiments. A similar construct was generated in which the Gly-Ala repeat of EBNA-1 was replaced by LANA-1, resulting in LANA-GFP_{ova}. From the latter clone a deletion construct was made from which the codons for the central acidic region, i.e. aa 360 to aa 911, were

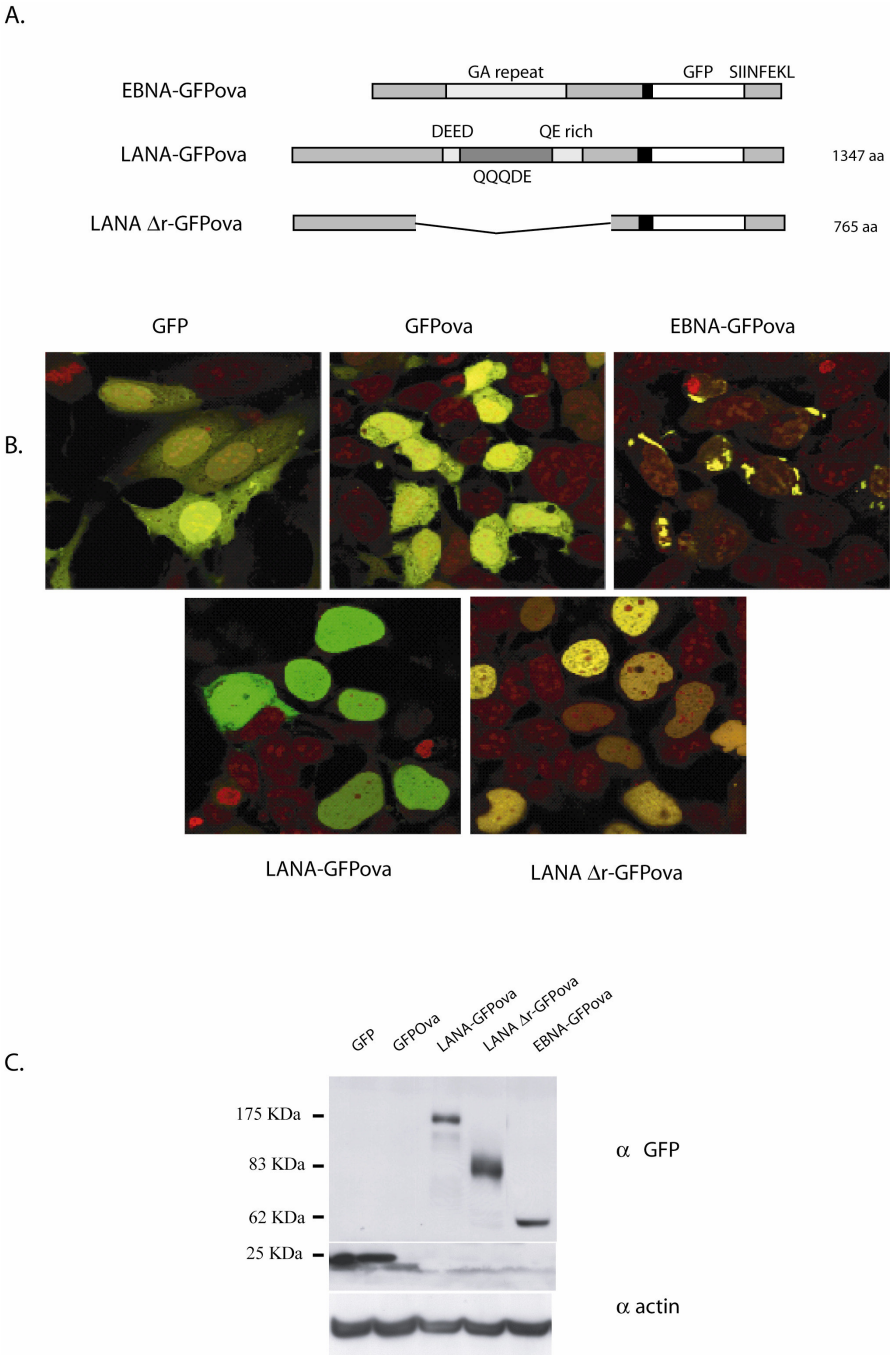

Figure 2

GFP fusion proteins. (A) Schematic diagram of pRRL-CMV-EBNA-GFP$_{ova}$, pRRL CMV-LANA-GFP$_{ova}$ and pRRL-CMV-LANAΔr-GFP$_{ova}$. (B) Cellular localization of modified GFP. Confocal microscopy pictures taken 60h after transfection in 911 cells. (C) Western blot analysis of protein extracts from 911 transfected cells expressing GFP / GFP$_{ova}$ / LANA-GFP$_{ova}$ / LANAΔr-GFP$_{ova}$ / EBNA-GFP$_{ova}$. Anti-GFP antibody is used for the detection, and loading control is provided by anti-Actin.

