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Kaposi's Sarcoma-Associated Herpesvirus Latency-Associated Nuclear Antigen 1 Mimics Epstein-Barr Virus EBNA1 Immune Evasion through Central Repeat Domain Effects on Protein Processing[∇]

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Kaposi's sarcoma-associated herpesvirus (KSHV/human herpesvirus 8 [HHV8]) and Epstein-Barr virus (EBV/HHV4) are distantly related gammaherpesviruses causing tumors in humans. KSHV latency-associated nuclear antigen 1 (LANA1) is functionally similar to the EBV nuclear antigen-1 (EBNA1) protein expressed during viral latency, although they have no amino acid similarities. EBNA1 escapes cytotoxic lymphocyte (CTL) antigen processing by inhibiting its own proteasomal degradation and retarding its own synthesis to reduce defective ribosomal product processing. We show here that the LANA1 QED-rich central repeat (CR) region, particularly the CR2CR3 subdomain, also retards LANA1 synthesis and markedly enhances LANA1 stability in vitro and in vivo. LANA1 isoforms have half-lives greater than 24 h, and fusion of the LANA1 CR2CR3 domain to a destabilized heterologous protein markedly decreases protein turnover. Unlike EBNA1, the LANA1 CR2CR3 subdomain retards translation regardless of whether it is fused to the 5' or 3' end of a heterologous gene construct. Manipulation of sequence order, orientation, and composition of the CR2 and CR3 subdomains suggests that specific peptide sequences rather than RNA structures are responsible for synthesis retardation. Although mechanistic differences exist between LANA1 and EBNA1, the primary structures of both proteins have evolved to minimize provoking CTL immune responses. Simple strategies to eliminate these viral inhibitory regions may markedly improve vaccine effectiveness by maximizing CTL responses.

Kaposi's sarcoma-associated herpesvirus (KSHV or human herpesvirus 8 [HHV8]) causes Kaposi's sarcoma (KS), primary effusion lymphomas (PELs), and a subset of multicentric Castleman's disease (8, 10, 49). The high rate of KSHV-related cancers among KSHV-infected immunosuppressed patients reflects the importance of cellular immune surveillance in controlling KSHV-related malignancies.

KSHV encodes structural and lytic replication proteins that are highly conserved with those found among other herpesviruses (44). KSHV also encodes a number of genes expressed during various forms of KSHV latency that are unique to KSHV and closely related rhadinoviruses. Despite apparent differences in sequence, many of these proteins share functional similarity with nonstructural proteins of other herpesviruses, particularly Epstein-Barr virus (EBV or HHV4). For example, KSHV encodes its own interleukin 6 (IL-6) homolog (37, 38), while EBV induces cellular IL-6 expression through glycoproteins gp350 and gp220 (52) and LMP1 (17). KSHV and EBV each dysregulate p53 and pRB1 (18, 27, 39, 40, 42) but do so through different mechanisms using unrelated viral

proteins. This pattern of functional correspondence between herpesviruses has been particularly useful in predicting new viral functions.

KSHV latency-associated nuclear antigen 1 (LANA1) and EBV nuclear antigen 1 (EBNA1) are, respectively, major latency proteins from these two gammaherpesviruses responsible for maintaining viral episomes in infected cells. LANA1 was first discovered as a latent virus antigen in infected cells recognized by KS patient sera (37). Highly specific serologic assays to detect KSHV infection based on antibody recognition of LANA1 are still in common use (22, 23, 31). LANA1 is encoded by the Orf73 gene and has a predicted molecular mass of 135 kDa but migrates aberrantly in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as a 222- to 234-kDa doublet (30, 43). In addition to the 222- to 234-kDa isoforms, a lower-shoulder multiplex of bands at ~150 to 180 kDa is present in infected cells (22). Aberrant LANA1 migration is most likely due to the physical characteristics of LANA1 but may also represent covalent posttranslational modifications. LANA1 is a highly acidic protein with an isoelectric point of 3.69 due to an acidic central repeat (CR) region. The CR region can be further subdivided into three subdomains based on differences in amino acid (aa) repeat sequences: CR1 (aa 321 to 428 in Orf73 of the BC-1 sequence [U75698]) (44), CR2 (aa 430 to 768), and CR3 (aa 769 to 937). These regions are imperfect repeats rich in glutamine (Q), glutamate (E), and aspartate (D), with a conserved leucine zipper domain

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TABLE 1. The sequences of primers used in PCR assays

Construct ^a	Polarity	Sequence ^b
N terminus	Sense	GAATTCATGGCGCCCCCGGAATGCGC
	Sense	TCGATATCATGGCGCCCCCGGAATGCGC
	Antisense	CCGATATCCTTATTGTTCATTGTATCCTT
C terminus	Sense	CGATATCATCTTGCACGGGTCGTCATCC
	Antisense	CCAAGCTTTGTCAATTCCTGTGGAGAGTC
CR1	Sense	CCGAATTCGACAAGGATGACAATGACAAT
	Sense	CCGATATCGACAAGGATGACAATGACAAT
	Antisense	CGATATCGCTCAACGTTTTGTTTCCATCG
CR2	Sense	GAATTCGGCGATGGAAACAAAACGTTGAGC
	Sense	GATATCGGCGATGGAAACAAAACGTTGAGC
	Antisense	GATATCCTCTGCTCCTGCTCCTCTGCT
CR3	Sense	GAATTCCTAGAGGAGCAGGAGCAGGAGTTA
	Sense	GATATCTTAGAGGAGCAGGAGCAGGAGTTA
	Sense	GGCAAGCTTTAATTAGAGGAGCAGGAGCAGGAGTAC
	Antisense	GATATCCAAGATTATGGGCTCTTCCACCGT
EGFP	Sense	CATGGATCCGCCACCATGGTGTAGCAAGGGC
	Antisense	CTTGAATTCCTGTACAGCTCGTCCATGC
	Sense	CATAAGCTTGTGAGCAAGGGCGAGGAGCTG
	Antisense	CTTCTCGAGCTGTACAGCTCGTCCATGC

^a Constructs were acquired from PCR using the indicated primers. Restriction enzyme sites are underlined.

^b Sequences of primers are based on BC-1 cells (U75698) (44) and pEGFP-C1 vector (Clontech).

present in CR3. Studies of this region have been hampered by difficulties in cloning the highly repetitious CR sequences.

Several important functions have been ascribed to LANA1. LANA1 is needed for viral episome maintenance and segregation (1, 2, 28, 35), with both its N and C termini required for these functions (3). The LANA1 N terminus binds nucleosomes through interaction with histones H2A and H2B (4) and facilitates viral DNA replication by binding to latent replication origins (LBS-1 and LBS-2) within the terminal repeat of the virus (24, 50). Additionally, LANA1 stabilizes β -catenin through glucocorticoid synthase kinase 3 β (GSK-3 β) (19, 20), inhibits p53-mediated apoptosis (18), and upregulates telomerase through Sp1 interactions (55).

