Herpesviruses are an ancient virus family whose members have long histories of coevolution with their host species (1). A hallmark of herpesvirus biology is the ability to colonize a naive host through the productive (lytic) infection of permissive cells, usually at a mucosal site of virus transmission, and thereafter to persist within that host as a nonproductive (latent) infection of a different specialized cell type. Persistence within the now-immune host is achieved through the down-regulation of all viral antigen expression in latently infected cells. Subsequently, occasional reactivations from latency can serve to reestablish the foci of virus replication at mucosal sites, providing a source of infectious virions for transmission to other individuals.

In the best studied system, HSV, a member of the \( \gamma_1 \) -herpesvirus subfamily establishing latency in neurons, the possibilities of successful reactivation are increased if the virus initially establishes a high latent virus genome load (2, 3); this in turn is reliant on the level of virus replication initially achieved during primary infection of the naive host (3, 4). Because the host CD8\(^+\) T cell response is the principle means of
controlling this initial replication, any factor restricting the efficiency of that control would be to the advantage of the virus. Indeed, HSV was the first herpesvirus in which a CD8+ T cell evasion mechanism was recognized. This involves an immediate early protein of the HSV lytic cycle, ICP47, which inhibits the peptide transporter associated with antigen processing (TAP) by acting as a pseudosubstrate preventing peptide binding and transport. Consequently, TAP cannot pump potentially antigenic peptides from proteasomal digestion in the cytoplasm into the endoplasmic reticulum for loading onto MHC class I molecules (5, 6). Other herpesviruses heavily dependent on virus replication to establish a latent viral load, such as the β-subfamily human and mouse cytomegaloviruses, have likewise been found to possess several CD8+ T cell evasion mechanisms targeting either TAP or MHC class I assembly (7, 8).

The situation has been less clear for lymphocryptoviruses (LCVs), the most recently evolved (γ1) herpesvirus genus, whose members are found only in Old World and some New World primate species and whose prototype is the EBV of humans. These viruses are orally transmitted, replicate in oropharyngeal epithelial sites and establish latency in B lymphocytes (for review see reference 9). When first colonizing the B cell system, LCVs use their unique growth-transforming ability to directly drive the expansion of latently infected B cells and only later down-regulate latent antigen expression to establish an immunologically silent infection of the memory B cell pool (10). This ability to expand the latently infected cell reservoir independent of lytic virus replication has raised debate as to whether LCVs might be under less intense immunological pressure to evade T cell control over the lytic cycle (11).

We were prompted to return to this question by two recent findings in the EBV system, both suggestive of an active immune evasion strategy in the lytic cycle. First, cells productively infected with EBV in vitro showed HLA class I down-regulation and reduced TAP function (12). Second, in vitro assays on latently infected target cells using CD8+ T cell clones specific for immediate early-, early-, or late-expressed EBV antigens indicated much poorer presentation as the lytic cycle progressed (13). In this paper, we show that EBV and other Old World LCVs have indeed acquired an evasion strategy that specifically targets the peptide transporter TAP, thus limiting the supply of antigenic fragments to MHC class I molecules and, ultimately, their presentation to CD8+ T cells.