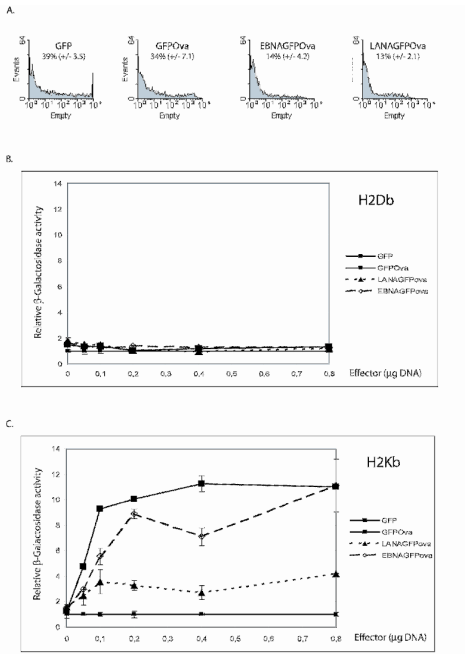

Figure 3
LANA acts as an *in-cis*-inhibitor of antigen processing. (A) FACS analysis of 293T transfected cells. 15h after transfection with 0.8 µg of GFP / GFP_{ova} / EBNA-GFP_{ova} / LANA-GFP_{ova} expressing vector, percentage of GFP positive cells is determined in order to evaluate the amount of target cells. Ratio 1:2 is used between GFP positive cells and B3Z hybridomas. (B)&(C) *LacZ* activity expressed from the B3Z CTL after co-culture with 293T transfected cells. GFP is used to normalized *LacZ* activity and arbitrary set to 1.

removed (Fig. 2A). In the LANA-1 fusion genes the codons for the nls sequences have been retained leading to predominant nuclear localization of the fusion proteins (Fig. 2B). EBNA-GFP_{ova} is present as the specific stippled perinuclear pattern described before. We checked the expression level of each fusion protein by western analysis. All constructs (GFP, GFP_{ova}, LANA-GFP_{ova}, LANAΔr-GFP_{ova}, EBNA-GFP_{ova}) are expressed at equal levels and the fusion proteins migrate at the position consistent with their expected molecular weights of 27, 28, 170, 85, and 64 kDa, respectively (Fig.2C).

To evaluate the ability of LANA-1 to inhibit the ova-peptide presentation *in-cis*, we co-transfected 293T cells with increasing amounts (0, 0.05, 0.1, 0.2, 0.4, 0.8 µg DNA) of the expression vectors for GFP, GFP_{ova}, EBNA-GFP_{ova}, and LANA-GFP_{ova} and either $H2K^b$ or $H2D^b$ MHC I expression vectors. The percentages of target cells expressing the GFP protein was determined by FACS analysis (Fig.3A). A ratio 1:2 between GFP-positive cells and B3Z hybridomas was used. Sixteen hours post transfection, no significant differences were noted either in percentage of GFP positive cells or in mean fluorescence intensity (MFI) (data not shown) between the different constructs. In the presence of $H2K^b$, we observed a strong response of B3Z against the GFP_{ova} expressing cells. The activation is specific since no *LacZ* activity was detected in the $H2D^b$ context or in the GFP expressing cells. Furthermore, the dose-response curve reaches a plateau already at 0.1 µg DNA with a maximal induction comprised between 8 and 12 times compared to the background signal (i.e. GFP without the OVA epitope). When LANA-1 is fused to GFP_{ova} we found a significant (60%-80%)