While EBV does not encode a protein with amino acid similarity to LANA1, the EBNA1 protein is functionally analogous. Like LANA1, EBNA1 is also a latent origin binding protein that acts to ensure EBV episome maintenance and segregation by tethering the EBV episome to the host cell chromosomes (25, 26). In contrast to the LANA1 CR region, which has a high QED composition, the EBNA1 central repeat region is composed of glycine-alanine repeats (GAR). Although the EBNA1 GAR has no amino acid similarity to that of the LANA1 CR, the corresponding gene sequences are highly homologous, since the two protein reading frames are frame shifted relative to each other (58). EBNA1 is also obligately expressed in EBV-infected cells and thus may be an immunologic target for cytotoxic T lymphocyte (CTL) immune responses.

The EBNA1 GAR has been shown to inhibit CTL antigen presentation in two ways. Masucci and colleagues have shown that the EBNA1 GAR domain inhibits proteasomal degradation of EBNA1 protein. Heterologous proteins fused to the GAR are resistant to proteasomal degradation (32). When the

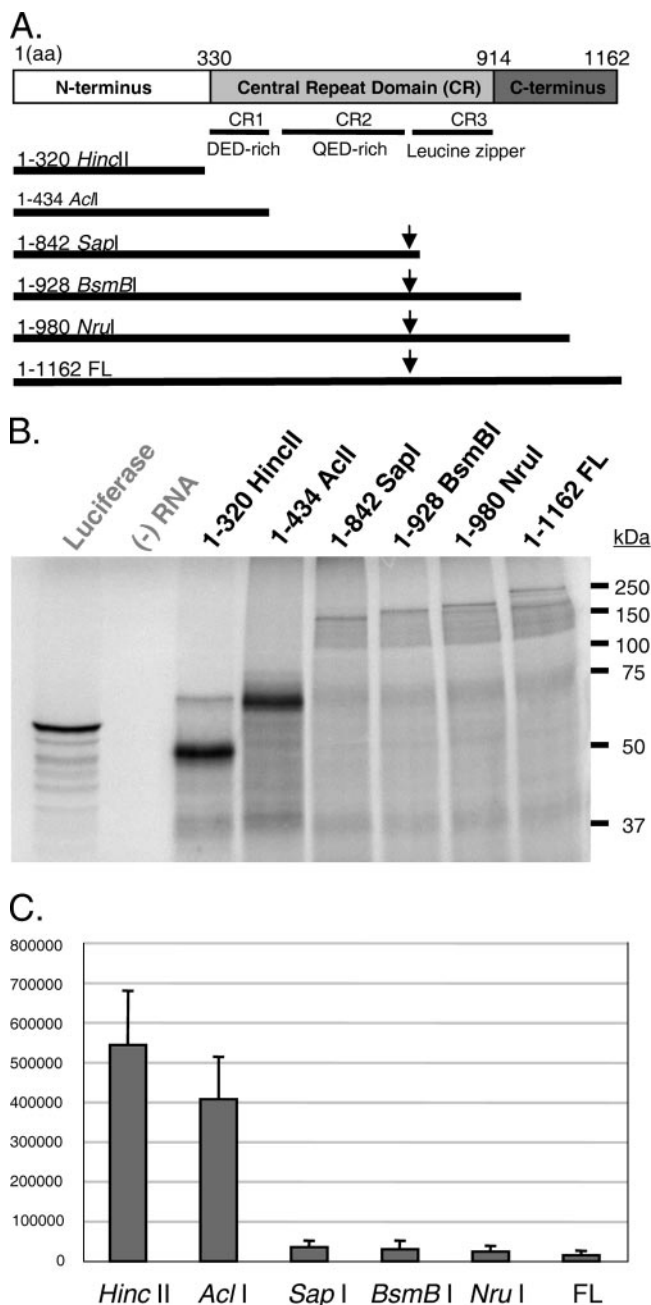


FIG. 1. Identification of LANA1 synthesis retardation domain. (A) Map of C-terminal deletion constructs for in vitro translation experiments. Arrows show sites of CR2CR3 junctions. (B) LANA1 mRNAs containing the central repeat region have reduced translation efficiency in uncoupled in vitro translation assays. Results are averages from three experiments. Translation reactions were performed using equimolar amounts of T7 polymerase transcribed-capped RNA. Luciferase RNA was translated (~61-kDa product) as a positive control. As a negative control, the reaction was performed without added RNA. (C) The amount of [³⁵S]methionine-labeled protein shown in panel B was quantified using ImageQuant software (Molecular Dynamics) and further standardized to the number of methionines in each construct. Lanes 1 to 320, HincII (4 methionines); 1 to 434, AclI (4 methionines); 1 to 842, SapI (4 methionines); 1 to 928, BsmBI (4 methionines); 1 to 980, NruI (5 methionines); 1 to 1162, FL (8 methionines).

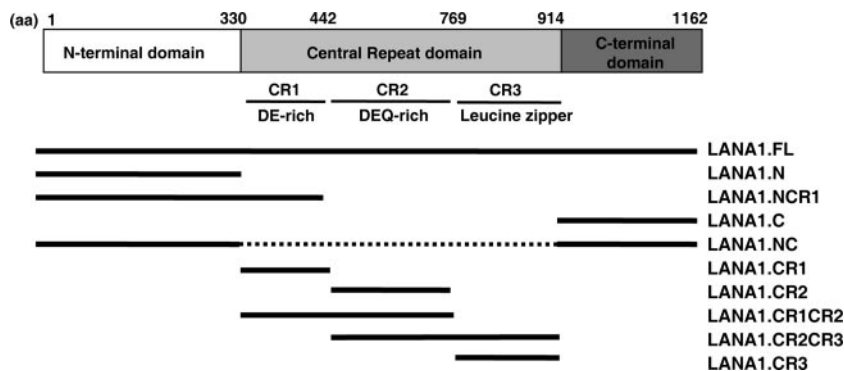


FIG. 2. Map of LANA1 subdomain clones.

25-aa GAR residue is cloned into a destabilized green fluorescent protein (GFP) construct, the GAR domain efficiently inhibits proteasomal degradation of the fusion protein (15), and even an 8-aa GAR residue is sufficient to retard IκBα degradation (48). The precise mechanism for proteasomal inhibition remains unclear, but evidence suggests that the GAR domain does not prevent protein polyubiquitination (48). As expected from its ability to escape proteasomal processing, EBNA1 protein has a markedly prolonged half-life (16).

