Targeting of EBNA1 for Rapid Intracellular Degradation Overrides the Inhibitory Effects of the Gly-Ala Repeat Domain and Restores CD8+ T Cell Recognition*

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Epstein-Barr virus (EBV)-encoded nuclear antigen 1 (EBNA1) includes a unique glycine-alanine repeat domain that inhibits the endogenous presentation of cytotoxic T lymphocyte (CTL) epitopes through the class I pathway by blocking proteasome-dependent degradation of this antigen. This immune evasion mechanism has been implicated in the pathogenesis of EBV-associated diseases. Here, we show that cotranslational ubiquitination combined with N-end rule targeting enhances the intracellular degradation of EBNA1, thus resulting in a dramatic reduction in the half-life of the antigen. Using DNA expression vectors encoding different forms of ubiquitinated EBNA1 for *in vivo* studies revealed that this rapid degradation, remarkably, leads to induction of a very strong CTL response to an EBNA1-specific CTL epitope. Furthermore, this targeting also restored the endogenous processing of HLA class I-restricted CTL epitopes within EBNA1 for immune recognition by human EBV-specific CTLs. These observations provide, for the first time, evidence that the glycine-alanine repeat-mediated proteasomal block on EBNA1 can be reversed by specifically targeting this antigen for rapid degradation resulting in enhanced CD8+ T cell-mediated recognition *in vitro* and *in vivo*.

Epstein-Barr virus (EBV)[1] establishes a highly immunogenic growth-transforming infection of B lymphocytes associated with the co-ordinate expression of virus-encoded nuclear antigens (referred to as EBNA1–EBNA6). This latent growth-transforming infection elicits a strong CD8+ cytotoxic T lymphocyte (CTL) response directed against all the nuclear antigens except EBNA1 (1–3). EBNA1 is the only viral protein regularly detected in all EBV-associated malignancies (reviewed in Ref. 4). It is now well established that EBNA1 is poorly recognized by the CD8+ CTL-mediated surveillance that prevents the uncontrolled proliferation of EBV-infected B cells *in vivo* (1–3). Furthermore, mammary carcinoma cells transfected with EBNA1 are poorly immunogenic in mice, whereas strong immunogenicity was induced by expression of other EBV proteins that are highly immunogenic in humans, suggesting that CTL evasion occurs across species (5).

Studies by Levitskaya *et al.* (6) showed that an internal glycine-alanine repeat (GAr) domain of EBNA1 acts as a cis-inhibitor of MHC class I-restricted presentation. They proposed that the sequence within the EBNA1 GAr domain may influence the folding pattern of this antigen and affect its capacity to associate with various components of the ubiquitin/proteasome pathway, including ubiquitin conjugation enzymes and/or regulatory subunits of the proteasome. More recent studies by Sharipo *et al.* (7) have shown that although the GAr sequence allows ubiquitination when inserted into the IκB protein sequence, the polyubiquitinated protein is unable to form stable complexes with the proteasome. Based on these observations, it was argued that the GAr domain forms β-sheets that are resistant to unfolding and that block entry into the proteasomal complex.

Prompted by these observations, we constructed a series of EBNA1 expression vectors to determine whether any strategies could be devised to override this GAr-mediated inhibition of MHC class I-restricted presentation of EBNA1. Based on our understanding of proteasomal targeting of cellular and viral proteins (8, 9), these expression vectors were specifically designed to target EBNA1 through the ubiquitin/proteasome pathway to enhance its intracellular degradation. Here, we report that covalent linking of EBNA1 with ubiquitin and targeting to the N-end rule pathway dramatically enhances its intracellular degradation and restores CD8+ T cell recognition.

EXPERIMENTAL PROCEDURES

Construction of Ubiquitin-EBNA1 Chimeras—Full-length EBNA1 was cloned into the expression vector pcDNA3.1 (Invitrogen, San Diego, CA) to generate EBNA1 (Fig. 1A), which encodes nonubiquitinated EBNA1 protein. To generate noncleavable ubiquitin conjugates of EBNA1 in pcDNA3.1, a vector was constructed expressing the EBNA1 coding sequence, EBNA1, fused in-frame to the C terminus of the human ubiquitin-coding sequence. The C-terminal glycine residue (Gly[76]) of human ubiquitin was mutated to an alanine residue (Ala[76]),

[1] The abbreviations used are: EBV, Epstein-Barr virus; EBNA1, EBV-encoded nuclear antigen 1; Ub, ubiquitin; CTL, cytotoxic T lymphocyte; GFP, green fluorescent protein; Cbz-L3, Cbz-leu-leu-leucinal; GAr, glycine-alanine repeat; PT, posttransfection; FLR, the epitope FLRGRAYGL.

which diminishes cleavage of the fusion protein (8), generating Ub-Ala/Met-EBNA1 (Fig. 1*B*). In addition, we constructed an expression vector, Ub-Ala/Arg-EBNA1 (Fig. 1*C*) in which the ubiquitin-Ala[76] conjugate of EBNA1 was expressed in such a way that the N-terminal residue of EBNA1 was modified from Met[1] to Arg[1]. The latter substitution applies the principles of N-end rule targeting and has been shown to decrease the *in vivo* half-life of a protein from greater than 10 h to 3 min (10). For comparison, Arg[1]-EBNA1 was also fused in-frame to the C terminus of unmodified ubiquitin-Gly[76] to generate the expression vector Ub-Gly/Arg-EBNA1 (Fig. 1*D*). Similarly, Met[1]-EBNA1 was also fused in-frame to the C terminus of unmodified ubiquitin-Gly[76] to generate the expression vector Ub-Gly/Met-EBNA1 (Fig. 1*F*). In addition, we constructed an EBNA1 construct in which the GAr domain was deleted, EBNA1-GAr-del (Fig. 1*J*). The above constructs included insertion of a well defined HLA-B8-restricted, EBNA-3 CTL epitope, FLRGRAYGL (referred to as FLR) (21) into the *Sac*II site at nucleotide position 1853 of EBNA1, to allow evaluation of endogenous processing of EBNA1 by using specific CTL clones. To assess expression of EBNA1, EBNA1-GAr-del, and Ub-EBNA1, each of the inserts from the above pcDNA3.1 constructs was subcloned in-frame with a sequence coding for green fluorescent protein (pEGFP-N1, CLONTECH, Palo Alto, CA); EBNA1-GFP (Fig. 1*E*), Ub-Gly/Met-EBNA1-GFP (Fig. 1*F*), Ub-Ala/Met-EBNA1-GFP (Fig. 1*G*), Ub-Ala/Arg-EBNA1-GFP (Fig. 1*H*), Ub-Gly/Arg-EBNA1-GFP (Fig. 1*I*), and EBNA1-GAr-del-GFP (Fig. 1*J*).