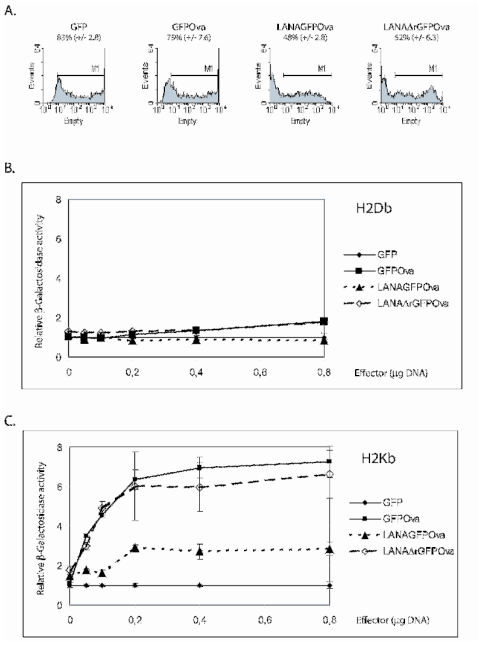

Figure 4
The acidic region from LANA-1 is required for a maximal inhibitory effect. (A) FACS analysis of 293T transfected cells 30h after transfection. (B) & (C) β−galactosidase activity. Similar experiment as described in figure 3 with 293T cells transfected with GFP / GFP_{ova} / LANA-GFP_{ova} / LANAΔr-GFP_{ova} expressing vectors

inhibition of *LacZ* activity (Fig.3C). Surprisingly, we noticed that the CTL were able to recognize the EBNA-GFP_{ova} expressing cells (5 to 10-fold *LacZ* induction and no statistical differences were observed at 0.8 µg between GFP_{ova} and EBNA-GFP_{ova} expressing cells).

Similar assays were conducted to further examine this inhibitory effect of LANA-1. 293T cells were co-transfected with increasing amounts of GFP, GFP_{ova}, LANA-GFP_{ova} or LANAΔr-GFP_{ova} expression vectors and mixed with B3Z cells (Fig 4). Again B3Z cells did not recognize the Ova epitope in $H2D^b$ context, and LANA-1 can inhibit the ova presentation (more than 60% of inhibition compared to GFP_{ova} transfected cells with 0.1 and 0.8 µg DNA) (Fig.4B). Interestingly, Fig 4C shows that after deletion of the central region, LANA-1 loses its capacity to inhibit the response of the hybridoma against OVA. Indeed, the difference between GFP_{ova} and LANAΔr-GFP_{ova} transfected cells is not significantly different ($p = 0.0772, 0.2745, 0.8154, 0.4121$, and 0.6345, respectively, at 0.05, 0.1, 0.2, 0.4, and 0.8 µg DNA).

Taken together these results suggest that LANA-1 from the KSHV can function as an *in-cis* acting inhibitor of antigen processing. This activity is dependent on the presence of the large central acidic-repeat region.

Discussion

In KSHV LANA-1 is one of the subset proteins expressed during the latency phase. Also, it is expressed at high level in all Kaposi sarcoma (KS) associated malignancies [38] [39]. Here we demonstrate that LANA-1 can prevent the presentation of linked antigens by MHC I. This may facilitate LANA-1 to prevent eradication of LANA-1 producing cells by the cellular immune system. So far, only the K3 and K5 gene products of KSHV were know to be involved in immune evasion[7]. The K3 and K5 gene products block the endocytosis of MHC I molecules[3] and the down-regulate accessory proteins involved in T-cell stimulation[4]. The fact that LANA-GFP$_{ova}$ can escape recognition by the OVA-peptide specific B3Z hybridoma suggests that LANA-1 is immunologically invisible. The central role of LANA-1 in latency and long-persistence of KSHV genome in mammalian cells, is supported by LANA disruption studies after transposon-based mutagenesis on a KSHV BAC[38].

Functional similarities between the latency-associated proteins of EBV (EBNA-1) and MHV (ORF73), demonstrate that KSHV kept a similar but still unknown mechanism to control latency. However, it appears that the presence of Gly-Ala repeat is not a prerequisite to facilitate evasion of the immune system during latency since MHV-68 ORF73 lacks the long alanine stretch. Although surprisingly, our results indicate that the Gly-Ala repeat, in this system, is unable to protect the OVA presentation, these results are consistent with IFNγ release assay performed with EBNA1-GFP or EBNA1-GAr-del-GFP[35] (GAr deleted form).

Despite the controversy around this inhibitory effect[32] [36], we and others, have already demonstrated, under certain conditions, that the Gly-Ala repeat can be very efficient as well[21], suggesting that the effectiveness of immune evasion is transgene and context-dependent. Another hypothesis we cannot exclude is the proteolysis of our fusion protein that then generates unprotected OVA epitopes. Interestingly, it seems that in addition to the 80kDa band observed on the western-blot, EBNA-GFP$_{ova}$ can generate a smaller product (around 30kDa) that could carry the OVA epitope but would not be linked to the protective Gly-Ala repeat (data not shown).