Increasing evidence suggests that a second mechanism involving rapid protein degradation immediately after initial peptide synthesis may be the primary CTL epitope generation mechanism. Up to 20% of all newly made cellular proteins are misfolded (so-called defective ribosomal products or DRiPs) and undergo immediate proteasomal degradation and CTL surveillance sampling (47). EBNA1 also inhibits this mechanism of antigen processing. The GAR has been shown by Fahrenus and colleagues to retard EBNA1 translation, increasing its stability and reducing unfolded protein turnover (57). Recently, Tellam and colleagues have confirmed that the presentation efficiency of CD8⁺ T-cell epitopes from EBNA1 is primarily determined by translation efficiency rather than intracellular stability (53). Positional cloning has been used to distinguish the relative contributions of degradation inhibition versus synthesis retardation to EBNA1 CTL immunoevasion, since GAR inhibition of DRiP processing occurs primarily when the GAR is cloned into the N terminus of a protein (57), whereas GAR inhibition of proteasomal processing occurs regardless of N- or C-terminal orientation of the GAR sequence (32, 33). At present, it is not known whether there are common molecular steps involved in these two distinct biological processes, protein translation and protein degradation, that are targeted by the GAR.

Although EBNA1 and LANA1 do not share amino acid homology, we show here that the LANA1 CR region behaves similarly to the EBNA1 GAR by inhibiting proteasomal degradation and retarding protein synthesis both in vitro and in vivo. During the course of these studies, Zaldumbide and colleagues demonstrated that the LANA1 central repeat region, like the EBNA1 GAR, inhibits CTL peptide antigen processing when fused to an Ova peptide antigen (58). LANA1 has a prolonged half-life, and low-molecular-weight LANA1 isoforms have half-lives exceeding 24 h. We have generated a series of LANA1 clones that

allows us to study the metabolism of distinct regions of this protein. In this study, we show that the CR repeats inherently confer some degree of synthesis retardation and that a LANA1 peptide motif at the junction between LANA1 CR2 and CR3, rather than a nucleic acid secondary structure, markedly retards peptide synthesis. Our study provides insights into the mechanism of CTL immunity evasion described by Zaldumbide et al. (58). These findings also suggest that repeat structures of viral proteins may play a broader role than previously realized in blunting adaptive immune responses to latent viral proteins during chronic viral infections.

MATERIALS AND METHODS

Plasmids. LANA1 constructs containing the N terminus, the C terminus, the central repeat domains, and various combinations were generated by PCR with a BC-1 DNA template using the primers shown in Table 1. CR2-EcoRV-CR3 has an EcoRV enzyme site engineered (addition of two amino acids) between CR2 and CR3. The LANA1 N-terminal fragment was inserted into CR2-EcoRV-CR3 to generate CR2-N-CR3. To construct the LANA1_{TAA} plasmid, a stop codon (TAA) was introduced at the junction (aa 768 to 769) between CR2 and CR3 in full-length LANA1. All PCR products of LANA1 were cloned into pCMV-Tag2B (Stratagene), and the sequences of all constructs were confirmed to be wild-type BC-1 by DNA sequencing (44). To ensure that in vitro translation products representing only central repeat regions (aa 330 to 914), which are devoid of methionines, could be detected, the vector pCMV-Tag2B (ATG twice) was constructed by substituting two methionines (ATG, shown in bold type) between the BamHI and the EcoRI sites (underlined) in the multiple cloning site (5'-GGATCCCGGGGCTGCAGGAATTC-3' to 5'-GGATCCATGGGGCTGATGGGAATTC-3') of the parental pCMV-Tag2B vector. For LANA1 fragments fused C terminally and N terminally to enhanced GFP (EGFP), EGFP was amplified from the pEGFP-C1 vector template (Clontech) by PCR using primers listed in Table 1. The EGFP PCR product was then inserted into the BamHI/EcoRI or HindIII/XhoI sites of various pCMV-Tag2B LANA1 constructs. Full-length EBNA1 plasmid (7) was introduced into the EcoRI/EcoRV site of pCMV-Tag2B vector, providing a T3 promoter for performing in vitro transcription.

To examine inhibition of GFP turnover by the CR2CR3 subdomain, pd1EGFP-N1 (a kind gift from Don Ganem) encoding a destabilized EGFP with an estimated half-life (*t*_{1/2}) of 1 h was used for constructing fusions to CR2-CR3 at the N terminus of EGFP. For this, we modified the pd1EGFP-N1 multiple cloning site (MCS) and Kozak sequence for easy subcloning of LANA1 constructs from pCMV-Tag2B and to ensure synthesis of the full gene product. The modified MCS sequence is as follows, with the EcoRI, EcoRV and HindIII sites underlined: 5'-GAGCTCGCCACCATGGCAGAATTCGAGATATCGCAAAGCTTGTG-3'. For the generation of CR2CR3-EGFP, plasmid pCMV-Tag2B-CR2CR3 was digested with EcoRI/HindIII, and the 1.5-kb insert was ligated into similarly modified pd1EGFP-N1 vector.

Immunoprecipitation of LANA1. BJAB cells were grown in RPMI 1640 medium (Sigma) supplemented with 1% glutamine and 10% fetal bovine serum

