

# Influence of translation efficiency of homologous viral proteins on the endogenous presentation of CD8<sup>+</sup> T cell epitopes

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**A significant proportion of endogenously processed CD8<sup>+</sup> T cell epitopes are derived from newly synthesized proteins and rapidly degrading polypeptides (RDPs). It has been hypothesized that the generation of rapidly degrading polypeptides and CD8<sup>+</sup> T cell epitopes from these RDP precursors may be influenced by the efficiency of protein translation. Here we address this hypothesis by using the Epstein–Barr virus–encoded nuclear antigen 1 protein (EBNA1), with or without its internal glycine–alanine repeat sequence (EBNA1 and EBNA1ΔGA, respectively), which display distinct differences in translation efficiency. We demonstrate that RDPs constitute a significant proportion of newly synthesized EBNA1 and EBNA1ΔGA and that the levels of RDPs produced by each of these proteins directly correlate with the translation efficiency of either EBNA1 or EBNA1ΔGA. As a consequence, a higher number of major histocompatibility complex–peptide complexes can be detected on the surface of cells expressing EBNA1ΔGA, and these cells are more efficiently recognized by virus–specific cytotoxic T lymphocytes compared to the full–length EBNA1. More importantly, we also demonstrate that the endogenous processing of these CD8<sup>+</sup> T cell epitopes is predominantly determined by the rate at which the RDPs are generated rather than the intracellular turnover of these proteins.**

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Abbreviations used: DRiP, defective ribosomal product; EBNA1, EBV–encoded nuclear antigen 1 protein; LCL, lymphoblastoid cell line; RDP, rapidly degrading polypeptide.

The interaction of an MHC–peptide complex with a TCR on CD8<sup>+</sup> CTL is a crucial step toward the activation of virus–specific T cell responses (for review see references 1, 2). Peptide epitopes bound to MHC molecules are derived from viral proteins synthesized within the infected cells, which determine the specificity of the interaction between the TCR and MHC molecules (3). It is now firmly established that CTL recognition of virus–infected cells is dependent on the intracellular degradation of virally encoded proteins so that sufficient MHC–peptide complexes can be generated (4–6). In addition to intracellular degradation, the steady–state concentration of viral proteins and efficiency of endogenous processing also determine epitope production (7).

Indeed, the importance of these crucial steps in the generation of MHC–peptide complexes is highlighted by the fact that pretreatment of virus–infected cells with proteasome inhibitors (e.g., lactacystin) blocks the endogenous processing of peptide epitopes resulting in inhibition of T cell recognition (8–10).

A major caveat of the above concept is that the majority of antigenic peptides are derived from viral proteins, which are extremely stable. A classic example of one such protein is the EBV–encoded nuclear antigen 1 (EBNA1), which not only inhibits its self–synthesis but also blocks its proteasomal degradation (11, 12). Recent studies from our laboratory and others have shown that in spite of the highly stable nature of this protein in B cells, immunodominant epitopes can be efficiently generated (13–15). An extensive analysis of the endogenous processing

J. Tellam and M.H. Fogg contributed equally to this work.

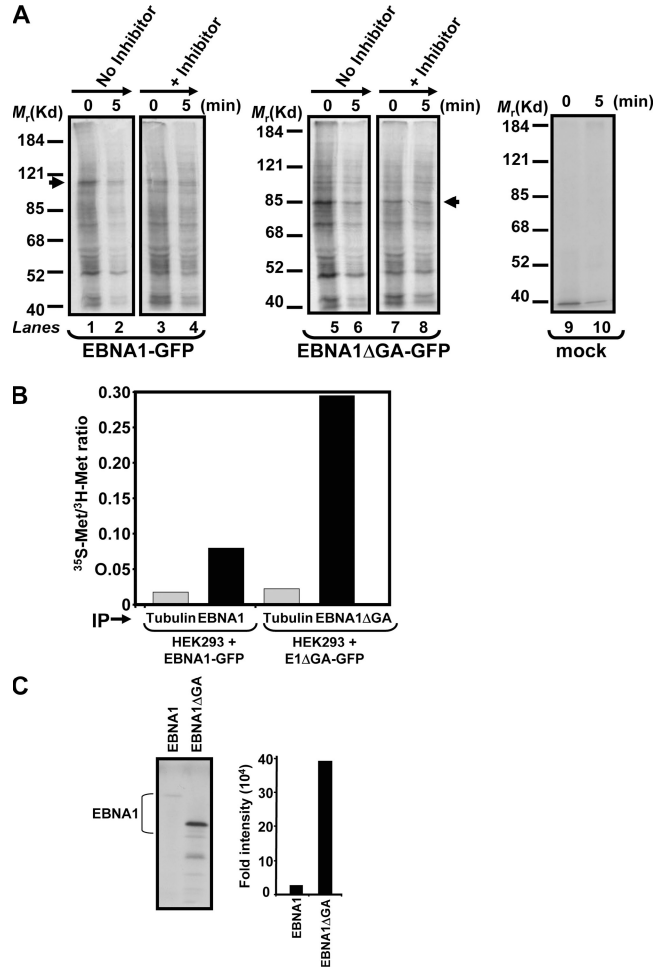
of EBNA1 revealed that antigenic epitopes from this protein are not necessarily derived from the degradation of the full-length stable protein, but rather are processed from newly synthesized polypeptides which are rapidly degraded (13, 14). These observations were consistent with the defective ribosomal products (DRiPs) hypothesis proposed by Yewdell et al. (16, 17). Studies performed by these authors have shown that about one-third of newly synthesized proteins are degraded within 15 min after expression and peptide-dependent maturation of class I molecules in the endoplasmic reticulum can be significantly blocked when protein translation is suppressed with protein synthesis inhibitors (10, 18).

More importantly, there is now sufficient evidence to indicate that a substantial proportion of MHC class I-peptide complexes are derived from proteins that are expressed and degraded within very short periods (i.e., <2 h) (10). Collectively, these observations strongly suggest that protein translation efficiency may play a crucial role in determining the efficiency by which MHC-peptide complexes are generated endogenously. To test this hypothesis we have compared the endogenous presentation of CD8<sup>+</sup> T cell epitopes from the EBV-encoded EBNA1 protein with and without its internal glycine-alanine repeat, which display distinct differences in translation efficiency (19). Our analysis showed that the translation efficiency of these proteins directly correlated with the efficiency by which the rapidly degrading polypeptides are generated and consequently effects the presentation of MHC-peptide complexes on the cell surface and immune recognition by virus-specific T cells.