Transfection of EBNA1 and Ub-EBNA1 Expression Constructs—Recombinant EBNA1 and Ub-EBNA1 constructs were transiently transfected into either an EBV-negative Burkitt's lymphoma B-cell line (DG75), an EBV-transformed lymphoblastoid cell line (LCL-KK/B95.8), or an EBV-negative keratinocyte cell line (SVMR6). Exponentially growing DG75 or KK/B95.8-LCL cells (5×10^6) were transfected in growth medium with 10 μg of DNA using the Bio-Rad Gene Pulser (960 μF, 250 V, 0.4 cm gap electrode, 300 μl assay volume; 25EC). SVMR6 keratinocytes were also transfected with the pcDNA3.1 expression constructs (Fig. 1, *A–D*) using LipofectAMINE (Invitrogen Life Technologies, Inc.) according to the manufacturer's instructions. Two proteasome inhibitors, Cbz-leu-leu-leucinal (Cbz-L3) and *N*-acetyl-leucyl-leucyl-norleucinal, were added to the cells at a final concentration of 10 and 2 μg/ml, respectively, 24 h after the commencement of transfection. The efficiency of transfection and the effect of proteasome inhibitors on the transfected cells were assessed by FACScan (Becton Dickinson, San Jose, CA) set to measure GFP fluorescence and analyzed with the Cellquest software (Becton Dickinson).

Degradation of EBNA1 and Ub-EBNA1 Proteins in DG75 Cells—DG75 cells were transiently transfected with EBNA1-GFP, Ub-Gly/Met-EBNA1-GFP, Ub-Ala/Met-EBNA1-GFP, Ub-Ala/Arg-EBNA1-GFP, Ub-Gly/Arg-EBNA1-GFP, EBNA1-GFP-GAr-del, and pEGFP-N1 expression vectors as described above. At 30 h posttransfection, cyclohex-imide (25 μg/ml) was added to 8×10^6 cells. Equal aliquots of cells were removed at time points 0 min, 30 min, 1 h, and 2 h; lysed in SDS-polyacrylamide gel electrophoresis sample dye; and resolved under reducing conditions on a 7.5% SDS-polyacrylamide gel.

Detection of EBNA1 and Ub-EBNA1 by Immunoblotting—Cell lysates were prepared by homogenizing 1×10^7 cells suspended in 0.2 ml of 1% SDS/phosphate-buffered saline with a 27 gauge needle. The samples were run on SDS-polyacrylamide gel electrophoresis and electroblotted onto a nitrocellulose membrane, (Hybond C, Amersham Pharmacia Biotech) and incubated with anti-GFP serum (1:2000) or anti-actin antibody (1:1000).

DNA Immunization—DNA was purified using the Qiagen endotoxin-free maxi-prep kit. 6–8-week-old female BALB/c (H-2d) mice were immunized intramuscularly three times, at 14-day intervals, with 50 μg of plasmid DNA. This was dissolved in endotoxin free phosphate-buffered saline at a concentration of 1 μg/ml, and 50 μl was injected into the rear quadriceps muscle using a 28 gauge needle. For the constructs EBNA1, Ub-Ala/Met-EBNA1, and Ub-Ala/Arg-EBNA1, five mice were used in each treatment group. For the pcDNA3.1 vector control and Ub-Gly/Arg-EBNA1, three mice per group were used. Five weeks after the final DNA immunization, mice were killed by cervical dislocation, and spleens were removed for *in vitro* restimulation of CTLs.

Assay for EBNA1-specific CTL Response—Splenocytes (16×10^6) from each mouse were seeded into four wells of a 24-well plate and mixed with peptide-coated (10 μg/ml) -irradiated (2000 rads) LPS blasts (derived from naive Balb/c splenocytes) at a responder to stimulator ratio of 3:1. The H-2K^d-restricted, EBNA1 peptide epitope is VYGGSKTSL (12). After 5 days, the responder cells were harvested, and viable cells were counted. ^{51}Cr-labeled P815 target cells (a H-2d-positive mastocytoma cell line) were incubated in the presence or ab-

sence of the EBNA1 peptide epitope and used as targets in a standard cytotoxicity assay (11).

RESULTS

Covalent Linkage of Ubiquitin with EBNA1 Dramatically Reduces the Intracellular Stability of EBNA1—There is now convincing evidence that proteasomal degradation of intracellular proteins is critically dependent on the covalent linkage of ubiquitin via its C-terminal glycine residue (Gly[76]) to μ-amino groups on lysine residues on the protein substrate, followed by rapid polyubiquitination through the attachment of additional ubiquitin molecules (13–15). Because previous studies have suggested that inefficient targeting of EBNA1 through the ubiquitin/proteasome pathway may be responsible for poor endogenous processing, we reasoned that covalent linkage of EBNA1 to ubiquitin may allow efficient targeting of this protein to the proteasomal complex. To further enhance the proteasomal targeting of our ubiquitinated EBNA1 constructs, we have mutated the Gly[76] residue of ubiquitin to Ala[76], which is known to diminish the cleavage of the fusion protein by greater than 90% (16) while still permitting the fused ubiquitin to act as a substrate for polyubiquitination. In addition to the above modification, we have constructed expression vectors in which ubiquitin conjugates of EBNA1 are expressed in such a way as to generate proteins with N-terminal amino acids other than methionine. Previous studies showed that changing the N-terminal amino acid of a protein (N-end rule targeting) (17) can result in a markedly altered stability of that protein (10, 18–20).