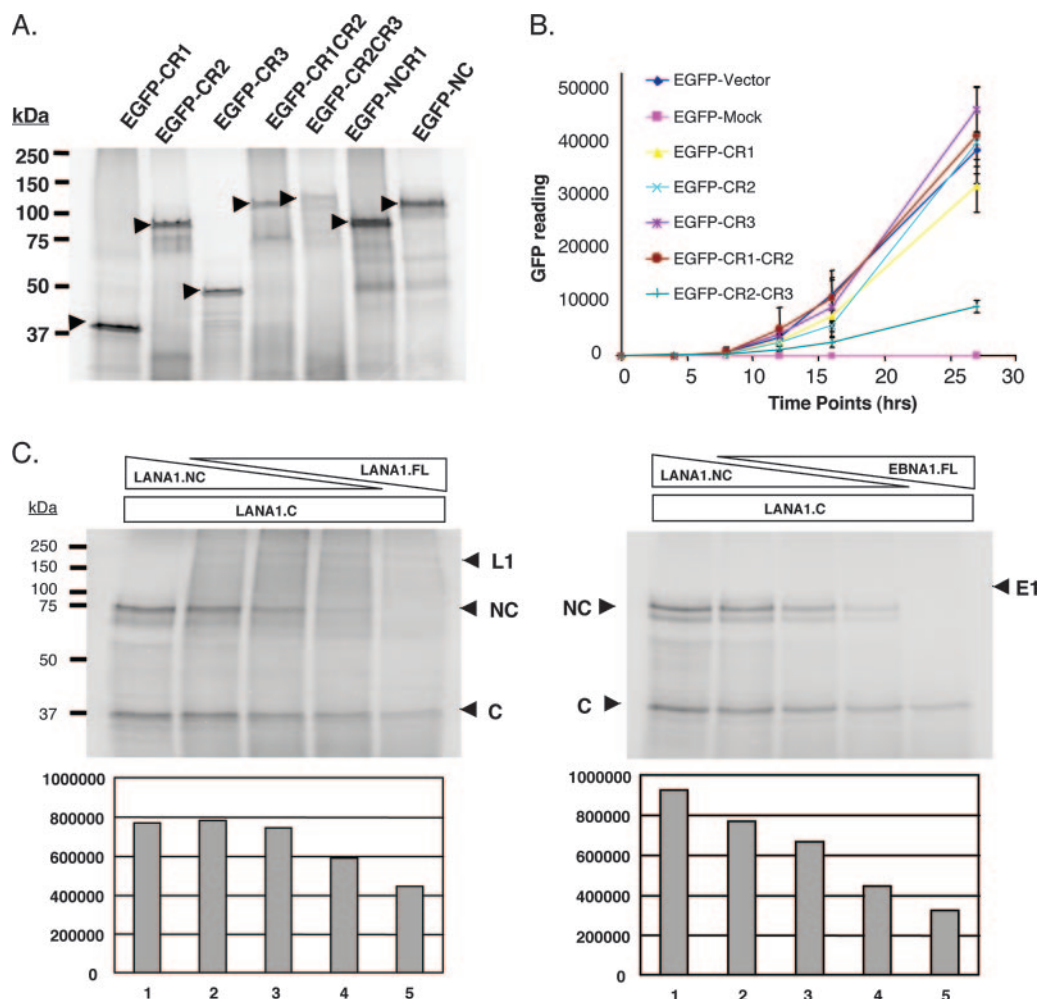


FIG. 3. Effect of LANA1 repeat regions on protein synthesis. (A) Maximal translation retardation occurs when CR2 is joined to CR3. Each LANA1 domain was fused at the C terminus to EGFP and translated using equimolar amounts of RNA. All three central repeat domains (EGFP-CR1, EGFP-CR2, and EGFP-CR3) individually show similar rates of translation to the EGFP-NC construct entirely lacking the central repeat domain. Arrows show expected sizes for each construct. Note that the combined CR1CR2 construct may also have reduced translation compared to other comparably sized fragments, but translation retardation is most pronounced for the CR2CR3 construct. (B) CR2CR3 retards *in vivo* accumulation of a heterologous EGFP fusion protein. 293 cells were transfected with EGFP-fused LANA1 CR1, CR2, CR3, CR1-CR2, and CR2-CR3 to determine the effect of LANA1 fragments on protein synthesis *in vivo*. Cells were harvested at different time points (0, 4, 8, 12, 16, and 27 h), and lysates were quantitatively assayed for GFP using a fluorescence reader. (C) Translational retardation by LANA1 and EBNA1 occurs *in trans* at high expression levels. In this experiment, synthesis of constant amounts of LANA1-C (C-terminal region) was measured in the presence of increasing amounts of full-length LANA1 and EBNA1. To ensure equal total amounts of RNA, increasing amounts of full-length LANA1 (upper left panel) or EBNA1 (upper right panel) RNA (0, 0.5, 1.0, 1.5, and 2.0 pM) were added with parallel decreasing amounts (2.0, 1.5, 1.0, 0.5, and 0 pM) of LANA1-NC (lacking the central repeat region) and constant amounts (1 pM) of LANA1-C (C-terminal region). Amounts of [³⁵S]methionine-labeled LANA1-C were quantitated by ImageQuant software and reveal reduced LANA1-C synthesis as full-length LANA1 and EBNA1 amounts are increased (lower panels). Representative results of three experiments are shown.

(FBS). BC-1, BCP-1, and BCBL-1 cells were grown in ATCC complete growth medium (RPMI 1640 medium with 2 mM L-glutamine, 4.5 g/liter glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, and 20% fetal bovine serum). Cells were washed twice in phosphate-buffered saline (PBS), harvested, and lysed in buffer (0.15 M NaCl, 50 mM Tris-HCl [pH 7.4], 0.5% NP-40) supplemented with protease inhibitors. Cell extracts were centrifuged at 13,000 rpm at 4°C for 10 min, and supernatants were incubated overnight at 4°C with CMA-810, a mouse anti-LANA1 monoclonal antibody (13). Protein A/G agarose beads (Amersham) were incubated with the lysates for 2 h at 4°C with gentle shaking. The beads were collected by centrifugation, washed four times with the lysis buffer, and resuspended in SDS loading buffer. The immunoprecipitates were eluted from the beads by incubation at 95°C for 5 min. The eluted proteins were separated by SDS-PAGE. Immunoblotting was subsequently performed with rat anti-LANA1 antibody (ABI).

Cycloheximide treatment on BCBL-1 cells. BCBL-1 cells were treated with 100 µg/ml of cycloheximide (Sigma) or an equal volume of dimethyl sulfoxide (DMSO) diluent alone. Cells (6.5×10^5 cells/ml) were harvested at each experimental time point indicated and lysed in buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate supplemented with proteinase inhibitors. Cell extracts were separated by 6% SDS-polyacrylamide gel electrophoresis. Loading was equalized according to cell number. Immunoblotting was performed using mouse anti-LANA1 antibody (CMA-810) and rabbit anti-IRF3 antibody (Santa Cruz).

Inhibition of protein turnover. 293 cells were grown in Dulbecco's minimum essential medium (DMEM) supplemented with 10% FBS and transfected with Lipofectamine-2000 (Invitrogen), with either d1EGFP or CR2CR3-d1EGFP constructs. Twenty-four hours after transfection, cells were grown in DMEM

with 100 $\mu\text{g/ml}$ cycloheximide (CHX; Sigma) to inhibit further protein synthesis or in an equal volume of DMSO diluent as control. Samples were collected at 1, 2, 3, 6, and 12 h after CHX treatment and at 6 and 12 h for control. Fluorescence images were captured at 0 and 12 h after CHX treatment. Harvested cells were lysed in buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.1% SDS, 3 mM EDTA, 1% Triton X-100, 1 mM NaF, and 1 mM Na orthovanadate) supplemented with proteinase inhibitors. Proteins were separated on 10% SDS gels and transferred onto nitrocellulose membranes. Immunoblotting was performed with anti-GFP antibodies (Zymed) and subsequently detected by ECL (Amersham).

In vitro transcription/translation. pcDNA.LANA1 C-terminal truncations were generated by digesting and linearizing a pcDNA full-length LANA1 construct at the following restriction sites: HincII (320 aa), AclI (434 aa), SapI (842 aa), BsmBI (928 aa), NruI (980 aa), and XhoI orFL (1,162 aa). LANA1 constructs in pCMV-Tag2 vector were linearized with PvuI enzyme. To generate capped RNA, DNA templates were in vitro transcribed using T3 or T7 RNA polymerase and an mMESAGE mMachine high-yield capped RNA transcription kit (Ambion). RNA concentrations were determined by UV spectroscopic measurement at 260 nm, and molar equivalents of each RNA were calculated (based on nucleotide length and concentration) for use in uncoupled in vitro translation with a rabbit reticulocyte lysate system (Promega) and [^{35}S]methionine (Amersham). Reaction products were resolved on SDS-PAGE gel, dried, exposed overnight to screens, and read using a PhosphorImager SI model (Molecular Dynamics).