**RESULTS**

**Detection of EBNA1-specific rapidly degrading polypeptides**

Previous studies have shown that in spite of a cis-inhibitory effect of the GAR domain on the translation of EBV-encoded EBNA1 (11), CD8<sup>+</sup> T cell epitopes from this protein can be efficiently generated and presented on the surface of virus-infected cells (13–15). Extensive analysis of endogenous processing revealed that most of these epitopes were derived from newly synthesized protein rather than long-lived pools of EBNA1 protein. It was hypothesized that DRiPs, which are by-products of rapidly degrading polypeptides (RDPs) of newly synthesized EBNA1 protein, may constitute a major source of these epitopes (13). To provide proof of this hypothesis, we transfected HeLa cells with expression vectors encoding GFP-tagged EBNA1 with or without the GAR domain (referred to as EBNA1-GFP and EBNA1ΔGA-GFP). These cells were left untreated or pretreated with 50 μM of the proteasome inhibitors MG132 and lactacystin during the final 30 min of a 60-min starvation in Met-free medium preceding a short [<sup>35</sup>S]methionine pulse (2 min) and chase (10, 18). GFP immunoprecipitates from the lysed extracts were analyzed by SDS-PAGE (Fig. 1 A). These experiments showed two important observations. First, newly synthesized EBNA1-GFP and EBNA1ΔGA-GFP showed a smear of protein bands ranging from 120 to 40 kD, and the intensity of these bands was relatively stronger in cells expressing EBNA1ΔGA-GFP.



**Figure 1. EBNA1 and EBNA1ΔGA translation efficiency and rapidly degrading polypeptides.** (A) Detection of EBNA1 rapidly degrading polypeptides. HeLa cells treated with 50 μM of both MG132 and lactacystin during the final 30 min of a 60-min starvation in Met-free media were radiolabeled for 2 min and chased for 5 min. EBNA1-GFP immunoprecipitates were subjected to SDS-PAGE and autoradiography. Mol wt markers in kD are shown on the left. Arrows indicate full-length EBNA1-GFP and EBNA1ΔGA-GFP. (B) Measurement of EBNA1 synthesis. HEK293 cells transfected with either EBNA1-GFP or EBNA1ΔGA-GFP were metabolically labeled for 12–14 h in growth medium containing 20 μCi/ml of [<sup>3</sup>H]methionine followed by a 30-min pulse with 100 μCi of [<sup>35</sup>S]methionine. Cells were lysed and immunoprecipitated with either anti-GFP or antitubulin. Quantitation of EBNA1-GFP, EBNA1ΔGA-GFP, or tubulin synthesis was determined by measuring the [<sup>35</sup>S] to [<sup>3</sup>H] ratio for each protein by liquid scintillation counting. (C) In vitro translation assay of EBNA1 and EBNA1ΔGA. pcDNA3.1 expression constructs encoding either EBNA1 or EBNA1ΔGA were transcribed and translated in vitro with T7 RNA polymerase using a coupled transcription-translation reticulocyte lysate system supplemented with [<sup>35</sup>S]methionine. Band intensities were quantified by densitometric analysis of the imaging data and graphed.

Second, a more rapid degradation of these polypeptides was observed in cells not treated with proteasome inhibitors. In contrast, pretreatment of the transfectants with MG132 and lactacystin blocked the degradation of these polypeptides.

No GFP-specific protein bands were observed under similar conditions from mock-transfected cells (Fig. 1 A). These observations strongly suggest that the observed polypeptide smears represent EBNA1-specific RDPs and not cellular proteins binding nonspecifically to anti-GFP immunocomplexes.

To further explore the difference in the translation efficiencies of EBNA1 and EBNA1 $\Delta$ GA, we measured absolute rates of synthesis relative to a common standard protein using a double label protocol (20). HEK293 cells were transfected with expression vectors encoding either EBNA1-GFP or EBNA1 $\Delta$ GA-GFP, and 24 h after transfection the cells were labeled with a low concentration of [<sup>3</sup>H]methionine (12–14 h), followed by a short pulse (30 min) with [<sup>35</sup>S]methionine. The amount of tritium label per cell serves as the standard against which to measure the efficacy of incorporation during the pulse. By examining the ratio of label for a standard protein by immunoprecipitation (e.g., tubulin), we estimated the rates of synthesis for EBNA1-GFP and EBNA1 $\Delta$ GA-GFP. Total incorporation of [<sup>35</sup>S]methionine during the pulse relative to the total amount of [<sup>3</sup>H]methionine allowed us to determine whether EBNA1 $\Delta$ GA-GFP was synthesized at a rate greater, equal, or less than the full-length EBNA1-GFP. Representative data from one of these experiments is presented in Fig. 1 B. As expected, the rate of synthesis for tubulin was very similar in HEK293 cells transfected with expression vectors encoding EBNA1-GFP or EBNA1 $\Delta$ GA-GFP. In contrast, the rate of synthesis for EBNA1 $\Delta$ GA-GFP was 3.7 times higher compared with full-length EBNA1-GFP. These observations were further supported by an *in vitro* translation assay that demonstrated increased translation of EBNA1 $\Delta$ GA compared with EBNA1 (Fig. 1 C). These experiments collectively provide strong evidence that a substantial fraction of newly synthesized EBNA1 protein is composed of RDPs and that the deletion of the GAr domain domain dramatically increases the translation efficiency of these polypeptides.

#### **Influence of translation efficiency of EBNA1 and EBNA1 $\Delta$ GA on endogenous processing of a model CD8<sup>+</sup> T cell**