Fig. 1 shows a schematic representation of the EBNA1 constructs used in this study. To determine the intracellular kinetics of ubiquitinated EBNA1, an EBV-negative B cell line (DG75) was transiently transfected with expression vectors pEGFP-N1, EBNA1-GFP, Ub-Gly/Met-EBNA1-GFP, Ub-Ala/Arg-EBNA1-GFP, Ub-Gly/Arg-EBNA1-GFP, Ub-Ala/Met-EBNA1-GFP, or EBNA-GAr-del-GFP, and protein expression was analyzed by SDS-polyacrylamide gel electrophoresis following incubation with cycloheximide. The intensity of EBNA1-GFP band was measured by densitometric analysis. Representative data from this analysis are shown in Fig. 2. Full-length EBNA1-GFP and GFP were highly stable in these cells, and even after a 120-min chase, >90% of the protein was detectable (Fig. 2, *A* and *G*). In contrast, cells transfected with the expression vectors Ub-Ala/Arg-EBNA1-GFP, Ub-Gly/Arg-EBNA1-GFP, Ub-Ala/Met-EBNA1-GFP, or EBNA-GAr-del-GFP showed a dramatic reduction in the stability of EBNA1, with almost 50% of the protein being degraded within the first 60 min (Fig. 2, *C–F,* and Fig. 3). By 120 min, 60–80% of the protein was degraded. EBNA1-GFP protein in cells transfected with the Ub-Gly/Met-EBNA1-GFP construct was relatively stable, with only 20% degradation within the first 60 min (Figs. 2*B* and 3). Densitometric analysis indicated that the half-life for the native form of the EBNA1 was >120 min, whereas the half-life for the ubiquitinated forms with strong degradation signals and also the GAr-deleted form of EBNA1 was between 60 and 120 min (Fig. 3). To demonstrate that the expression of the Ub-EBNA1-GFP protein does not effect cell viability, the immunoblots were also probed with a specific antibody to demonstrate the stability of actin over the time course of the experiment. Representative data from the EBNA1-GAr-del cycloheximide-treated transfectants are shown in Fig. 2*H*. A similar pattern of stable actin expression was also observed for the other transfectants (data not shown). Notably, the cells transfected with Ub-Ala/Met-EBNA1-GFP construct showed two distinct species, whereas other ubiquitinated forms of EBNA1 showed a single species (Fig. 2). Both of these species showed an identical pattern of degradation. It is possible that the low
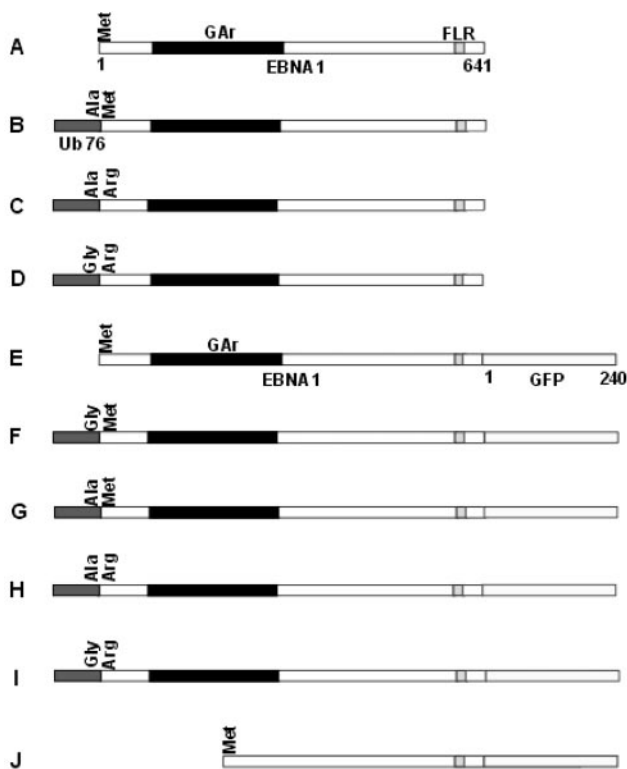
FIG. 1. **Schematic description of EBNA1 and Ub-EBNA1 expression constructs.** Ten plasmids expressing EBNA1 were generated in either pcDNA3.1 (*A–D*) or in pEGFP-N1 (*E–J*) as described under "Experimental Procedures." *A,* full-length EBNA1; *B,* EBNA1 as a covalent fusion with the ubiquitin gene (the native C-terminal amino acid residue of the Ub moiety has been mutated from Gly[76] to Ala[76] (Ub-Ala/Met-EBNA1)); *C,* EBNA1 as a covalent fusion with Ub-Ala[76] in which the N-terminal residue for EBNA1 is substituted from methionine to arginine (Ub-Ala/Arg-EBNA1); and *D,* EBNA1 as a noncovalent fusion with unmodified Ub-Gly[76] in which EBNA1 has the unstable N-terminal arginine residue (Ub-Gly/Arg-EBNA1). In addition, the above constructs were fused in-frame to GFP at the C-terminal end. *E,* native full-length EBNA1 (EBNA1-GFP); *F,* Ub-Gly/Met-EBNA1-GFP; *G,* Ub-Ala/Met-EBNA1-GFP; *H,* Ub-Ala/Arg-EBNA1-GFP; *I,* Ub-Gly/Arg-EBNA1-GFP; *J,* GAr-deleted EBNA1 (EBNA1-GAr-del). The above constructs include insertion of a HLA-B8-restricted EBNA3 CTL epitope, FLRGRAYGL, within the EBNA1 C-terminal sequence, which allows analysis of endogenous processing of EBNA1 using FLR-specific CTL clones.

molecular weight polypeptide may be derived from a distinct cleavage event.

To confirm these results, each of the transfectants were incubated with the proteasome inhibitor Cbz-L3. As shown in Fig. 4, full-length EBNA1 fusion proteins were readily detected in DG75 cells transfected with the EBNA1-GFP and Ub-Gly/Met-EBNA1-GFP expression constructs, and their expression remained unaffected following treatment of the transfected cells with the proteasome inhibitor Cbz-L3. In contrast, cells transfected with the Ub-Ala/Met-EBNA1-GFP, Ub-Ala/Arg-EBNA1-GFP, or Ub-Gly/Arg-EBNA1-GFP constructs displayed low levels of full-length EBNA1-GFP fusion proteins, indicating that specific targeting of EBNA1 through the proteasomal pathway dramatically reduced the stability of this protein. Incubation of these transfected cells with Cbz-L3 led to the detection of significantly enhanced levels of EBNA1-GFP (Fig. 4). Thus, Cbz-L3 prevents intracellular degradation of the ubiquitinated EBNA1-GFP fusion proteins targeted through the proteasomal pathway.