Analysis of GFP fluorescence. 293 cells were transfected with LANA1 fused to EGFP constructs using Lipofectamine-2000 (Invitrogen). Cells were harvested at time points 0, 4, 8, 12, 16, and 27 h after transfection. Cells (1.7×10^6) were lysed in buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.1% SDS, 3 mM EDTA, 1% Triton X-100, 1 mM NaF, and 1 mM Na orthovanadate) supplemented with protease inhibitors. For GFP analysis, 50 μg of protein in 100 μl of PBS was analyzed by a fluorescence reader (SAFIRE microplate reader, TECAN).

RESULTS

Survey for synthesis retardation by subdomains of LANA1 protein. To determine if LANA1 retards its own synthesis, 3' truncations at sites shown in Fig. 1A were transcribed in vitro by using equimolar amounts of RNA through uncoupled transcription-translation reactions. To minimize variability, C-terminal deletion constructs were derived from an identical LANA1 full-length parental construct, using unique or infrequent restriction enzyme sites within LANA1. Most of these fragments lack stop codons, and single-pass translation predominates in the reaction. Robust translation is present for constructs containing the N terminus and CR1 region, but translation is retarded for the SapI, BsmBI, NruI, and full-length fragments (Fig. 1B and C).

Synthesis retardation by the LANA1 CR2CR3 repeat region. To confirm that the CR subdomain(s) retards protein synthesis, CR regions were cloned in fusion with N-terminal EGFP using pCMV-Tag2B vector, and the translation efficiency of each construct was measured (Fig. 2). These constructs possess identical 5' translation-initiation sequences as well as 3' EGFP sequences, with identical 3' stop codons. All mRNAs were capped, and equimolar amounts were used in translation reactions. Individual CR domains (CR1, CR2, and CR3), as well as regions CR1CR2, NCR1, and NC, are efficiently translated (Fig. 3A). When the CR2 and CR3 domains are joined, however, in vitro translation is markedly inhibited. This was confirmed in vivo by transient transfection of these constructs into 293 cells and by measuring protein synthesis using EGFP fluorescence (Fig. 3B). CR1, CR2, CR3, and CR1CR2 GFP fusion proteins show kinetics of synthesis similar to those of the EGFP vector alone, but the fusion of CR2CR3 markedly decreases GFP expression, suggesting that the junction between

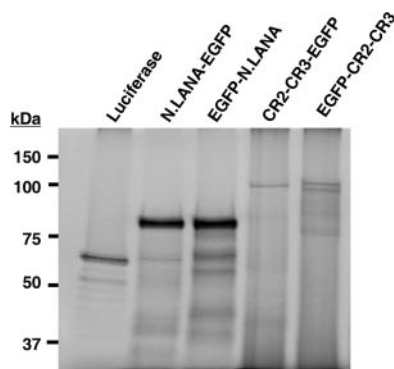


FIG. 4. LANA1 CR2-CR3 fragment retards synthesis in vitro at both the N- and the C-terminal positions. The CR2-CR3 LANA1 fragment fused to GFP either at the N or C terminus prevents translation of heterologously fused protein. In comparison, LANA-N fused at the N-terminal or C-terminal position to GFP is efficiently translated. Representative results of three experiments are shown.

CR2 and CR3 is responsible for the protein synthesis retardation.

Synthesis retardation by the EBNA1 GAr has previously been found to occur in *cis* during EBNA1 translation (57). To determine if LANA1 behaves similarly, in vitro cotranslation was performed using constant amounts of LANA1-C mRNA together with increasing amounts of full-length LANA1 RNA and decreasing amounts of LANA1-NC RNA, so that total amounts of RNA are equal. Increasing amounts of full-length LANA1 RNA resulted in slight decreases in LANA1-C translation (Fig. 3C, left panel). Similar effects are seen when full-length EBNA1 RNA is substituted for LANA1 (Fig. 3C, right panel). Both full-length proteins were poorly translated, consistent with their innate translation inhibition properties. We thus cannot rule out a minor effect of in *trans* inhibition of protein synthesis by either LANA1 or EBNA1 in this experiment.

The EBNA1 GAr also has been reported to principally inhibit synthesis when cloned to the 5' position of a heterologous protein. In contrast, we find that the LANA1 CR2CR3 motif markedly retards translation regardless of whether it is 5' or 3' to a heterologous gene construct, suggesting fundamental differences between CR2CR3 and GAr (Fig. 4).

Mechanism for translational retardation of LANA1. While EBNA1 and LANA1 have no amino acid similarity, the gene sequences of the two viral repeat sequences are highly homologous to each other but are frameshifted for translation. One appealing possibility is that these two proteins have similar mRNA secondary structures with common effects on protein synthesis retardation. We sought to test this possibility using modified CR2 and CR3 constructs of LANA1 in uncoupled in vitro translation reactions. In vitro translation was measured for constructs having an EcoRV site insertion (GATATC) and a large (LANA1 N-terminal region) insertion between CR2 and CR3, as well as a protein in which CR2 and CR3 (CR3CR2) are reversed (Fig. 5A). Both short and large insertions disrupt the autoinhibitory activity of CR2CR3. Reversing the order of the domains (CR3CR2) also abrogates inhibition and allows efficient translation (Fig. 5B). These results confirm that the site of