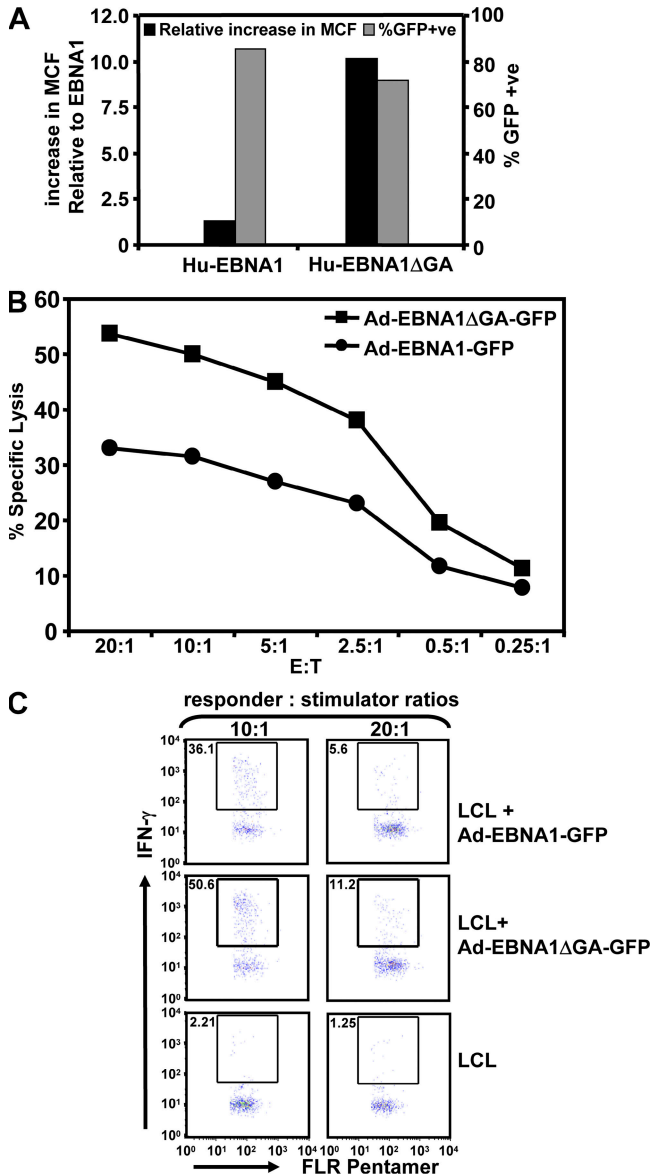
The data presented in Fig. 1 provide strong evidence that translation efficiency of the EBNA1 protein with or without its internal GAr domain can directly influence the rate of production of RDPs. Since previous studies have proposed that the majority of the CD8<sup>+</sup> T cell epitopes may be derived from RDPs, we designed a series of experiments to determine whether increased production of RDPs enhances the presentation of these epitopes. We assessed the endogenous processing and surface presentation of two different model CD8<sup>+</sup> T cell epitopes inserted within EBNA1-GFP and EBNA1 $\Delta$ GA-GFP. These epitopes were the H-2K<sup>b</sup>-restricted CTL epitope from ovalbumin, SIINFEKL (referred to as SIIN), and the HLA B8-restricted CTL epitope, FLRGRAYGL (referred to as FLR) from an EBV-encoded EBNA3 protein. In the first set of these experiments H-2K<sup>b</sup>-expressing HEK293 cells were transiently transfected with either EBNA1-GFP or EBNA1 $\Delta$ GA-GFP expression constructs each with or without

the SIIN epitope. At 48 h after transfection these cells were assessed for cell surface expression of H-2K<sup>b</sup>-SIIN complexes using a monoclonal antibody (25-D1.16) that recognizes the SIIN epitope bound to H-2K<sup>b</sup> molecules (21). Representative data from one such experiment is presented in Fig. 2 A.

Consistent with the data presented above, a higher level of 25-D1.16 antibody binding was observed when these cells were transfected with an expression construct encoding EBNA1 $\Delta$ GA-GFP compared with EBNA1-GFP. To further confirm these observations using an antigen presentation assay based on CTL recognition, full-length EBNA1 and EBNA1 $\Delta$ GA were expressed in an HLA B8-positive human keratinocyte (SVMR6) cell line with subsequent assessment of CTL lysis of these cells by an FLR-specific CD8<sup>+</sup> T cell clone, LC13 (22). To ensure that the level of antigen expression was not limiting, SVMR6 cells were infected with recombinant adenovirus expressing EBNA1 or EBNA1 $\Delta$ GA at a multiplicity of infection of 80:1. Data presented in Fig. 2 B show that lysis of target cells expressing EBNA1 $\Delta$ GA by LC13 T cells was 1.8–2-fold higher compared with the cells expressing full-length EBNA1. To further confirm this observation, we tested the endogenous presentation of CD8<sup>+</sup> T cell epitopes using B cells as antigen-presenting cells. HLA B8-positive lymphoblastoid cell line (LCLs) transformed with the B95.8 virus (this virus encodes a mutated sequence of the FLR epitope) were infected with a recombinant adenovirus (MOI 80:1) encoding either EBNA1 or EBNA1 $\Delta$ GA and then used as stimulators to activate FLR-specific CD8<sup>+</sup> T cell responses from an HLA B8 EBV-seropositive individual. EBNA1 and EBNA1 $\Delta$ GA include a model FLR epitope within the coding sequence. Responder to stimulator ratios of 10:1 and 20:1 were used in these assays. The activation of FLR-specific T cells was assessed using a combination of HLA B8-FLR pentamer and costaining for intracellular IFN- $\gamma$  expression. Representative data from one such experiment are presented in Fig. 2 C. These studies showed that IFN- $\gamma$  production by FLR-specific T cells was 1.4–2-fold higher when LCLs expressing EBNA1 $\Delta$ GA were used as stimulators. The differences in the levels of T cell stimulation were similar to the data obtained with cytotoxicity assays (Fig. 2 B).

#### **Endogenous processing of HLA B35-restricted epitopes from recombinant EBV-infected LCLs expressing EBNA1 and EBNA1 $\Delta$ GA**

Because most of the data presented in Fig. 2 were based on model epitopes inserted into the coding sequence of EBNA1, it was important to demonstrate that the effect of protein translation can also be observed for an epitope derived from EBNA1 itself. To address this issue we constructed a recombinant EBV isolate carrying an EBNA1 $\Delta$ GA sequence from a B95-8 EBV BAC using lambda red recombinase as previously described (23). The EBV BAC comprising either full-length EBNA1 or EBNA1 $\Delta$ GA was transferred into HEK293 cells, and stable transformants were induced for virus replication by treatment with phorbol ester, butyrate, and infection with a recombinant BZLF1 adenovirus. Viral supernatants



**Figure 2. Endogenous processing of CD8<sup>+</sup> T cell epitopes from EBNA1 and EBNA1ΔGA.** (A) Detection of H-2K<sup>b</sup>-SIIN complexes on the surface of 293KbC2 cells transfected with constructs expressing EBNA1 or EBNA1ΔGA. Data shown are the percentage of GFP-positive events (right axis) and the relative increase in median fluorescence for GFP-positive events from transfectants with SIIN-expressing constructs over controls without SIIN, with the value for EBNA1ΔGA made relative to that for EBNA1, which was set to 1 (left axis). (B) CTL recognition of an endogenously processed HLA B8-inserted epitope (FLR) in EBNA1 and EBNA1ΔGA. SVMR6 keratinocytes infected with a recombinant adenovirus encoding either EBNA1 (Ad-EBNA1) or EBNA1ΔGA (Ad-EBNA1ΔGA) were used as targets in a standard <sup>51</sup>Cr-release assay to assess CTL activity. An HLA B8-restricted FLR-specific CTL clone, LC13, was used as an effector in this assay. The multiplicity of infection was 80:1 for this assay, whereas the E to T ratio varied from 20:1 to 0.25:1. These data are representative of three separate experiments. (C) Ex vivo stimulation of FLR-specific CD8<sup>+</sup> T cells by EBNA1 and EBNA1ΔGA. HLA B8-positive LCLs were infected with expression vectors encoding EBNA1-GFP or EBNA1ΔGA-GFP (both modified to include FLR encoding sequences;