Consistent with the immunoblot assays, GFP fluorescence analysis by FACScan of DG75 cells transfected with the expression vectors encoding EBNA1-GFP, Ub-Ala/Met-EBNA1-

GFP, Ub-Ala/Arg-EBNA1-GFP, or Ub-Gly/Arg-EBNA1 also showed a similar pattern of expression in the presence or absence of the Cbz-L3 inhibitor. Representative data from this analysis are shown in Fig. 5. The number of GFP-positive cells following transfection with the EBNA1-GFP construct remained constant over the 48-h period and increased slightly following treatment with proteasome inhibitor Cbz-L3. In contrast, a reduction in the number of GFP-positive cells was seen over the 48 h period when DG75 cells were transfected with Ub-Ala/Met-EBNA1, Ub-Ala/Arg-EBNA1, or Ub-Gly/Arg-EBNA1, whereas the number of GFP-positive cells increased significantly in the presence of the proteasome inhibitor (Fig. 5). It is important to point out that the number of GFP-positive cells following transfection with ubiquitin-conjugated EBNA1 constructs was consistently lower compared with cells transfected with the EBNA1-GFP construct. This was presumably due to the enhanced degradation of EBNA1 through the proteasome pathway. To ensure that these results were not influenced by the transfection efficiency of the individual constructs, we conducted a longitudinal analysis (at the indicated time points) of GFP expression for each construct in the presence or absence of Cbz-L3. Data from this analysis are presented as the relative percentage of GFP-positive cells (Fig. 6). It is clear from this analysis that the number of GFP-positive cells following transfection with the EBNA1-GFP construct remained almost constant from 24 h posttransfection (PT) to 48 h PT. On the other hand, a gradual reduction in relative GFP-positive cells was observed for Ub-Ala/Met-EBNA1, Ub-Ala/Arg-EBNA1, and Ub-Gly/Arg-EBNA1 transfectants. This reduction was more pronounced in DG75 cells transfected with the Ub-Gly/Arg-EBNA1 construct. A reversal in this reduction of GFP-positive cells was seen following treatment of these transfectants with Cbz-L3 (Fig. 6). Although some increase in relative GFP-positive cells was seen for EBNA1-GFP transfectants in the presence of Cbz-L3, this increment was clearly more enhanced in ubiquitin-conjugated EBNA1 constructs. A similar effect on GFP expression was also observed in the presence of another proteasome inhibitor *N*-acetyl-leucyl-leucyl-norleucinal (data not shown). These data clearly demonstrate that covalent linkage to ubiquitin and N-end rule targeting of EBNA1 overrides the GAr-mediated inhibitory effect on the proteasome-mediated intracellular degradation and thus may enhance endogenous presentation of CTL epitopes from EBNA1.

Covalent Ubiquitin Linkage and N-end Rule Targeting Dramatically Enhance the EBNA1-specific CTL Response in Vivo—To test the hypothesis that the rapid degradation of EBNA1 through the ubiquitin-proteasomal pathway may enhance *de novo* CTL responses *in vivo*, five different groups of BALB/c mice were immunized with plasmid DNA vectors encoding either EBNA1, Ub-Ala/Met-EBNA1, Ub-Ala/Arg-EBNA1, Ub-Gly/Arg-EBNA1, or pcDNA3.1 vector alone. Following immunization, the CTL response against an H-2K[d]-restricted EBNA1 CTL epitope (VYGGSKTSL) (12) was assessed. As shown in Fig. 7, mice immunized with the EBNA1 plasmid consistently showed low to undetectable levels of CTL activity against the VYGGSKTSL epitope (Fig. 7*A*). Detection of low levels of EBNA1-specific responses in mice immunized with native forms of EBNA1 is consistent with the earlier observation in humans, in which occasional EBNA1-specific responses have also been detected in healthy virus carriers (22). In contrast, a significantly enhanced EBNA1-specific CTL response was observed following immunization with expression vectors encoding ubiquitinated EBNA1 (Ub-Ala/Met-EBNA1, Ub-Ala/Arg-EBNA1, and Ub-Gly/Arg-EBNA1) (Fig. 7, *B–D*, respectively). No EBNA1-specific CTL response was detected in

FIG. 2. **Ubiquitination enhances intracellular degradation of EBNA1.** DG75 cells were transfected with expression constructs EBNA1-GFP (*A*), Ub-Gly/Met-EBNA1-GFP (*B*), Ub-Ala/Arg-EBNA1-GFP (*C*), Ub-Gly/Arg-EBNA1-GFP (*D*), EBNA1-GAr-del-GFP (*E*), Ub-Ala/Met-EBNA1-GFP (*F*), and the control plasmid pEGFP-N1 (*G*). At 30 h posttransfection, the cells were degraded over a 2-h time course in the presence of cycloheximide as described under "Experimental Procedures." The stability of actin was monitored over the time course for each of the above expression constructs, and a representative immunoblot is shown in *H*. Molecular weight standards are indicated at the side of each panel.
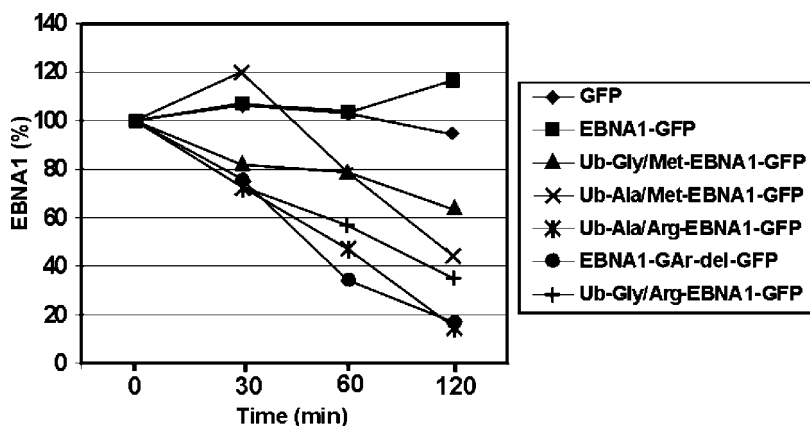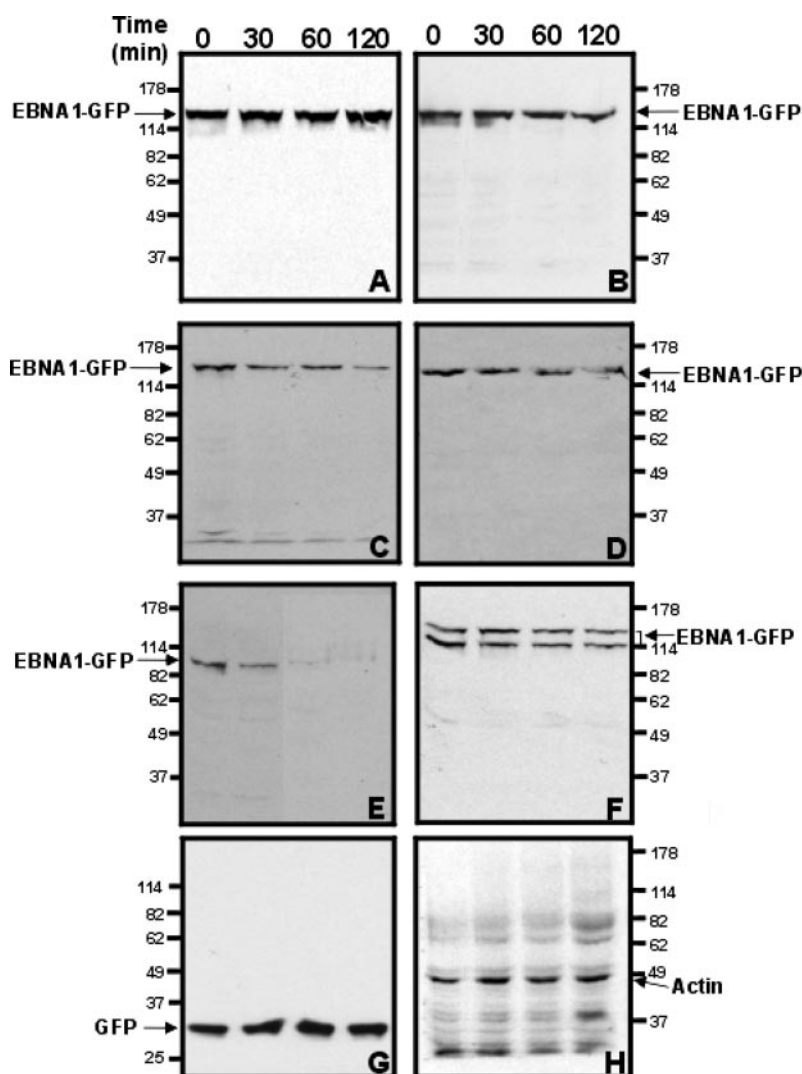
FIG. 3. **Densitometric analysis of EBNA1-GFP expression.** Band intensities were quantified by analysis of the imaging data and plotted as a relative percentage of the signal at time 0 for EBNA1-GFP, Ub-Gly/Met-EBNA1-GFP, Ub-Ala/Met-EBNA1-GFP, Ub-Ala/Arg-EBNA1-GFP, Ub-Gly/Arg-EBNA1-GFP, and EBNA1-GAr-del-GFP.