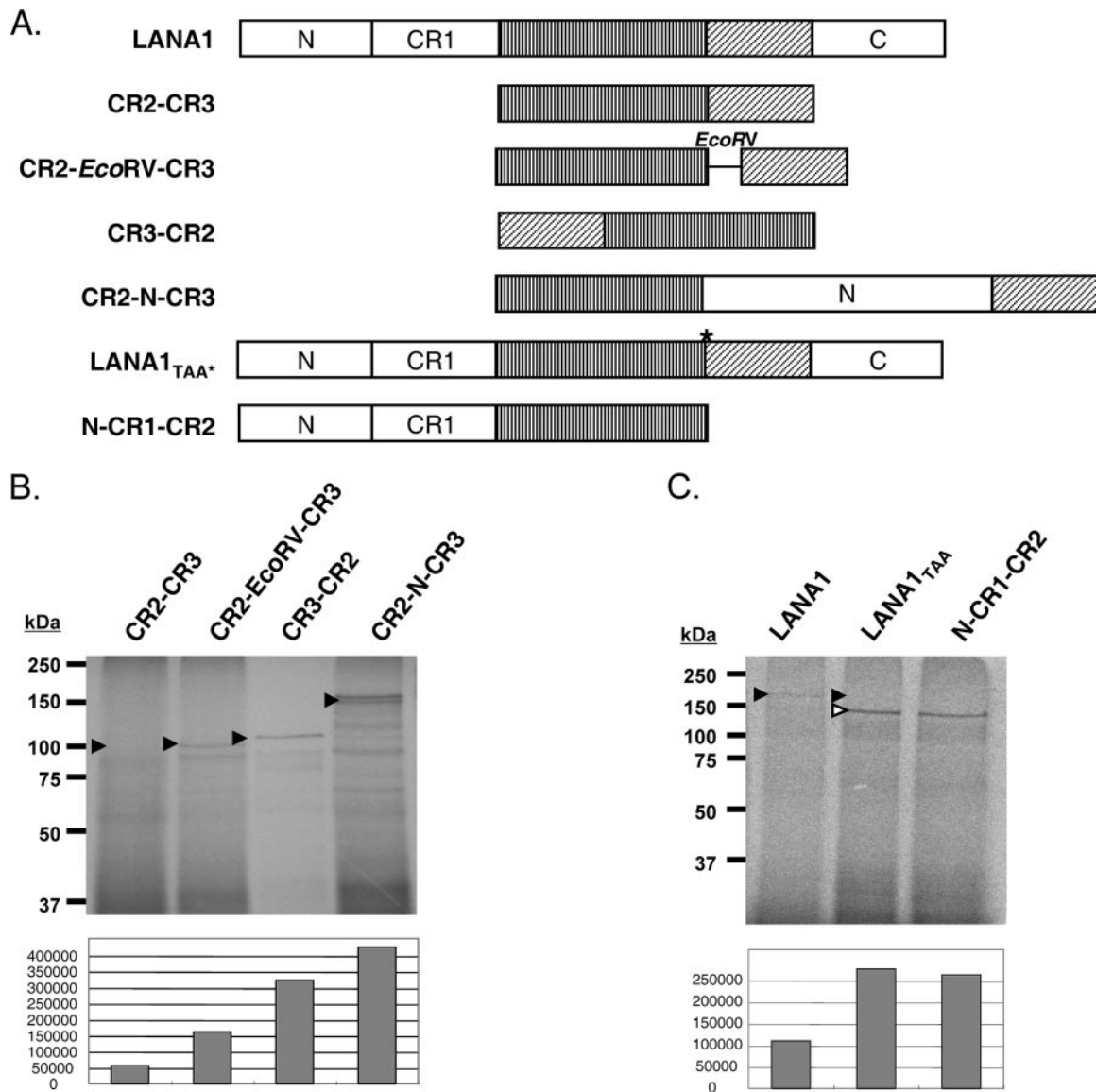


FIG. 5. Disruption of a protein motif located at the junction of CR2 and CR3 reduces translational retardation. (A) Schematic diagram of CR2 and CR3 constructs for in vitro translation experiments. (TAA is a stop codon). (B) Disruption of the junction between CR2 and CR3 increases protein translation efficiency. In this representative experiment (of three), CR2-CR3 translation is compared to CR2-CR3 constructs having a small insert between CR2 and CR3 (CR2-EcoRV-CR3, 6 nt), a large insert (CR2-N-CR3, 1,002 nt), or having the CR3 and CR2 in reverse orientation (CR3-CR2). Each construct has markedly increased translation efficiency compared to that of CR2-CR3. (C) Protein rather than mRNA structure is critical for CR2-CR3 translation retardation. Protein translation was truncated by cloning the TAA stop codon between CR2 and CR3 (*) in the full-length LANA1 gene. Arrowheads show expected size of full-length LANA1 (black) and actual size of LANA1_{TAA*} (white). Translation efficiency for this construct was comparable to that of a construct truncated 3' to the CR2 sequence (N-CR1-CR2). Representative results of three experiments are shown.

the autoinhibitory domain lies between CR2 and CR3 and suggest that even small changes in this structure prevent peptide translation inhibition.

To test whether mRNA structure is likely to be responsible for LANA1 translation inhibition, a TAA stop codon was engineered in the full-length protein at nucleotides (nt) 768 to 769, between CR2 and CR3. This construct will allow efficient transcription of mRNA, but translation will be truncated before synthesis of CR3. In vitro translation of this construct (Fig. 5A, LANA1_{TAA*}) was compared to parental LANA1 as

well as a construct truncated between CR2 and CR3 (Fig. 5A, N-CR1-CR2). Figure 5C shows that LANA1_{TAA*} synthesis is markedly more efficient than parental LANA1 protein and similar to that of the N-CR1-CR2 protein, suggesting that the inhibitory effect of LANA1 is determined by the peptide sequence present at the junction between CR2 and CR3. While our data suggest that disruption of the CR2CR3 junction enhances CR2CR3 translation, we cannot exclude the possibility that other portions of the central repeat region also contribute to LANA1 translation inhibition.

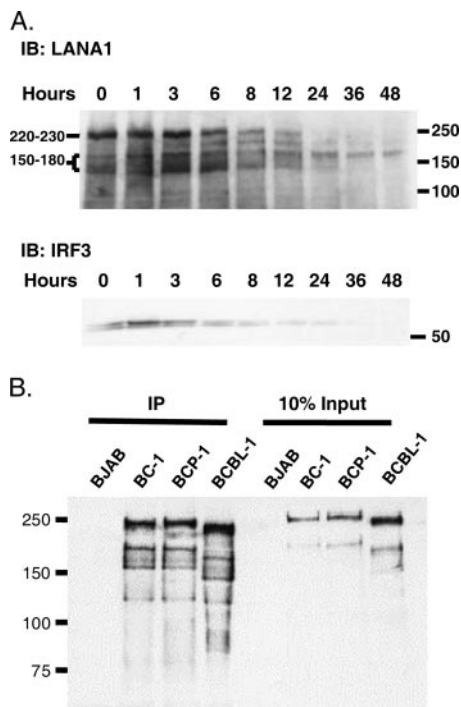


FIG. 6. LANA1 turnover in KSHV-infected cell line BCBL-1. (A) LANA1 immunoblot after cycloheximide treatment in BCBL-1 cells. BCBL-1 cells were treated with cycloheximide (100 μ g/ml) to abolish new protein synthesis at different time points, and immunoblotting (IB) was performed using mouse LANA1 antibody (upper panel). The \sim 220- to 230-kDa bands of LANA1 are detectable at 12 h after treatment, but the \sim 150- to 180-kDa LANA1 bands (LANA1 isoforms) can be detected for up to 48 h. LANA1 half-life is prolonged (\sim 24 h) compared to that of cellular IRF3 protein (\sim 8 h). The membrane was stripped and reprobed with cellular IRF3 antibody for comparison (lower panel). Representative results of two experiments are shown. (B) Immunoprecipitation (IP) of LANA1 from PEL cell lines. LANA1 was immunoprecipitated with mouse monoclonal antibody (CMA-810) and detected with rat monoclonal antibody (ABI). Both the \sim 150- to 180-kDa and the \sim 222- to 234-kDa bands were detected by both antibodies and show polymorphic variations among cell lines. The BJAB control cell line shows no protein reactivity.