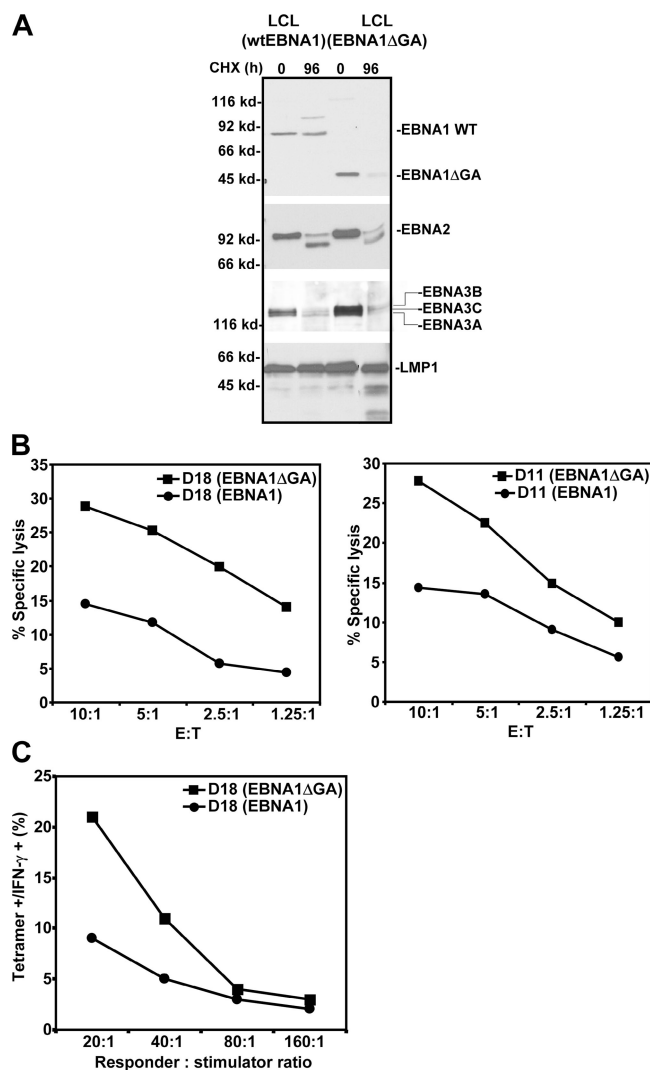
were collected from these transfectants and were used for generating two different sets of LCLs from HLA B35<sup>+</sup> individuals (referred to as D11 and D18). In the first instance, we assessed the expression of EBV latent genes in these LCLs (Fig. 3 A). LCLs expressing full-length EBNA1 or EBNA1ΔGA showed comparable levels of all EBV latent antigens. To assess the intracellular stability of these latent proteins, these LCLs were incubated with cycloheximide (5 μg/ml), and the expression of these proteins was assessed after 96 h using SDS-PAGE and immunoblotting. As reported previously, full-length EBNA1 was comparably more stable than EBNA1ΔGA (13), whereas the other EBV latent proteins showed very similar intracellular stabilities.

Having established that these cell lines expressed the full complement of EBV latent genes including EBNA1, we next assessed the endogenous presentation of an HLA B35-restricted CTL epitope (HPVGEADYFEY), which is encoded within the EBNA1 sequence (residue number 407–417). A HPV-specific CTL line was used as effector cells in a standard <sup>51</sup>Cr-release assay. Data presented in Fig. 3 B clearly show that LCLs from both D11 and D18 donors, transformed with EBV encoding EBNA1ΔGA, were recognized by HPV-specific CTLs more efficiently compared with the paired LCLs carrying the full-length EBNA1 sequence. The difference in the level of CTL lysis for both cell lines was almost twofold, which was very similar to the levels observed in the earlier assays using the FLR-inserted epitope (Fig. 2). To further confirm these observations we also assessed the endogenous presentation of the HPV epitope using an intracellular cytokine assay. HPV-specific T cells were stimulated with D18 LCLs at different responder to stimulator ratios, and then the cells were assessed for HLA B35-HPV pentamer binding and costained for intracellular IFN-γ expression. Data presented in Fig. 3 C show that D18 LCLs expressing EBNA1ΔGA were more efficient in inducing IFN-γ expression in HPV-specific T cells compared with paired LCLs expressing full-length EBNA1. These data indicate that CTL epitopes encoded within EBNA1ΔGA are more efficiently processed and presented through the class I pathway, and this presentation correlates with the enhanced translation efficiency of EBNA1ΔGA.

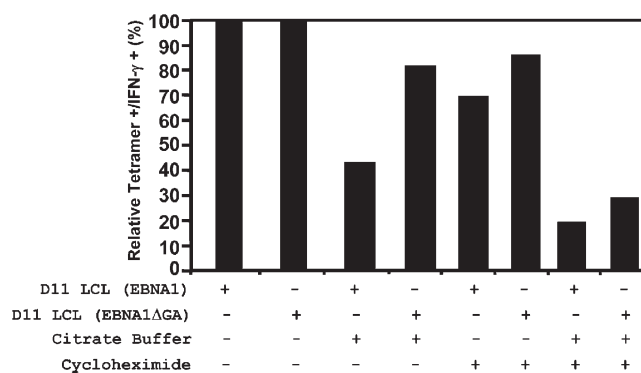
Although the data presented in Figs. 2 and 3 indicated that translation efficiency of target antigens directly impacts on the endogenous processing and presentation of CD8<sup>+</sup> T cell epitopes, it is also possible that the intracellular turnover of EBNA1 or EBNA1ΔGA may also contribute toward endogenous processing and presentation. Previous studies from our laboratory have shown that EBNA1ΔGA displays a shorter half-life in human B cells compared with full-length

(see Materials and methods). These cells were used as stimulators to activate FLR-specific CD8<sup>+</sup> T cells in fresh PBMCs. Responder to stimulator ratios of 10:1 and 20:1 were used in this assay. After a 6-h incubation, FLR-specific CD8<sup>+</sup> T cells were assessed for intracellular IFN-γ expression using a Cytofix/Cytoperm kit (BD Biosciences).