mice immunized with the pcDNA3.1 vector alone (data not shown). Epitope-specific lysis by CTLs recovered from the ubiquitinated EBNA1 mice was, on average, 2–3-fold higher than that of CTLs recovered from mice immunized with EBNA1. These results support the conclusions drawn from the data shown in Figs. 2 and 5, as well as demonstrating that ubiquitination of EBNA1 enhances the presentation of CTL epitopes within EBNA1, which in turn results in the induction of strong epitope-specific CTL responses *in vivo*.

Specific Targeting of EBNA1 through the Ub-Proteasome Pathway Restores Endogenous Processing of HLA Class I-re-

stricted CTL Epitopes within EBNA1—Although data presented in Fig. 7 clearly showed that rapid degradation of EBNA1 can enhance *in vivo* CTL responses in a murine model, it was important to further confirm these observations by using human class I-restricted EBV-specific CTLs. As shown in Fig. 1, each of the EBNA1 constructs included an HLA B8-restricted CTL epitope, FLR, within the EBNA1 C-terminal sequence, which allowed the study of endogenous processing of EBNA1 using FLR-specific human CTL clones. An EBV-negative, HLA B8-positive human keratinocyte line (SVMR6) was transfected with expression vectors encoding EBNA1, Ub-Ala/
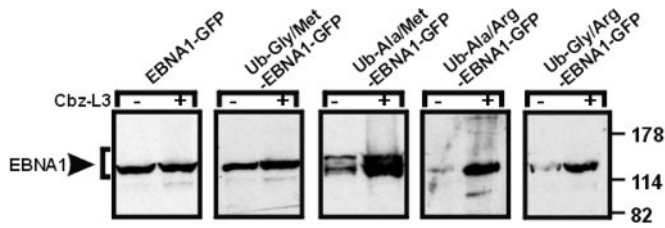
FIG. 4. **Effect of the proteasomal inhibitor Cbz-L3 on the stability of EBNA1.** Duplicate aliquots of DG75 cells were transfected with control plasmid pEGFP-N1 (*lanes 1* and *2*) or expression constructs EBNA1-GFP, Ub-Gly/Met-EBNA1-GFP, Ub-Ala/Met-EBNA1-GFP, Ub-Ala/Arg-EBNA1-GFP, and Ub-Gly/Arg-EBNA1-GFP; 36 h later, the proteasome inhibitor Cbz-L3 was added at a final concentration of 10 μM for 12 h to one of each duplicate. Cell lysates were separated by electrophoresis for immunoblotting with the GFP-specific antibody. The absence (−) or presence (+) of Cbz-L3 is indicated.

Met-EBNA1, and Ub-Ala/Arg-EBNA1 or transfected with the vector control pcDNA3.1. These transfectants were maintained in RPMI 1640/10% fetal calf serum medium supplemented with G418. Following G418 selection for 2–4 weeks, EBNA1 expression in transfectants was confirmed and tested for endogenous processing of CTL epitopes in a standard ^{51}Cr-release cytotoxicity assay using a panel of three FLR-specific CTL clones. Representative data from several different experiments are shown in Fig. 8*A*. Consistent with previous studies (22), SVMR6 cells transfected with the expression vector encoding EBNA1 were poorly recognized by FLR-specific CTL clones, as were SVMR6 cells transfected with the pcDNA3.1 expression vector. This lack of immune recognition of EBNA1 transfectants by FLR-specific CTL clones was not due to inadequate antigen expression, as immunoblot analysis clearly showed strong protein expression in these transfectants (data not shown). In contrast, significantly higher lysis of SVMR6 transfectants expressing ubiquitinated forms of EBNA1 (Ub-Ala/Met-EBNA1 or Ub-Ala/Arg-EBNA1) was observed following exposure to FLR-specific CTL clones LC13, DD1, and GA18 (Fig. 8*A*). It is important to point out that this increased lysis is in accordance with the decreased stability of ubiquitinated forms of EBNA1 (as shown in Figs. 2, 5, and 6), which results in lower levels of EBNA1 expression in SVMR6 cells transfected with the Ub-Ala/Arg-EBNA1 construct. Similar results were also obtained with a B cell line transfected with these expression vectors (data not shown). The FLR-specificity of this CTL lysis was confirmed by the lack of immune recognition by another HLA B8-restricted CTL clone, LC7, which is specific for the QAKWRLQTL epitope (3) from EBV (Fig. 8*A*). CTL lysis of transfectants expressing ubiquitinated EBNA1 (Ub-Ala/Arg-EBNA1) was blocked following addition of the proteasome inhibitor, Cbz-L3 (Fig. 8*B*). Similarly, inhibition of epitope specific lysis of transfectants expressing ubiquitinated EBNA1 (Ub-Ala/Arg-EBNA1) protein was demonstrated following incubation with an anti-class I antibody (W6/32) (Fig. 8*B*).