LANA1 degradation inhibition. Since the EBNA1 GAR inhibits protein turnover as well as protein synthesis, we next examined the potential role of the QED central repeat domain in degradation inhibition. To investigate LANA1 protein stability, BCBL-1 cells were treated with cycloheximide to block new protein synthesis, and the level of LANA1 expression was measured by immunoblotting over time (Fig. 6). The half-life of the \sim 222- to 234-kDa isoform of LANA1 is somewhat prolonged ($t_{1/2}$, \sim 12 to 24 h) compared to that of the endogenous cellular protein IRF3 ($t_{1/2}$, \sim 6 h). The \sim 150- to 180-kDa isoforms, however, are remarkably stable, with a half-life of \sim 48 h, which exceeds the technical timescale of the experiment due to cycloheximide cytotoxicity.

Although full-length LANA1 has only a modest increase in stability, lower-molecular-weight isoforms are remarkably stable. The precise identities of the \sim 150- to 180-kDa isoforms are under investigation, but preliminary studies reveal that they are not degradation products of full-length LANA1 (data not shown). To determine whether shoulder isoforms are

present in all viral isolates, we examined LANA1 in naturally infected BC-1, BCBL-1, and BCP-1 cell lines. LANA1 was immunoprecipitated with a mouse monoclonal antibody (CMA-810) and detected with a rat monoclonal antibody (ABI). Both full-length and \sim 150- to 180-kDa shoulder bands were detected, with polymorphic variations among cell lines (Fig. 6B). Each shoulder band is stable, and the bands consistently vary with the full-length protein size among different polymorphic forms of LANA1.

Inhibition of proteasomal degradation by the LANA1 CR2CR3 repeat region. By analogy with the EBNA1 GAR, we sought to determine if CR2CR3 could contribute to LANA1 degradation inhibition in vivo. Heterologous fusion proteins were generated with CR2CR3 fused to a destabilized enhanced green fluorescence protein (d1EGFP-N1) encoded by ornithine decarboxylase PEST sequences (9). In the presence of cycloheximide, the half-life of the parental d1EGFP-N1 protein is 1 to 2 h, but when CR2CR3 is cloned to the N terminus of this protein, its half-life is extended to 4 to 6 h (Fig. 7A), suggesting that CR2CR3 antagonizes PEST degradation. This is confirmed by fluorescence microscopy in which fluorescence is nearly absent for the parental construct when d1EGFP-N1 plasmid is transfected into 293 cells and treated with cycloheximide for 12 h (Fig. 7B). In contrast, robust fluorescence remains present after 12 h of cycloheximide treatment for the CR2CR3-dsEGFP fusion protein. Similar to the EBNA1 GAR, the LANA1 CR2CR3 domain appears to antagonize both efficient peptide translation and proteasomal degradation.

DISCUSSION

Our findings, together with data published by others (58), suggest that the primary sequence of the LANA1 central repeat region inhibits cellular surveillance for LANA1 CTL epitopes (Fig. 8). Although our findings are largely consistent with similar studies performed with EBNA1, they raise several unanswered questions. First, proteasomal degradation and protein synthesis are biologically distinct and unrelated processes. We find that the LANA1 CR2CR3 domain behaves similarly to the EBNA1 GAR domain to retard both processes. This suggests that these viral peptides might target an as-yet-unidentified common molecular mechanism present in normal proteasomal processing and protein translation.

Second, the LANA1 CR2CR3 and EBNA1 GAR, despite their obvious common gene ancestry, have no amino acid similarities because of frame shifting. A potential explanation for these common effects is that both genes express a similar mRNA structure that retards ribosomal processing. Our LANA1 construct containing a stop codon at the CR2CR3 junction suggests instead that the mechanism for protein translation is peptide based. We cannot formally exclude the possibility, however, that changing a coding codon to a stop codon disrupts a common mRNA structure. An alternative explanation that we are actively investigating is that programmed frame shifting generates similar protein structures for both EBNA1 and LANA1. While these questions remain unresolved, it is apparent that both EBV and LANA1 have evolved specific primary protein structures to reduce sampling by the CTL epitope surveillance mechanism.

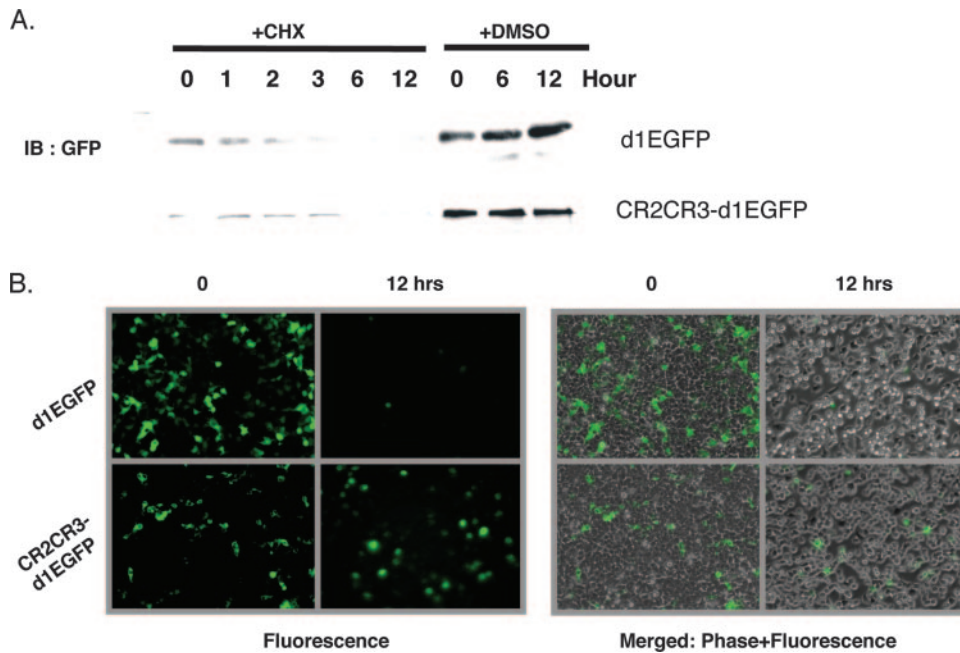


FIG. 7. LANA CR2CR3 repeat region inhibits proteosomal processing. (A) The LANA1 CR2CR3 region was cloned as a fusion protein with destabilized enhanced green fluorescent protein (d1EGFP) and expressed in 293 cells. Cycloheximide (CHX) treatment was used to prevent de novo protein synthesis, and protein levels were determined by immunoblotting (IB) with anti-GFP mouse antibody. (B) GFP fluorescence in 293 cells is markedly higher for CR2CR3-d1EGFP expression after 12 h of cycloheximide treatment than for d1EGFP alone. Merged phase-contrast images show similar numbers of cells for both CR2CR3-d1EGFP and d1EGFP expression conditions. Representative results of three experiments are shown.