**Figure 3. Endogenous processing of EBNA1 epitopes in B cells infected with recombinant EBV.** (A) Immunoblot of EBV latent gene expression of B cells immortalized with a wild-type EBV BAC (LCL wtEBNA1) or an EBV BAC carrying a GAR-deleted EBNA1 (LCL EBNA1 $\Delta$ GA). Expression without and after 96 h of cycloheximide treatment is shown. EBNA1, EBNA-2, and LMP1 immunoblots were probed with the OTX1, PE2, and S12 monoclonal antibodies. EBNA-3 expression was detected with EBV immune human sera. (B) CTL recognition of an endogenously processed HLA B35-restricted epitope (HPV) in LCL wtEBNA1 and LCL EBNA1 $\Delta$ GA. LCLs transformed with recombinant EBV encoding either EBNA1 or EBNA1 $\Delta$ GA (D18 and D11) were used as targets in a standard  $^{51}\text{Cr}$ -release assay to assess CTL activity. An HLA B35-restricted HPV-specific CTL line was used as the effector in this assay. (C) Ex vivo stimulation of HPV-specific CD8 $^{+}$  T cells by LCLs transformed with recombinant EBV encoding either EBNA1 or EBNA1 $\Delta$ GA (D18). These LCLs were used as stimulators to activate HPV-specific CD8 $^{+}$  T cells. Responder to stimulator ratios of 20:1, 40:1, 80:1, and 160:1 were used in this assay. After a 6-h incubation, HPV-specific CD8 $^{+}$  T cells were assessed for intracellular IFN- $\gamma$  expression using a Cytotfix/Cytoperm kit (BD Biosciences).



**Figure 4. Effect of MHC-peptide stripping and cycloheximide treatment on ex vivo intracellular IFN- $\gamma$  production by specific T cells generated to the EBNA1 epitope (HPVGEADYFEY).** PBMCs from an HLA B35-positive donor were incubated with HLA B35 $^{+}$  donor LCLs, D11 (encoding either full-length EBNA1 or EBNA1 $\Delta$ GA), which had been untreated or treated with citrate phosphate (pH 3) buffer, cycloheximide (50  $\mu\text{M}$ ), or treated with both cycloheximide (50  $\mu\text{M}$ ) and citrate buffer. Data shown represents the relative HPV pentamer-positive population producing IFN- $\gamma$ .

EBNA1. Thus, it is possible that the rapid degradation of EBNA1 $\Delta$ GA may also enhance the endogenous presentation of the HPV epitope from these homologous proteins. To explore this possibility, we used a modified ex vivo stimulation assay described for Fig. 3 C. In this experiment, PBMCs from an HLA B35 $^{+}$  EBV-seropositive individual were stimulated with MHC-peptide-stripped D11 LCLs (encoding either full-length EBNA1 or EBNA1 $\Delta$ GA) that had been incubated in the presence or absence of cycloheximide to inhibit new protein synthesis. These cells were then assessed for HLA B35-HPV pentamer binding and costained for intracellular IFN- $\gamma$  expression. Data presented in Fig. 4 show that pretreatment of antigen-presenting cells with citrate buffer to strip MHC-peptide, followed by incubation with cycloheximide, considerably reduced the proportion of intracellular IFN- $\gamma$ -expressing T cells within the HLA B35-HPV pentamer-positive population by almost 70–80%. In contrast, LCLs treated with cycloheximide alone had only a minimal effect on the activation of pentamer-positive T cells, whereas citric acid treatment showed a differential effect on LCLs expressing either full-length EBNA1 or EBNA1 $\Delta$ GA which correlated with the rate of RDP expression. Pretreatment of LCLs with cycloheximide and citric acid had minimal effect on the ability of these cells to present synthetic HPV peptide (unpublished data). Furthermore, immediate fixing of cells after cycloheximide treatment showed a similar pattern of T cell activation, although IFN- $\gamma$  expression by these cells was reduced because of the paraformaldehyde treatment of antigen-presenting cells (unpublished data). These observations indicate that regardless of the intracellular half-life, the endogenously processed epitopes from EBNA1 and EBNA1 $\Delta$ GA proteins are predominantly derived from newly synthesized protein. Thus, these results imply that the difference in presentation efficiency of CD8 $^{+}$  T cells epitopes from either

EBNA1 or EBNA1 $\Delta$ GA is primarily determined by their translation efficiency rather than their intracellular stability.

## DISCUSSION

The interaction of the adaptive cellular immune system with virus-infected cells determines the outcome of primary viral infection. In particular, CD8<sup>+</sup> T cells continuously scan the surface of virus-infected cells to detect foreign peptides bound to MHC class I molecules and eliminate these cells either by direct lysis or by secreting cytokines/chemokines which are aimed at limiting the replication of the virus (for review see references 1, 2). In the face of this hostile effector system, viruses have adopted numerous strategies to evade CD8<sup>+</sup> T cells (24, 25). This is particularly highlighted in the case of persistent viruses like EBV, where the virus selectively suppresses the expression of immunodominant antigens (26, 27). Although this may be an ideal mechanism for the virus to escape immune recognition, this strategy can also limit their capacity to maintain a successful latent infection. To overcome this dilemma, viruses have evolved strategies to limit the availability of the antigen by inhibiting protein translation and/or by blocking proteasomal degradation (28). EBV-encoded EBNA1 is a classic example of a viral protein which not only blocks its degradation by proteasomes but also inhibits its translation (11, 12). It has been hypothesized that by limiting its translation, EBNA1 restricts the generation of CD8<sup>+</sup> T cell epitopes in virus-infected cells. Recent studies have indicated that in spite of this inhibitory effect, some CD8<sup>+</sup> T cell epitopes from EBNA1 can be presented on the cell surface of EBV-infected B cells (13–15). Extensive analysis of the endogenous processing of EBNA1 revealed that these epitopes are predominantly generated from DRiPs, which are the byproducts of newly synthesized rapidly degrading polypeptides (13). RDPs constitute ~30% of the polypeptides synthesized in mammalian cells and degraded by proteasomes within 30 min of synthesis (16, 29). The importance of RDPs in T cell recognition has also been emphasized in studies based on other viral infections and tumor antigens, which also showed that endogenously processed CD8<sup>+</sup> T cell epitopes were generated from newly synthesized proteins rather than from the long-lived pool of viral antigens in the cell (30–32).