To exclude the possibility that the observed FLR-specific CTL recognition was due to exogenously transferred EBNA1 protein or peptides, coculture experiments were conducted. SVMR6 transfectants were mixed with a ^{51}Cr-labeled, EBV-transformed lymphoblastoid cell line (HLA B8-positive LC/B95.8, which expresses a mutated form of FLR epitope) to test for transfer of EBNA1 protein or FLR epitope from the transfected SVMR6. The FLR-specific CTL clone LC13 was then added, and lysis of ^{51}Cr-labeled LC/B95.8 cells was assessed. No lysis of LC/B95.8 cells was observed under these conditions. Under identical conditions ^{51}Cr-labeled SVMR6 transfectants or peptide sensitized LC/B95.8 cells were efficiently recognized (data not shown). Collectively, these observations demonstrate that the GAr-mediated inhibitory effect on intracellular deg-

radation of EBNA1 can be reversed by specifically targeting this protein through the ubiquitin/proteasome degradation pathway.

DISCUSSION

Adaptation of a successful latent infection by lymphocryptoviruses, such as EBV, within the cells of the immune system has been achieved by evolving unique mechanisms to negate the hostile effects of the host immune system (2, 3, 5). One classic example is the ability of the EBNA1 protein to inhibit endogenous presentation of CTL epitopes derived from this protein (6, 7, 12, 22, 23). This inhibitory effect is mediated by a repeat sequence of glycine and alanine residues included within the EBNA1 sequence (6). Recent studies have suggested that the GAr forms β-sheets that are resistant to unfolding and thus block the entry of EBNA1 protein into the proteasome complex (7). Because EBNA1 plays a crucial role in the maintenance of the EBV episome in latently infected normal and malignant cells, protection from CTL-mediated immune control may allow the reservoir of latently infected cells to seed other cellular compartments, where EBV can replicate and be transmitted. More importantly, the evasion of CTL-mediated immune control may also explain how EBV-positive BL cells, which express only EBNA1, can survive in immunocompetent hosts (reviewed in Refs. 2 and 4). Thus, any strategies that are capable of overriding the GAr-mediated inhibitory effect on the intracellular degradation of EBNA1 may have important implications for developing therapeutic protocols for the treatment of EBV-associated malignancies.

In the present study, we have targeted EBNA1 through the ubiquitin-proteasome pathway to enhance its intracellular degradation. This approach was primarily designed on the earlier insights into proteasomal targeting of cellular and other viral proteins (8). Considering the importance of stable ubiquitination on intracellular degradation of cellular proteins, we reasoned that covalent linkage of ubiquitin combined with N-end rule targeting may allow efficient unfolding of EBNA1 and subsequent degradation by the proteasomal complex. This hypothesis was also supported by the previous studies of Levitskaya *et al.* (23), who showed that the EBNA1 polypeptide is poorly ubiquitinated *in vitro*. Indeed, we show here that this approach is extremely effective in overriding the GAr-mediated inhibitory effect on EBNA1 degradation in human cells. Due to effective proteasomal degradation, covalently linked ubiquitinated forms of EBNA1 are rapidly degraded in transfected cells, but this low expression is completely reversed in the presence of a ubiquitin-proteasome inhibitor Cbz-L3. Kinetic analysis indicated that covalent linking of ubiquitin in combination with N-end rule targeting dramatically reduces the intracellular half-life of EBNA1. These results provided a first indication that EBNA1 may not be completely resistant to proteasomal degradation as previously proposed. More importantly, the data also suggested that rapid turnover may lead to the presentation of class I-restricted CTL epitopes within EBNA1.

To explore this possibility, we first tested the efficacy of ubiquitinated EBNA1 to induce epitope-specific CTL responses *in vivo* in a murine model. Expression vectors for three different forms of ubiquitinated EBNA1 and a native EBNA1 were used to immunize different groups of mice. Distinct forms of ubiquitinated EBNA1 allowed us to test whether the covalent linkage and/or N-end rule targeting of EBNA1 was essential to enhance the priming of the EBNA1-specific CTL response *in vivo*. As expected, immunization with expression vectors encoding native forms of EBNA1 failed to generate any significant CTL response, whereas all three expression vectors encoding different forms of ubiquitinated EBNA1 induced dramatically
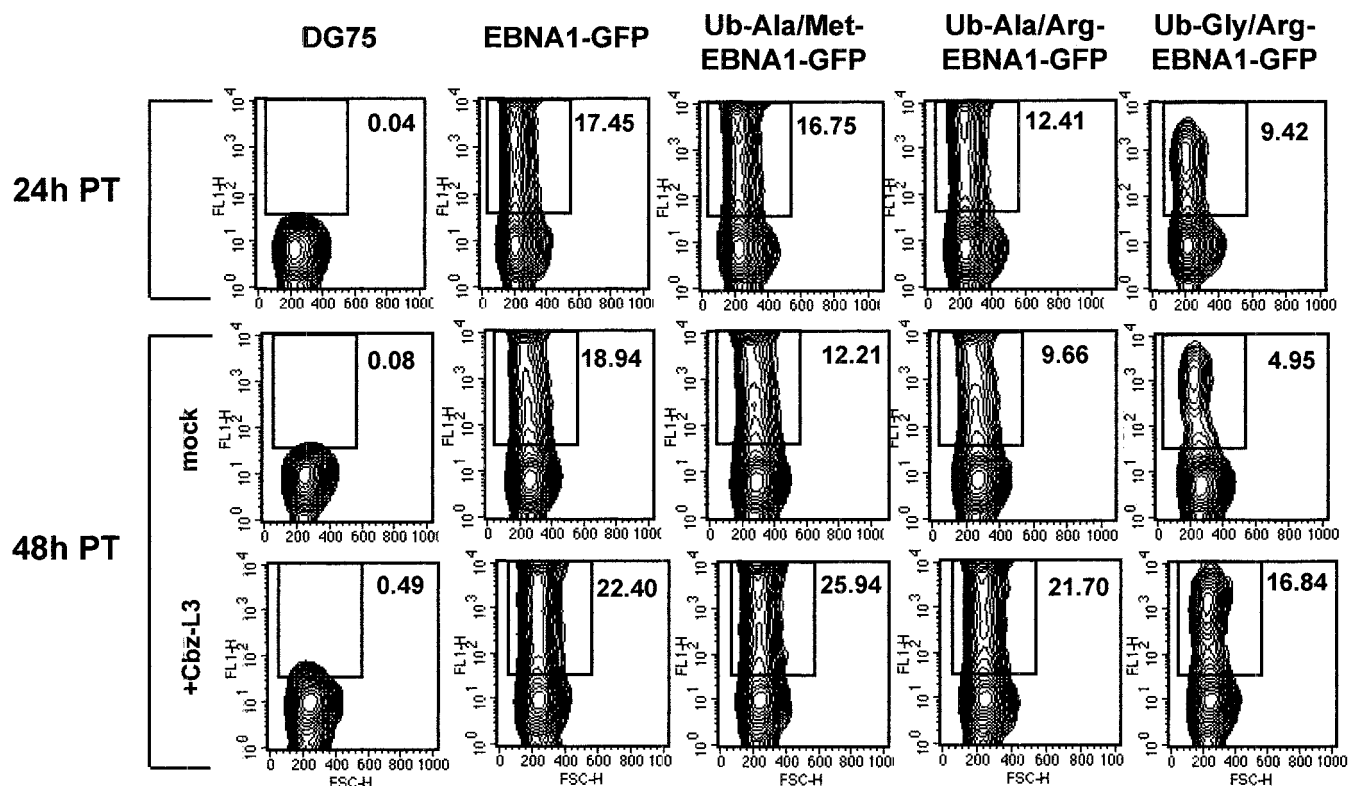
FIG. 5. **FACScan analysis of GFP fluorescence in DG75 cells transfected with expression vectors encoding EBNA1-GFP, Ub-Ala/Met-EBNA1-GFP, Ub-Ala/Arg-EBNA1-GFP, or Ub-Gly/Arg-EBNA1-GFP.** The number of GFP-positive cells were initially assessed at 24 h PT. At the 24-h time point, these transfectants were split into two wells. Cells in one well were treated with Cbz-L3 (10 μM), whereas transfectants in the other well were left untreated. After 24 h (*i.e.* 48 h PT) GFP-positive cells were again assessed by FACScan. The percentage of GFP-positive cells is indicated in each panel.