KSHV possesses multiple mechanisms to evade both adaptive and innate immune surveillance. During gammaherpesvirus latency, most viral protein synthesis is silenced to reduce opportunities for cellular immune recognition. Lytic replication results in the expression of a number of viral proteins that block innate and adaptive immune recognition. Innate immune signaling is blunted by KSHV proteins such as vIL-6, vIRF1, and Orf45 that antagonize interferon signaling in infected cells

(11, 21, 59). KSHV MIR1 and MIR2 proteins are ubiquitin ligases encoded by Orfs K3 and K5 that target major histocompatibility complex class I (MHC-I) and accessory immune receptor proteins, including the gamma interferon receptor and CD1d, for cytoplasmic internalization and degradation (14, 29, 34, 45). Virus-encoded chemokines also act to polarize immune recognition toward ineffective Th2 rather than Th1 immune responses (36, 51). These immunity evasion proteins

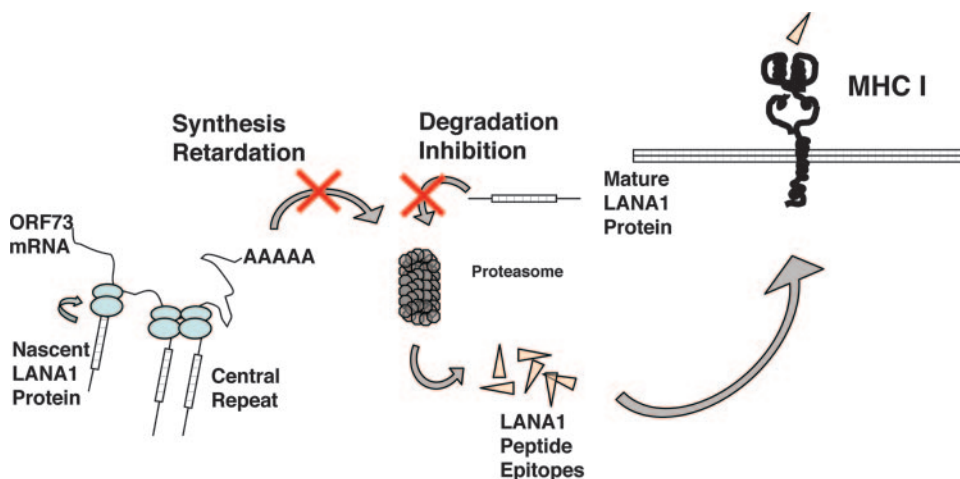


FIG. 8. Mechanism for CTL immune evasion by the QED central repeat domain of LANA1. The LANA1 central repeat domain may inhibit peptide processing and MHC-I antigen presentation through two distinct mechanisms: (i) synthesis retardation during initial translation from Orf73 mRNA, in which the QED-containing peptide region slows down protein translation, thereby reducing misfolded protein turnover; and (ii) degradation inhibition, in which mature protein proteasomal processing is inhibited by the QED-containing motifs.

are mainly, but not exclusively, expressed during lytic replication and might themselves serve as CTL antigens. Restriction of viral protein expression to only a small subset of proteins critical for maintaining the viral episome during latency appears to be the principal means by which KSHV evades host immune recognition during persistent infection.

Although latency is an effective means of reducing viral antigen targets that might be recognized by the immune system, LANA1 for KSHV and EBNA1 for EBV must be synthesized to maintain the replicating viral episome. Our data, together with similar studies by others, suggest that these proteins have evolved mechanisms to remain largely invisible to the immune system that would otherwise eliminate latently infected cells. Stability of the LANA1 protein helps to explain a widely recognized phenomenon in which LANA1 protein is expressed at easily detected levels in infected cells but Orf73 mRNA is minimally expressed and difficult to detect by Northern blotting (46). An important conclusion from our study is that EBV EBNA1 is not unique in its ability to evade CTL immune surveillance, and it is likely that other persistent viral infections will be found that have evolved similar mechanisms to escape CTL sampling.

The central repeat domain of LANA1 (CR1-CR2-CR3) has been shown by Zaldumbide and colleagues (58) to inhibit CTL antigen presentation when it is cloned into heterologous fusion proteins. Investigation of this phenomenon is hampered by the difficulty of fine cloning such a highly repetitious sequence. We localized one likely subregion for this effect to the CR2-CR3 domain, specifically the junction between these two repeat regions, and show that this peptide both inhibits PEST degradation and retards protein synthesis.

Although the immunity evasion properties of CR2CR3 are broadly similar to those reported for the EBNA1 GAR, we find evidence for distinct differences as well. Unlike the EBNA1 GAR, CR2CR3 retards peptide translation when cloned to both the amino- and the carboxyl-terminal sequences of a heterologous protein. In contrast to finding by Yin et al. (57), we reproducibly find evidence that both the GAR and CR2CR3 inhibit translation in *trans*, although differences in experimental conditions are likely to explain this discrepancy since *trans* inhibition occurs only at relatively high levels of EBNA1 and LANA1 cDNA translation. Differences in *in vitro* compared to *in vivo* conditions may in part account for this discrepancy. In these experiments, total cDNA was balanced, suggesting that competition for translation does not explain this effect. The precise mechanism by which these different viral proteins inhibit two distinct cellular processes involved in CTL antigen processing requires additional investigation.

The LANA1 sequence may provide clues to how the CR2CR3 domains evade proteasomal processing. Expanded poly-glutamine (Q) repeats can form stable β sheets mediating intermolecular contacts between repeat sequences (6, 41). Consequently, cells expressing such proteins often contain insoluble aggregates that are predominantly composed of tightly packed poly(Q)-containing proteins. While soluble poly(Q) proteins containing a strong degradation signal are efficiently degraded independent of the length of the repeat (54), once captured in aggregates, both poly(Q) proteins and coaggregating proteins are stable despite the presence of strong degradation signals. Thus, the formation of aggregates resistant to

proteasomal degradation initiates the accumulation of poly(Q) proteins and poly(Q)-interacting proteins. It is unknown whether the poly(Q) sequences in the LANA1 central repeat are responsible for a similar protein stabilization. In addition, due to difficulties in manipulating this region, we only described a degradation inhibition effect for the combined CR2CR3 region. It is possible that other portions of the central repeat region (e.g., CR1) show similar activity.

Even prior to the AIDS epidemic, Kaposi's sarcoma was one of the most common tumors in sub-Saharan Africa (12). KS tumor rates have dramatically increased with the AIDS pandemic, and KS is the most commonly reported cancer in some African cancer registries (5, 56). The only viable measure for controlling this viral infection is the development of effective preventive or therapeutic vaccines. KSHV is largely latent in KS tumors and presents a limited repertoire of antigen targets that might be recognized by vaccine-induced immunity. Our study suggests the possibility of a novel vaccine strategy for controlling KSHV infection in which CTL inhibitory domains of LANA1 are eliminated. Successful delivery of this engineered antigen might prime a CTL response sufficient to be active for both preventive and therapeutic vaccinations.

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