Even if the proteasome plays a central role in class I antigen generation, more than 70% of the antigenic peptides secreted comes from Defective Ribosomal Products (DRiPs) constituting the primary source of CD8$^+$T cell peptides [39]. This could indicate that the inhibition in the case of LANA-1 could also rely on a more accurate control (quantitative or qualitative) of the protein synthesis –described before for EBNA-1[40]. On the other hand, some particular characteristics previously reported, as a possible posttranslational modification like sumoylation[18,34], that could interfere with antigen generation by stabilizing LANA-1, or the specific charge structure of the protein (basic N and C-terminal ends and acidic central region) [18]could be some other pathways to investigate.

Remarquably, it is interesting to note that even if EBV is so far the only characterized gamma herpes virus that kept a GA repeat during the evolution, a frame shift on this repeat

can generate a new repeat that presents more than 65% of homology with the central region of LANA-1 (Fig.5). These results combined to the one recently described by *Bennett et al*, on ORF73 from MHV68 [1] showed that these viruses developed different strategies aiming at a similar goal, the escape of the immune system.

A last attracting prospect for this work, besides the fundamental understanding of Herpes virus actions, is the possible utilization of such a sequence in gene therapy approaches. One of the major obstacles in gene therapy is T cell mediated destruction of transgene expressing cells. Until now one of the potential candidates that can be used to stealth proteins for the immune system is the Gly-Ala repeat of EBNA-1. Unfortunately, as mentioned previously, recent studies on EBNA-1 reveal its weakness, so it becomes more and more important to find new inhibitory sequences of antigen processing in order to develop "stealth-gene library".

In this perspective LANA-1 can be an alternative to EBNA-1 as *in-cis*-inhibitor of antigen processing since these two proteins does not have any effect *in-trans* on the OVA presentation.

Materials and Methods

Constructs

To generate GFP_{ova} expressing vector, we introduced the Ova epitope (SIINFEKL) by mutagenic PCR (stratagene Kit) on pRRL-CMV-GFP vector using following primers:

Forward: 5'-ACGAGCTGTACAAGAGCATAATTAATTTCGAAAAGCTCTAAGCGGCCGCGTC-3'

Reverse: 5'-GACGCGGCCGCTTTAGAGCTTTTCGAAATTAATTATGCTCTTGTACAGCTCGTC-3'

To check the *in-cis*-inhibitory effect of EBNA-1 and LANA-1 repeats, we fused respectively amino acids 1 to 430 and 1 to 1082 to GFP_{ova}.

pRRL-CMV-EBNA-GFP_{ova} has been generated using a EBNA fragment from pLXRN-EBNA-Luciferase (previously described [21]) in pRRL-CMV-GFP_{ova}.

pRRL-LANA-GFP_{ova} has been generated replacing a SalI/SalI EBNA fragment from pRRL-CMV-EBNA-GFP_{ova} by a HindIII/XcmI fragment from pCDNA3.1 myc-LANA-1 (gift from Kenneth Kaye), creating pRRL-CMV-LANA-GFP_{ova}.

pRRL-CMV-LANAΔr-GFP_{ova} construct was generated by deleting the central region by mutagenic PCR using following primers:

Forward: 5'-GATGACAATGACAATAAGGATATCTTAGAGGAGGTGGAAGAG-3'

Reverse: 5'-CTCTTCCACCTCCTCTAAGATATCCTTATTGTCATTGTCATC-3'