Considering the importance of RDPs in CD8<sup>+</sup> T cell recognition, it can be postulated that endogenous processing and presentation of MHC class I-restricted epitopes must be influenced by the rate at which proteins are synthesized (28). Thus, proteins with a slower rate of synthesis may generate RDPs less efficiently compared with proteins, which are more efficiently synthesized. Although this hypothesis seems quite plausible, there is no evidence demonstrating a direct relationship between protein translation, RDPs, and endogenous CD8<sup>+</sup> T cell epitope processing. In the present study, two homologous viral proteins have been used to demonstrate the influence of protein translation on CD8<sup>+</sup> T cell recognition. Comparison of the *in vivo* translation of EBNA1 and EBNA1 $\Delta$ GA revealed that these proteins differentially expressed RDPs and the degradation of these polypeptides

could be blocked by the addition of proteasome-specific inhibitors. As a consequence, CD8<sup>+</sup> T cell epitopes from EBNA1 $\Delta$ GA were more efficiently processed, and cells expressing EBNA1 $\Delta$ GA were lysed at higher levels by EBV-specific CTLs compared with cells expressing poorly translated full-length EBNA1. Although this data was generated using a GFP tag at the COOH terminus, it would be interesting to look for EBNA1 and EBNA1 $\Delta$ GA RDPs with a GFP tag at the NH<sub>2</sub> terminus. The endogenous processing of CD8<sup>+</sup> T cell epitopes from EBNA1 $\Delta$ GA is not determined by its intracellular stability but rather by the rate at which newly synthesized polypeptides are produced. Although it is not possible to completely rule out the possibility that some of the CD8<sup>+</sup> T cell epitopes are derived from the degradation of full-length EBNA1 or EBNA1 $\Delta$ GA protein, it is unlikely that this pathway contributes substantially toward the overall pool of epitopes. This argument is based on the observation that blocking new protein synthesis after MHC-peptide stripping considerably reduced the *ex vivo* activation potential of antigen-presenting cells.

Collectively the data presented in this study provides evidence linking protein translation and endogenous processing of CD8<sup>+</sup> T cell epitopes. These observations may have important implications for understanding the hierarchy of T cell responses directed toward multiple antigens within a complex virus and for designing better therapeutic strategies for virus-associated diseases. It is hypothesized that individual proteins within a complex virus, which are more efficiently translated, may provide a larger repertoire of T cell determinants and thus will be more frequently recognized by healthy virus carriers. Furthermore, it may be more advantageous to design therapeutic strategies that prime T cell responses toward viral proteins more efficiently synthesized in human cells. Indeed, previous studies by Liu et al. have shown that a DNA vaccine based on codon-modified human papillomavirus type 16 E7, which was expressed at higher levels compared with the wild-type E7, significantly enhanced CTL induction and antitumor activity (33).

## MATERIALS AND METHODS

**Cell lines.** Cell lines were routinely maintained in RPMI 1640 supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, and 100  $\mu$ g/ml streptomycin plus 10% FCS (referred to as growth medium). EBV-transformed lymphoblastoid cell lines were maintained in growth medium and used as targets for various T cell assays. Two different human epithelial cell lines (SVMR6 and HEK 293) (34–36) were used for EBNA1 half-life analysis or CTL assays. In addition, HEK293 cells stably expressing the mouse class I allele H-2K<sup>b</sup> (referred to as 293KbC2) were also used in the study (37).

### Generation of recombinant EBV encoding EBNA1 and EBNA1 $\Delta$ GA.

A recombinant EBV encoding EBNA1 $\Delta$ GA was constructed from a B95-8 EBV BAC using lambda red recombinase as previously described (23). In brief, the EBNA1 amino terminus (aa residues 1–327) was first deleted from a wild-type BAC. The truncated EBNA1 was repaired with a DNA fragment containing a chloramphenicol selectable marker at –10 nt relative to the EBNA1 translational start site and EBNA1 aa residues 1–89. The chloramphenicol cassette was subsequently removed by the use of Flp recombinase. The EBNA1 $\Delta$ GA sequence was confirmed by nucleotide sequencing. The wild-type B95-8 (encoding full-length EBNA1) or EBNA1 $\Delta$ GA BAC was transferred to 293 cells using an auxotrophic, invasive *Escherichia coli*, and stable

transformants were induced for virus replication by treatment with phorbol ester, butyrate, and infection with a recombinant BZLF1 adenovirus. Viral supernatants were collected and used to generate LCLs from healthy virus carriers (23).

**Generation of EBNA1 expression constructs.** Full-length EBV-encoded EBNA1 and EBNA1 $\Delta$ GA were cloned into the expression vector pcDNA3.1 (Invitrogen) (34). EBNA1 and EBNA1 $\Delta$ GA sequences were also subcloned in-frame with a sequence coding for GFP (pEGFP-N1; Clontech) to generate EBNA1-GFP and EBNA1 $\Delta$ GA-GFP. To enable the analysis of endogenous processing of these proteins, sequences encoding a previously defined HLA B8-restricted, EBNA3 epitope, FLRGRAYGL (referred to as FLR), and a H-2K<sup>b</sup>-restricted epitope SIINFEKL (referred to as SIIN) were inserted into the EBNA1 constructs. The FLR epitope sequence was inserted at the SacII site at nucleotide position 1853 of EBNA1, whereas SIIN was inserted into EBNA1-GFP constructs between the 3' end of the EBNA1 sequence and the start of the GFP sequence. Recombinant adenovirus encoding EBNA1 and EBNA1 $\Delta$ GA were generated by a highly efficient, ligation-based protocol of Adeno-X System (Clontech) (13, 38).

**Pulse-chase experiments for detection of RDPs.** For detection of EBNA1 RDPs, HeLa cells ( $2 \times 10^5$ ) were transiently transfected with either EBNA1-GFP or EBNA1 $\Delta$ GA-GFP in duplicate. 24 h after transfection the cells were incubated at 37°C for 60 min in Met-free medium with the addition of two proteasome inhibitors, 50  $\mu$ M MG132 (Merck Biosciences) and 50  $\mu$ M lactacystin (Merck Biosciences), for the final 30 min. The cells were then pulsed for 2 min with 100  $\mu$ Ci of [<sup>35</sup>S]methionine and chased for 0 and 5 min in growth medium containing 2 mg/ml L-methionine. Cells were then lysed in Tris-buffered saline including 1% Triton X-100 and protease inhibitors, precleared with protein A Sepharose, and lysates were immunoprecipitated with antibody to GFP (Invitrogen). Immunoprecipitated samples were subjected to SDS-PAGE and analyzed by autoradiography.

**Measurement of EBNA1 and EBNA1 $\Delta$ GA protein synthesis.** HEK293 cells ( $4 \times 10^5$ ) were transiently transfected with either EBNA1-GFP or EBNA1 $\Delta$ GA-GFP in duplicate. 24 h after transfection the cells were metabolically labeled at 37°C for 12–14 h in growth medium containing 20  $\mu$ Ci/ml of [<sup>3</sup>H]methionine (Amersham Biosciences) (20). The cells were then washed in phosphate-buffered saline and incubated in methionine-free growth medium for 30 min at 37°C preceding a 30-min pulse with 100  $\mu$ Ci of [<sup>35</sup>S]methionine (GE Healthcare). After the pulse, cells were lysed in Tris-buffered saline, including 1% Triton X-100 and protease inhibitors, precleared with protein A Sepharose, and lysates were immunoprecipitated with an antibody to GFP (Invitrogen) or a monoclonal antibody to  $\beta$ -tubulin (Sigma-Aldrich). Immunoprecipitated samples were added to 10 ml of scintillant fluid, Ultima Gold (PerkinElmer) and counted on a Packard Liquid Scintillation Analyzer, Tri-carb 2100TR.