FIG. 6. **Longitudinal analysis of GFP-fluorescence in DG75 cells transfected with expression vectors encoding EBNA1-GFP, Ub-Ala/Met-EBNA1-GFP, Ub-Ala/Arg-EBNA1-GFP, or Ub-Gly/Arg-EBNA1-GFP.** GFP-positive cells were assessed at 24, 28, 32, 36, and 48 h PT in the absence or presence of the proteasome inhibitor Cbz-L3 (10 μM). Results are expressed as the percentage of GFP-positive cells relative to the number of fluorescing cells at 24 h PT for each transfectant.
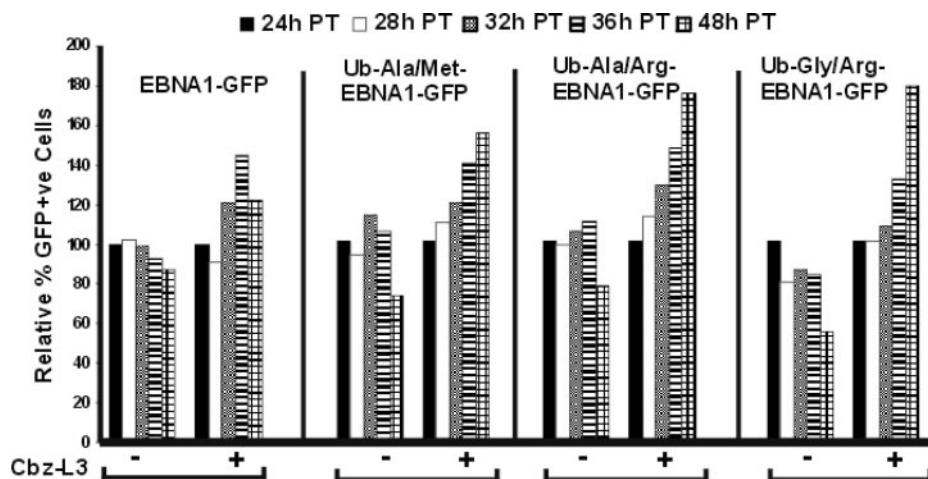
enhanced CTL responses to the EBNA1 epitope. Although overall, CTL induction with the three different forms of ubiquitinated EBNA1 was quite comparable, it is important to mention here that animals immunized with Ub-Ala/Arg-EBNA1 and Ub-Gly/Arg-EBNA1 (the two EBNA1 constructs with an unstable N-terminal residue) consistently induced slightly higher CTL responses. These results are consistent with our earlier conclusions that rapid intracellular degradation enhances CTL epitope presentation from EBNA1 and thus overrides the GAr-mediated inhibitory effect. The only limitation of these results is that one may argue that this CTL priming may involve exogenous loading by myocytes of intact EBNA1 antigen to professional antigen presenting cells through cross priming. Indeed, Blake *et al.* (22) have previously shown that the exogenous presentation of class I-restricted epitopes within EBNA1