All the fusions were checked by complete sequencing and immunoblot (Fig 2B).

```
Smith-Waterman score: 725;  42.424% identity (66.391% similar) in 363 aa overlap (634-
989:3-351)

           610       620       630       640       650       660
LANA   EPQQREPQQREPQQREPQQREPQQQDEQQQDEQQQDEQQQDEQQQDEQQQDEQQQDEQQQ
                                    .. ..::... :.:. .  ::. :. :. .
GZ                                MDEDGEEDEDEEAEDQEPRAAQDQGQDIEMVS
                                          10        20        30

           670       680       690       700       710       720
LANA   DEQQQDEQQQDEQQQDEQQQDEQQQDEQQQDEQQQDE-QQQDEQQQDEQQQDEQQQDEQQ
        . .. .     .    .:..::... :.:. :.:.: :.:.: :. .. :. :.:.: :
GZ     GDPKNVQVALAAKGPTVEQEQEQEREGQEQEGQEQEEGQEQEEGQEGQEGQEGQEQEEGQ
              40        50        60        70        80        90

           730       740       750       760       770       780
LANA   QDEQQQDEQEQQDEQEQQDEQEQQDEQQQDEQQQQDEQQQQDEQQQQDEQQQQDEQQQQD
       ..:. :. :: :...: :...: :. :.:.: :. .: :.:.: :.:.: :.   :.:..
GZ     EQEEGQEGQEGQEQEEGQEQEEGQEGQEQEEGQEGQEGQEQEEGQEQEEGQEG--QEQEE
            100       110       120       130       140         150

           790       800       810       820       830       840
LANA   EQEQQEEQEQQEEQEQELEEQEQELEDQEQELEEQEQELEEQE-QELEEQEQELEEQEQE
        :: :: :::.: :::: .: .   : :::: .:::.  : :: :: : :::  : ::::
GZ     GQEGQEGQEQEEGQEQEGQEGQ---EGQEQEGQEQEEGQEGQEGQEQEGQEQ--EGQEQE
              160       170          180       190         200

              850       860       870       880       890
LANA   LEEQE-QELEEQEQELEEQEQELEE-QEQELEEQEVEEQEQEVEEQEQE-QEEQELEEVE
        .::: :: .::: . : :::: .: :::: : :: .::::.  : :::: :: :: :  :
GZ     GQEQEGQEGQEQEGQ-EGQEQEGQEGQEQE-EGQEGQEQEEGQEGQEQEGQEGQEQEGQE
         210       220       230       240       250       260

      900       910       920       930       940       950
LANA   EQEQEQEEQEEQELEEVEEQEEQELEE-VEEQEEQELEEVEEQEQQELEEVEEQEQQGVE
        :::: .: .:::  . .: .::::.:  :::  :  .::: :   .:.  ::    . :.
GZ     GQEQEGQEGQEQEEGQEQEGQEQEVEAGVEEAVEAGVEEVVEAGVEEV--VEAAGVEDVK
           270       280       290       300       310         320

      960       970       980       990      1000      1010
LANA   QQEQETVEEPIILHGSSSEDEME-VDYPVVSTHEQIASSPPGDNTPDDDPQPGPSREYRY
       .   :.:.::    :   . : .    :::: :
GZ     EPGGEVVKEP---GGEVVDVEKRGPGVPVVSHHHPGLHRAGPLQVEGHFSTL
             330         340       350       360       370

      1020      1030      1040      1050      1060      1070
LANA   VLRTSPPHRPGVRMRRVPVTHPKKPHPRYQQPPVPYRQIDDCPAKARPQHIFYRRFLGKD
```

Figure 5

Amino acid alignment of LANA-1 and EBNA-1 frame shift. The EBNA-1 messenger contains a large nested open reading frame. Translation starting at an alternative initiator AUG downstream of the canonical initiation codon would yield an acidic protein (GZ) that presents 42% identity and 66% homology with LANA-1 protein according to SSearch analysis software.

Cell lines

293T, U2OS, and 911 were grown in high glucose DMEM supplemented with 10% fetal bovine serum (Gibco BRL) and penicillin/Streptomycin, supplemented with 8% (vol/vol) fetal bovine serum, 100 IU of penicillin per ml, 100 µg of streptomycin per ml and 0.2% glucose

The B3Z indicator cells[11] (Kindly provided by R.E.M. Toes) were cultured in IMDM (Gibco) containing 8% (vol/vol) fetal bovine serum, 100 IU of penicillin per ml, 100 µg of streptomycin per ml, 28 µM β-mercaptoethanol and 500 µg hygromycin B per ml.

All cells were maintained at 37 C in a humidified atmosphere of 5% CO2 in air.

Transfection and FACS analysis

293T, U2OS and 911 cells were transfected at 70% confluency using the Calcium Phosphate co-precipitation technique[10]. Transfection in 6-well dishes and 24-well dishes are respectively performed with a total of 5µg and 2µg DNA/well.