**In vitro translation assays.** EBNA1 and EBNA1 $\Delta$ GA cDNA in pcDNA3.1 were transcribed and translated in vitro with T7 RNA polymerase using a coupled transcription/translation reticulocyte lysate system (Promega) supplemented with 250  $\mu$ Ci of [<sup>35</sup>S]-methionine (GE Healthcare). Lysates (5  $\mu$ l) were incubated for 5 min at 95°C with Laemmli sample buffer (20  $\mu$ l) and subjected to SDS-PAGE. Gels were fixed, incubated with Amplify (GE Healthcare), dried, and subjected to autoradiography.

**Detection of cell surface H-2K<sup>b</sup>-SIIN.** 293KbC2 cells were transiently transfected with EBNA1- and EBNA1 $\Delta$ GA-containing constructs using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. For each expression construct, the parent construct without SIIN was used as a background control. 48 h after transfection, cells were harvested and stained with SIIN-H-2K<sup>b</sup> complex-specific mAb 25D1.16 conjugated to Alexa Fluor 647 (Invitrogen) (10, 21) (a gift from M. Princiotta and J. Yewdell, National Institute of Allergy and Infectious Diseases, Bethesda, MD). Cells were washed and analyzed by flow cytometry (FACSCalibur,

BD Biosciences and Flowjo software, Tree Star Inc.) for GFP expression and 25D1.16 binding.

**In vitro cytotoxicity assays.** An EBV-specific CTL clone (LC13) was used as an effector in the CTL assays. In some experiments, target cells were transfected with expression vectors encoding EBNA1-GFP or EBNA1 $\Delta$ GA-GFP for 36 h or infected with recombinant adenovirus encoding EBNA1 or EBNA1 $\Delta$ GA and incubated for 14–16 h at 37°C. After incubation, cells were washed in growth medium and labeled with <sup>51</sup>Cr for 60 min. After labeling, cells were washed with growth medium and used as targets in a 5-h <sup>51</sup>Cr-release assay (39). These cells were then used as targets in the CTL assay.

**Cell surface MHC-peptide stripping and antigen presentation assays.** Cell surface MHC-peptide stripping and ex vivo antigen presentation assays were performed as described previously (34). In brief, HLA B8<sup>+</sup>ve LCLs were infected with recombinant adenovirus encoding EBNA1 or EBNA1 $\Delta$ GA and then incubated at 37°C overnight. After incubation, these HLA B8<sup>+</sup>ve-transfected LCLs or donor HLA B35<sup>+</sup>ve LCLs, D11 (encoding either full-length EBNA1 or EBNA1 $\Delta$ GA) were treated with citrate phosphate (pH3) buffer (0.131 M citric acid, 0.066 M Na<sub>2</sub>HPO<sub>4</sub>) for 2 min on ice. The suspension was then neutralized with a 100-fold dilution of growth medium, and cells were washed twice. Aliquots of cells were resuspended in 2 ml growth medium and incubated in the presence or absence of 50  $\mu$ M cycloheximide for 5 h. These LCLs were then mixed with peripheral blood mononuclear cells from either an HLA B8-positive individual or from an HLA B35-positive individual, respectively, at varying responder to stimulator ratios. The responding CD8<sup>+</sup> T cells were assessed for intracellular IFN- $\gamma$  expression using a Cytofix/Cytoperm kit (BD Biosciences).

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## REFERENCES

1. Yewdell, J.W., and S.M. Haeryfar. 2005. Understanding presentation of viral antigens to CD8<sup>+</sup> T cells in vivo: the key to rational vaccine design. *Annu. Rev. Immunol.* 23:651–682.
2. Yewdell, J.W., E. Reits, and J. Neefjes. 2003. Making sense of mass destruction: quantitating MHC class I antigen presentation. *Nat. Rev. Immunol.* 3:952–961.
3. Yewdell, J.W., U. Schubert, and J.R. Bennink. 2001. At the crossroads of cell biology and immunology: DRiPs and other sources of peptide ligands for MHC class I molecules. *J. Cell Sci.* 114:845–851.
4. Goldberg, A.L., P. Cascio, T. Saric, and K.L. Rock. 2002. The importance of the proteasome and subsequent proteolytic steps in the generation of antigenic peptides. *Mol. Immunol.* 39:147–164.
5. Goldberg, A.L., and K.L. Rock. 1992. Proteolysis, proteasomes and antigen presentation. *Nature.* 357:375–379.
6. Shastri, N., S. Schwab, and T. Serwold. 2002. Producing nature's gene-chips: the generation of peptides for display by MHC class I molecules. *Annu. Rev. Immunol.* 20:463–493.
7. Lehner, P.J. 2003. The calculus of immunity: quantitating antigen processing. *Immunity.* 18:315–317.
8. Rock, E.P., P.R. Sibbald, M.M. Davis, and Y.H. Chien. 1994. CDR3 length in antigen-specific immune receptors. *J. Exp. Med.* 179:323–328.
9. Cerundolo, V., A. Benham, V. Braud, S. Mukherjee, K. Gould, B. Macino, J. Neefjes, and A. Townsend. 1997. The proteasome-specific inhibitor lactacystin blocks presentation of cytotoxic T lymphocyte epitopes in human and murine cells. *Eur. J. Immunol.* 27:336–341.
10. Princiotta, M.F., D. Finzi, S.B. Qian, J. Gibbs, S. Schuchmann, F. Buttgerit, J.R. Bennink, and J.W. Yewdell. 2003. Quantitating