is not inhibited by GAr sequences. However, it is unlikely that the CTL responses in our study were induced by the uptake of intact ubiquitinated protein released by myocytes because, as stated above, ubiquitinated EBNA1 is rapidly degraded and is barely detectable in cells expressing this protein. This argument is supported by previous studies that have shown only high levels of antigen can efficiently cross-prime CD8+ T cells, whereas low dose antigens are ignored (24). We therefore favor the hypothesis that the mechanism underlying the CTL priming of EBNA1 involves uptake of DNA, rather than intact soluble protein by the antigen presenting cells in the draining lymph nodes, and that when this DNA encodes a ubiquitinated protein, an enhanced CTL response is observed. This argument is also supported by earlier studies on CTL priming with DNA immunization (25, 26).
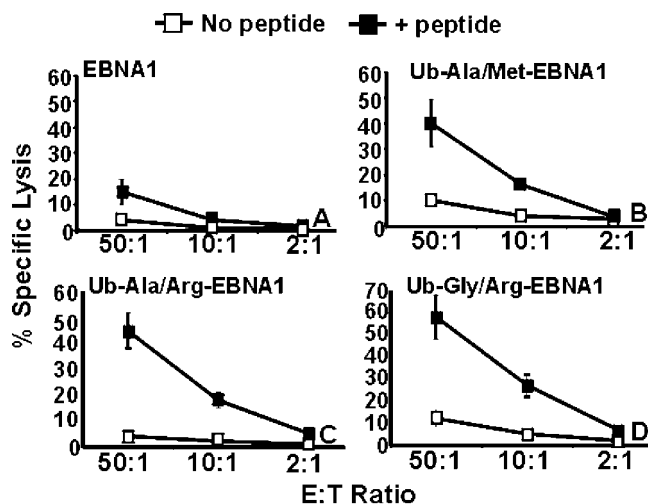
FIG. 7. *In Vivo* **induction of EBNA1-specific CTLs following immunization with Ub-EBNA1 DNA expression vectors.** BALB/c (H-2K^d) mice were immunized intramuscularly with 50 μg of the DNA vectors EBNA1 (*A*), Ub-Ala/Met-EBNA1 (*B*), Ub-Ala/Arg-EBNA1 (*C*), or Ub-Gly/Arg-EBNA1 (*D*), three times, at 14-day intervals. Splenocytes from immunized mice were stimulated for 5 days in the presence of the (H-2K^d)-restricted EBNA1 peptide (VYGGSKTSL). Stimulated splenocytes were then assayed for EBNA1 specific CTL activity against P815 cells pulsed with the (H-2K^d)-restricted EBNA1 peptide (■) or medium alone (□) at effector:target ratios of 50:1, 10:1, and 2:1. The data shown are representative of three separate experiments for *A–C* and one experiment for *D*.
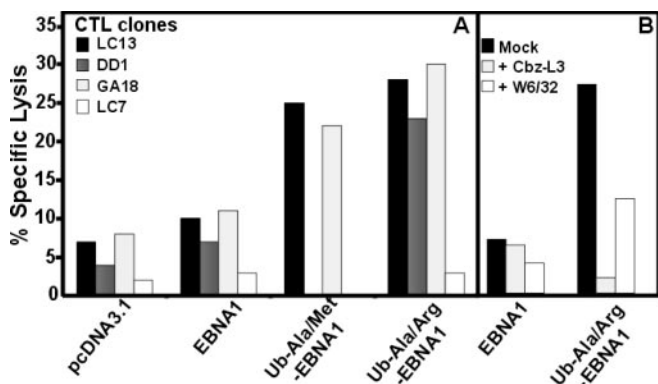

FIG. 8. **CTL recognition of endogenously expressed Ub-EBNA1 chimeric proteins.** *A,* an EBV-negative HLA B8-positive human keratinocyte cell line, SVMR6, was transfected with expression constructs pcDNA3.1, EBNA1, Ub-Ala/Met-EBNA1, and Ub-Ala/Arg-EBNA1. These transfectants were tested in a standard ^{51}Cr-release cytotoxicity assay against a panel of FLR-specific CTL clones (LC13, DD1, and GA18). As a control, these transfectants were also tested against another HLA B8 restricted CTL clone, LC7, which is specific for QAK-WRLQTL peptide. *B,* CTL recognition of Ub-EBNA1 is blocked by proteasome inhibitors. LCL-KK/B95.8 was transiently transfected with the expression constructs EBNA1 and Ub-Ala/Arg-EBNA1 and, after 36 h, with the proteasome inhibitor Cbz-L3 (10 μM). CTL recognition of Ub-EBNA1 is also inhibited in the presence of an anti-class I antibody. LCL-KK/B95.8 cells were transiently transfected with the expression constructs EBNA1 and Ub-Ala/Arg-EBNA1, and after 48 h, the cells were incubated in the absence and presence of the anti-class I antibody (W6/32) followed by testing in a standard ^{51}Cr-release cytotoxicity assay against the FLR clone LC13. Results are displayed as the percentage of specific lysis at an effector:target ratio of 5:1.

To further support our conclusions drawn from the murine model, we also tested endogenous presentation of CTL epitopes within EBNA1 using human EBV-specific CTL assays. This *in vitro* testing was based on the endogenous presentation of a well defined HLA B8-restricted CTL epitope (referred to as FLR) that was inserted into the EBNA1 sequence near the C

terminus. Consistent with our data from the murine model, human keratinocyte and B cell lines transfected with ubiquitinated forms of EBNA1 showed higher levels of CTL-mediated lysis compared with lysis of cells transfected with native EBNA1 alone. This lysis was almost entirely blocked by incubation with the proteasome inhibitor, Cbz-L3, suggesting that specific targeting of EBNA1 through the ubiquitin-proteasome pathway plays an important role in overriding the GAr-mediated inhibitory effect.

Based on the results presented in this study, we speculate that the GAr prevents not only proteasomal degradation but also ubiquitination of native EBNA1 in virus-infected cells. It is likely that the GAr-containing EBNA1 sequence folds in such a way that a stable association with ubiquitin is not possible and that this prevents polyubiquitination and hence results in inadequate targeting of this protein to the proteasomal complex. The covalent linkage of ubiquitin, combined with N-end rule targeting, overrides this blockage and allows efficient presentation of CTL epitopes from EBNA1. These observations are also supported by recent results based on the degradation studies using GAr-fused GFP reporter substrates (27). Overall, the data presented here have important implications for the development of strategies designed to control EBV-associated diseases. It is now well established that not only is EBNA1 expression critical for the long term persistence of EBV, but it also acts as a fundamental factor in the pathogenesis of many EBV-associated malignancies. Targeting this antigen through immunological or biochemical intervention(s) could provide a unique opportunity to manage the pathological consequences of many EBV-associated diseases. Indeed, data presented here provide, for the first time, encouraging evidence that it may be possible to develop therapeutic strategies to modulate the stability of EBNA1 in normal and malignant cells. These strategies may involve treatment of virus-infected cells with synthetic or biological mediators capable of enhancing stable ubiquitination and rapid intracellular degradation of EBNA1 *in vivo*. One such strategy recently proposed by Zhou *et al.* (28) may involve manipulation of the ubiquitin-dependent proteolytic machinery by targeting specific E3 ubiquitin-protein ligases to direct the degradation of otherwise stable cellular proteins, such as EBNA1, in mammalian cells. Potentially, this engineered proteolysis system could be used as a therapeutic method to counteract the proteasomal block conferred on EBNA1 through the *cis*-acting inhibitory GAr domain.

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Targeting of EBNA1 for Rapid Intracellular Degradation Overrides the Inhibitory Effects of the Gly-Ala Repeat Domain and Restores CD8+ T Cell Recognition

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