24h or 48h after transfection as indicated, cells were resuspended in PBS-/- and the percentage of GFP positive cells was measured by FACS analysis (FACScan Becton-Dickinson). GFP fluorescence was detected using a 530/30 nm bandpass filter (FL1 channel) following excitation with an argon ion laser source at 488 nm. Using a forward-scatter/side-scatter representation of events, a region was defined to exclude cellular debris from the analysis. A number of events/FL1 (which reflects the fluorescence intensity) histogram was then established according to this region, and percentages of GFP-positive cells were determined in comparison to the negative control (untreated cells). Data analysis was performed using CellQuest 3.1 software (Becton–Dickinson). For each sample, 10,000 events were collected.

Localization study

911 cells were cultured in 6 well-plates on coverslips. 48h after transfection, cells were fixed with paraformaldehyde (PFA) 2% Tween-20 0.5% and washed 3 times with PBS/tween 0.05%. Nuclear staining was performed using Propidium Iodide solution for 15 min. Slides were then analysed by Confocal Laser Scan Microscopy (CLSM, Leica DM-IRBE).

Western blot

Cells were treated with RIPA lysis buffer (50mM Tris pH 7.5, 150mM NaCl, 0.1% SDS, 0.5% DOC, 1% NP40 + Protease Inhibitors). Proteins were quantified using BCA kit and 50 µg protein was loaded. The samples were analysed on 15% (GFP/GFP_{ova}) or 8% (EBNA-GFP_{ova}/LANA-GFP_{ova}) polyacrylamide-SDS. Proteins were transferred to Immobilon-P (Immobilon-P transfer membrane (PVDF); Millipore, Etten-Leur, The Netherlands) and treated with anti-GFP (1:1000), and anti-Actin (1:5000, clone C4; ICN Biomedicals, Inc., Zoetemeer, The Netherlands). After incubation with a peroxidase-conjugated goat-anti-mouse

secondary antibody, the proteins were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Roosendaal, The Netherlands). All antibodies were diluted in TBST (0.2% Tween 20, 150mM NaCl, and 10mM Tris) with 5% nonfat dried milk (Protifar Plus; Nutricia BV, Zoetermeer, The Netherlands).

B3Z assay

Determination of Ova presentation was determined as previously described [28]. Briefly, 293T transfected cells were exposed to B3Z CTL. After o/n coculture plates were centrifuged 5 min at 1500 rpm, and lysed in luciferase Lysis Buffer (125 mM Tris-phosphate, pH 7.8, 10 mM CDTA, 10 mM DTT, 50% glycerol, 5% Triton X-100).

β-Galactosidase activity was determined by luminometry as well (Lumat LB9501 luminometer (Berthold, Wildbad, Germany)) using galactolight dual light kit (Tropix).

Each experiment was performed in duplicate and repeated at least 3 times. Statistical analyses were realized using unpaired T test on graphpad.com.

Reference List

1. **Bennett, N. J., J. S. May, and P. G. Stevenson**. 2005. Gamma-herpesvirus latency requires T cell evasion during episome maintenance. Plos Biology **3**:638-649.

2. **Borah, S., S. C. Verma, and E. S. Robertson**. 2004. ORF73 of herpesvirus saimiri, a viral homolog of Kaposi's sarcoma-associated herpesvirus, modulates the two cellular tumor suppressor proteins p53 and pRb. Journal of Virology **78**:10336-10347.

3. **Coscoy, L. and D. Ganem**. 2000. Kaposi's sarcoma-associated herpesvirus encodes two proteins that block cell surface display of MHC class I chains by enhancing their endocytosis. Proc. Natl. Acad. Sci. U. S. A **97**:8051-8056.

4. **Coscoy, L. and D. Ganem**. 2001. A viral protein that selectively downregulates ICAM-1 and B7-2 and modulates T cell costimulation. J. Clin. Invest **107**:1599-1606.

5. **Cotter, M. A. and E. S. Robertson**. 2002. Molecular biology of Kaposi's sarcoma-associated herpesvirus. Frontiers in Bioscience **7**:D358-D375.

6. **Denis, G. V., C. Vaziri, N. Guo, and D. V. Faller**. 2000. RING3 kinase transactivates promoters of cell cycle regulatory genes through E2F. Cell Growth Differ. **11**:417-424.

7. **Edelman, D. C.** 2005. Human herpesvirus 8 - A novel human pathogen. Virol. J. **2**:78.

8. **Friborg, J., W. P. Kong, M. O. Hottiger, and G. J. Nabel**. 1999. p53 inhibition by the LANA protein of KSHV protects against cell death. Nature **402**:889-894.