- protein synthesis, degradation, and endogenous antigen processing. *Immunity*. 18:343–354.
11. 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.
  12. 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. *Proc. Natl. Acad. Sci. USA*. 94:12616–12621.
  13. Tellam, J., G. Connolly, K.J. Green, J.J. Miles, D.J. Moss, S.R. Burrows, and R. Khanna. 2004. Endogenous presentation of CD8<sup>+</sup> T cell epitopes from Epstein-Barr virus-encoded nuclear antigen 1. *J. Exp. Med.* 199:1421–1431.
  14. 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<sup>+</sup> T lymphocytes. *J. Exp. Med.* 199:459–470.
  15. Lee, S.P., J.M. Brooks, H. Al-Jarrah, W.A. Thomas, T.A. Haigh, G.S. Taylor, S. Humme, A. Schepers, W. Hammerschmidt, J.L. Yates, et al. 2004. CD8 T cell recognition of endogenously expressed Epstein-Barr virus nuclear antigen 1. *J. Exp. Med.* 199:1409–1420.
  16. Yewdell, J.W., L.C. Anton, and J.R. Bennink. 1996. Defective ribosomal products (DRiPs): a major source of antigenic peptides for MHC class I molecules? *J. Immunol.* 157:1823–1826.
  17. Yewdell, J.W. 2005. Serendipity strikes twice: the discovery and re-discovery of defective ribosomal products (DRiPs). *Cell. Mol. Biol.* 51:635–641.
  18. Schubert, U., L.C. Anton, J. Gibbs, C.C. Norbury, J.W. Yewdell, and J.R. Bennink. 2000. Rapid degradation of a large fraction of newly synthesized proteins by proteasomes. *Nature*. 404:770–774.
  19. Blake, N.W., A. Moghaddam, P. Rao, A. Kaur, R. Glickman, Y.G. Cho, A. Marchini, T. Haigh, R.P. Johnson, A.B. Rickinson, and F. Wang. 1999. Inhibition of antigen presentation by the glycine/alanine repeat domain is not conserved in simian homologues of Epstein-Barr virus nuclear antigen 1. *J. Virol.* 73:7381–7389.
  20. Gonzalez-Garay, M.L., and F. Cabral. 1995. Overexpression of an epitope-tagged beta-tubulin in Chinese hamster ovary cells causes an increase in endogenous alpha-tubulin synthesis. *Cell Motil. Cytoskeleton*. 31:259–272.
  21. Porgador, A., J.W. Yewdell, Y. Deng, J.R. Bennink, and R.N. Germain. 1997. Localization, quantitation, and in situ detection of specific peptide-MHC class I complexes using a monoclonal antibody. *Immunity*. 6:715–726.
  22. Burrows, S.R., S.J. Rodda, A. Suhrbier, H.M. Geysen, and D.J. Moss. 1992. The specificity of recognition of a cytotoxic T lymphocyte epitope. *Eur. J. Immunol.* 22:191–195.
  23. Chen, A., M. Divisconte, X. Jiang, C. Quink, and F. Wang. 2005. Epstein-Barr virus with the latent infection nuclear antigen 3B completely deleted is still competent for B-cell growth transformation in vitro. *J. Virol.* 79:4506–4509.
  24. Gandhi, M.K., and R. Khanna. 2004. Human cytomegalovirus: clinical aspects, immune regulation, and emerging treatments. *Lancet Infect. Dis.* 4:725–738.
  25. Hilleman, M.R. 2004. Strategies and mechanisms for host and pathogen survival in acute and persistent viral infections. *Proc. Natl. Acad. Sci. USA*. 101(Suppl 2):14560–14566.
  26. Khanna, R., D.J. Moss, and M. Gandhi. 2005. Applications of emerging immunotherapeutic strategies for Epstein-Barr virus-associated malignancies. *Nature Clinical Practice. Oncology*. 2:138–149.
  27. Khanna, R., and S.R. Burrows. 2000. Role of cytotoxic T lymphocytes in Epstein-Barr virus-associated diseases. *Annu. Rev. Microbiol.* 54:19–48.
  28. Apcher, S., R. Fahraeus, and B. Manoury. 2004. Epstein-Barr virus: exploiting the immune system by interfering with defective ribosomal products. *Microbes Infect.* 6:1212–1218.
  29. Yewdell, J.W., and C.V. Nicchitta. 2006. The DRiP hypothesis decennial: support, controversy, refinement and extension. *Trends Immunol.* 27:368–373.
  30. Khan, S., R. de Giuli, G. Schmidtke, M. Bruns, M. Buchmeier, M. van den Broek, and M. Groettrup. 2001. Cutting edge: neosynthesis is required for the presentation of a T cell epitope from a long-lived viral protein. *J. Immunol.* 167:4801–4804.
  31. Probst, H.C., K. Tschannen, A. Gallimore, M. Martinic, M. Basler, T. Dumrese, E. Jones, and M.F. van den Broek. 2003. Immunodominance of an antiviral cytotoxic T cell response is shaped by the kinetics of viral protein expression. *J. Immunol.* 171:5415–5422.
  32. Ostankovitch, M., V. Robila, and V.H. Engelhard. 2005. Regulated folding of tyrosinase in the endoplasmic reticulum demonstrates that misfolded full-length proteins are efficient substrates for class I processing and presentation. *J. Immunol.* 174:2544–2551.
  33. Liu, W.J., F. Gao, K.N. Zhao, W. Zhao, G.J. Fernando, R. Thomas, and I.H. Frazer. 2002. Codon modified human papillomavirus type 16 E7 DNA vaccine enhances cytotoxic T-lymphocyte induction and anti-tumour activity. *Virology*. 301:43–52.
  34. Tellam, J., M. Sherritt, S. Thomson, R. Tellam, D.J. Moss, S.R. Burrows, E. Wiertz, and R. Khanna. 2001. Targeting of EBNA1 for rapid intracellular degradation overrides the inhibitory effects of the Gly-Ala repeat domain and restores CD8<sup>+</sup> T cell recognition. *J. Biol. Chem.* 276:33353–33360.
  35. Boukamp, P., R.T. Petrussevska, D. Breitkreutz, J. Hornung, A. Markham, and N.E. Fusenig. 1988. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *J. Cell Biol.* 106:761–771.
  36. Simmons, N.L. 1990. A cultured human renal epithelioid cell line responsive to vasoactive intestinal peptide. *Exp. Physiol.* 75:309–319.
  37. Tschärke, D.C., G. Karupiah, J. Zhou, T. Palmore, K.R. Irvine, S.M. Haeryfar, S. Williams, J. Sidney, A. Sette, J.R. Bennink, and J.W. Yewdell. 2005. Identification of poxvirus CD8<sup>+</sup> T cell determinants to enable rational design and characterization of smallpox vaccines. *J. Exp. Med.* 201:95–104.
  38. Yotnda, P., H. Onishi, H.E. Heslop, D. Shayakhmetov, A. Lieber, M. Brenner, and A. Davis. 2001. Efficient infection of primitive hematopoietic stem cells by modified adenovirus. *Gene Ther.* 8:930–937.
  39. Khanna, R., S.R. Burrows, M.G. Kurilla, C.A. Jacob, I.S. Misko, T.B. Sculley, E. Kieff, and D.J. Moss. 1992. Localization of Epstein-Barr virus cytotoxic T cell epitopes using recombinant vaccinia: implications for vaccine development. *J. Exp. Med.* 176:169–176.