9. **Garber, A. C., J. H. Hu, and R. Renne**. 2002. Latency-associated nuclear antigen (LANA) cooperatively binds to two sites within the terminal repeat, and both sites contribute to the ability of LANA to suppress transcription and to facilitate DNA replication. Journal of Biological Chemistry **277**:27401-27411.

10. **Graham, F. L. and A. J. van der Eb**. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456-467.

11. **Karttunen, J., S. Sanderson, and N. Shastri**. 1992. Detection of rare antigen-presenting cells by the lacZ T-cell activation assay suggests an expression cloning strategy for T-cell antigens. Proc. Natl. Acad. Sci. U. S. A **89**:6020-6024.

12. **Lan, K., D. A. Kuppers, S. C. Verma, and E. S. Robertson**. 2004. Kaposi's sarcoma-associated herpesvirus-encoded latency-associated nuclear antigen inhibits lytic replication by targeting Rta: a potential mechanism for virus-mediated control of latency. Journal of Virology **78**:6585-6594.

13. **Lan, K., D. A. Kuppers, S. C. Verma, N. Sharma, M. Murakami, and E. S. Robertson**. 2005. Induction of Kaposi's sarcoma-associated herpesvirus latency-associated nuclear antigen by the lytic transactivator RTA: A novel mechanism for establishment of latency. Journal of Virology **79**:7453-7465.

14. **Leight, E. R. and B. Sugden**. 2000. EBNA-1: a protein pivotal to latent infection by Epstein-Barr virus. Reviews in Medical Virology **10**:83-100.

15. **Levitskaya, J., M. Coram, V. Levitsky, S. Imreh, P. M. Steigerwaldmullen, G. Klein, M. G. Kurilla, and M. G. Masucci**. 1995. Inhibition of Antigen-Processing by the Internal Repeat Region of the Epstein-Barr-Virus Nuclear Antigen-1. Nature **375**:685-688.

16. **Levitskaya, J., A. Sharipo, A. Leonchiks, A. Ciechanover, and M. G. Masucci**. 1997. Inhibition of ubiquitin/proteasome-dependent protein degradation by the Gly-Ala repeat domain of the Epstein-Barr virus nuclear antigen 1. Proceedings of the National Academy of Sciences of the United States of America **94**:12616-12621.

17. **Marechal, V., A. Dehee, R. Chikhi-Brachet, T. Piolot, M. Coppey-Moisan, and J. C. Nicolas**. 1999. Mapping EBNA-1 domains involved in binding to metaphase chromosomes. Journal of Virology **73**:4385-4392.

18. **Moore, P. S. and Y. Chang**. 2001. Molecular virology of Kaposi's sarcoma-associated herpesvirus. Philosophical Transactions of the Royal Society of London Series B-Biological Sciences **356**:499-516.

19. **Moorman, N. J., D. O. Willer, and S. H. Speck**. 2003. The gammaherpesvirus 68 latency-associated nuclear antigen homolog is critical for the establishment of splenic latency. Journal of Virology **77**:10295-10303.

20. **Oksenhendler, E., E. Boulanger, L. Galicier, M. Q. Du, N. Dupin, T. C. Diss, R. Hamoudi, M. T. Daniel, F. Agbalika, C. Boshoff, J. P. Clauvel, P. G. Isaacson, and V. Meignin**. 2002. High incidence of Kaposi sarcoma-associated herpesvirus-related non-Hodgkin lymphoma in patients with HIV infection and multicentric Castleman disease. Blood **99**:2331-2336.

21. **Ossevoort, M., B. M. J. Visser, D. J. M. van den Wollenberg, E. I. H. van der Voort, R. Offringa, C. J. M. Melief, R. E. M. Toes, and R. C. Hoeben**. 2003. Creation of immune 'stealth' genes for gene therapy through fusion with the Gly-Ala repeat of EBNA-1. Gene Therapy **10**:2020-2028.

22. **Piolot, T., M. Tramier, M. Coppey, J. C. Nicolas, and V. Marechal**. 2001. Close but distinct regions of human herpesvirus 8 latency-associated nuclear antigen 1 are responsible for nuclear targeting and binding to human mitotic chromosomes. J. Virol. **75**:3948-3959.

23. **Radkov, S. A., P. Kellam, and C. Boshoff**. 2000. The latent nuclear antigen of Kaposi sarcoma-associated herpesvirus targets the retinoblastoma-E2F pathway and with the oncogene Hras transforms primary rat cells. Nat. Med. **6**:1121-1127.

24. **Rajcani, J. and M. Kudelova**. 2003. Gamma herpesviruses: Pathogenesis of infection and cell signaling. Folia Microbiologica **48**:291-318.

25. **Schulz, T. F.** 2001. KSHV/HHV8-associated lymphoproliferations in the AIDS setting. European Journal of Cancer **37**:1217-1226.

26. **Schwam, D. R., R. L. Luciano, S. S. Mahajan, L. Wong, and A. C. Wilson**. 2000. Carboxy terminus of human herpesvirus 8 latency-associated nuclear antigen mediates dimerization, transcriptional repression, and targeting to nuclear bodies. J. Virol. **74**:8532-8540.

27. **Sharipo, A., M. Imreh, A. Leonchiks, C. I. Branden, and M. G. Masucci**. 2001. cis-inhibition of proteasomal degradation by viral repeats: impact of length and amino acid composition. Febs Letters **499**:137-142.

28. **Shastri, N. and F. Gonzalez**. 1993. Endogenous generation and presentation of the ovalbumin peptide/Kb complex to T cells. J. Immunol. **150**:2724-2736.

29. **Spear, P. G. and R. Longnecker**. 2003. Herpesvirus entry: an update. Journal of Virology **77**:10179-10185.

30. **Sugden, B., K. Marsh, and J. Yates**. 1985. A Vector That Replicates As A Plasmid and Can be Efficiently Selected in Lymphoblasts-B Transformed by Epstein-Barr Virus. Molecular and Cellular Biology **5**:410-413.

31. **Szekely, L., C. Kiss, K. Mattsson, E. Kashuba, K. Pokrovskaja, A. Juhasz, P. Holmvall, and G. Klein**. 1999. Human herpesvirus-8-encoded LNA-1 accumulates in heterochromatin-associated nuclear bodies. Journal of General Virology **80**:2889-2900.

32. **Tellam, J., G. Connolly, K. J. Green, J. J. Miles, D. J. Moss, S. R. Burrows, and R. Khanna**. 2004. Endogenous presentation of CD8(+) T cell epitopes from Epstein-Barr virus-encoded nuclear antigen 1. Journal of Experimental Medicine **199**:1421-1431.

33. **Verma, S. C. and E. S. Robertson**. 2003. Molecular biology and pathogenesis of Kaposi sarcoma-associated herpesvirus. Fems Microbiology Letters **222**:155-163.

34. **Viejo-Borbolla, A., E. Kati, J. A. Sheldon, K. Nathan, K. Mattsson, L. Szekely, and T. F. Schulz**. 2003. A domain in the C-terminal region of latency-associated nuclear antigen 1 of Kaposi's sarcoma-associated herpesvirus affects transcriptional activation and binding to nuclear heterochromatin. Journal of Virology **77**:7093-7100.

35. **Voo, K. S., T. Fu, H. Y. Wang, J. Tellam, H. E. Heslop, M. K. Brenner, C. M. Rooney, and R. F. Wang**. 2004. Evidence for the presentation of major histocompatibility complex class I-restricted Epstein-Barr virus nuclear antigen 1 peptides to CD8+ T lymphocytes. J. Exp. Med. **199**:459-470.

36. **Voo, K. S., T. H. Fu, H. Y. Wang, J. Tellam, H. E. Helsop, M. K. Brenner, C. M. Rooney, and R. F. Wang**. 2004. Evidence for the presentation of major histocompatibility complex class I-restricted Epstein-Barr virus nuclear antigen 1 peptides to CD8(+) T lymphocytes. Journal of Experimental Medicine **199**:459-470.

37. **Yates, J. L., N. Warren, and B. Sugden**. 1985. Stable Replication of Plasmids Derived from Epstein-Barr Virus in Various Mammalian-Cells. Nature **313**:812-815.

38. **Ye, F. C., F. C. Zhou, S. M. Yoo, J. P. Xie, P. J. Browning, and S. J. Gao**. 2004. Disruption of Kaposi's sarcoma-associated herpesvirus latent nuclear antigen leads to abortive episome persistence. Journal of Virology **78**:11121-11129.

39. **Yewdell, J. W.** 2003. Hide and seek in the peptidome. Science **301**:1334-1335.

40. **Yin, Y., B. Manoury, and R. Fahraeus**. 2003. Self-inhibition of synthesis and antigen presentation by Epstein-Barr virus-encoded EBNA1. Science **301**:1371-1374.