RESULTS
Screening of γ1-herpesvirus–specific lytic cycle genes for inhibition of CD8+ T cell recognition
We reasoned that any specific immune evasion function associated with EBV replication would most likely map to a lytic cycle gene restricted to the γ1-herpesvirus (LCV) genus. Genomic sequences of HSV, CMV, and Kaposi’s sarcoma–associated herpesvirus, the prototypic α, β, and γ2 human herpesviruses, were aligned alongside those of two Old World γ1 viruses, EBV and rhesus LCV, and with the recently discovered New World γ1 virus of marmosets. This identified 11 LCV-specific genes that were present in the Old World viruses, some of which were also found in the New World γ1 viruses, EBV and rhesus LCV, and with the recently discovered New World γ1 virus of marmosets. This identified 11 LCV-specific genes that were present in the Old World viruses, some of which were also found in the New World virus. 4 out of these 11 genes are centrally positioned in the EBV genome; 2 of the 4, BLLF1 and BZLF2, encode viral envelope glycoproteins known to be involved in B cell entry, whereas the other 2, BLLF2 and BHLF1, were of particular interest because their functions are unknown. The other seven LCV-specific genes all lie within a 35-kb region at the right-hand end of the EBV genome and are identified as open blocks in the genome maps shown in Fig. 1. Two of these, BARF1 and BALF1, have homologies to the cellular CSF-1 receptor and bcl2 genes, respectively, implying functions other than the interception of antigen processing pathways, whereas a third gene, LF3, is deleted in the B95.8 virus strain that displays HLA class I down-regulation in the lytic cycle. The remaining unique genes in this region, BNLF2a, BNLF2b, BILF1, and BILF2, were selected for further study alongside BLLF2 and BHLF1.
All six genes were separately cloned into recombinant vaccinia virus vectors, as was the immune evasion gene from HSV, ICP47, as a positive control. These recombinants were then tested for their ability to inhibit CD8⁺ T cell killing when introduced into target cells along with a second vaccinia vector encoding an indicator target antigen. Such experiments were performed in the two cell types that EBV naturally infects in vivo, epithelial cells and B lymphocytes. Fig. 2 A (top) presents the results from a representative assay in the HLA-B*0801-positive epithelial cell line SVK using EBV nuclear antigen (EBNA) 3A as the target antigen and a CD8⁺ T cell clone specific for an HLA-B*0801-restricted EBNA3A epitope as the effector. Expression of one of the six EBV genes in question, BNLF2a, reduced the level of CD8⁺ T cell killing almost to background levels, as did the positive control ICP47. None of the other EBV genes tested (including BILF2, BLLF2, and BHLF1; not depicted) ever gave such an effect. Fig. 2 A (bottom) presents data from an essentially similar assay conducted in an EBV-transformed B lymphoblastoid cell line (LCL). In this case, the cells were infected with a vaccinia virus encoding, as an indicator antigen, an invariant chain–tagged form of the EBNA3C protein that is processed by the HLA class I and class II pathways, thereby allowing both CD8⁺ and CD4⁺ T cell recognition to be assayed simultaneously on the same target cells. As shown in Fig. 2 A (middle), expression of BNLF2a (and of ICP47) reduced target cell killing by an HLA-B*2705-restricted EBNA3C-specific CD8⁺ T cell clone to the background level seen on unmanipulated LCL targets. This background level represents recognition of preexisting epitopes derived from EBNA3C expressed from the resident EBV genome. In contrast, neither vaccinia BNLF2a nor any of the other proteins tested had any effect on the presentation of invariant chain–tagged EBNA3C to a DQ5-restricted EBNA3C-specific CD4⁺ T cell clone (Fig. 2 A, bottom).

Fig. 2 B shows the results of subsequent experiments conducted using EBV-transformed B cell lines with different HLA alleles to assess any allele-specific effects of BNLF2a on CD8⁺ T cell recognition. These assays used several different EBV latent (EBNA2 and EBNA3B) and lytic (BMLF1, BMRF1, BZLF1, and BGLF4) target antigens encoding epitopes recognized in the context of HLA-A (A*0201 and A*0201), HLA-B (B*0801, B*2705, and B*3801), and HLA-C (C*0101) molecules. We observed marked inhibition of target cell killing by BNLF2a throughout such experiments (Fig. 2 B).

**EBV BNLF2a causes cell-surface HLA class I down-regulation**

To determine the mechanism of BNLF2a-mediated inhibition of CD8⁺ T cell recognition, BNLF2a containing a hemagglutinin (HA) epitope tag (BNLF2aHA) was expressed in the melanoma cell line MelJuSo (MJS) through retroviral transduction. Initially, we performed cytotoxicity assays on the transduced cells to confirm that retrovirus-mediated expression of BNLF2aHA in MJS cells recapitulates the BNLF2a-induced phenotype described in the previous section. Fig. 3 A shows the results of a cytotoxicity assay using the HLA-B*0801-positive MJS cells expressing either BNLF2aHA or GFP as targets. Infection of MJS cells with recombinant vaccinia virus expressing the EBV BZLF1 antigen resulted in recognition by BZLF1-specific CD8⁺ T cells (Fig. 3 A, top), but this was abrogated in BNLF2aHA-expressing MJS cells (Fig. 3 A, bottom). However, when the target cells were sensitized with the relevant synthetic epitope peptide from BZLF1 (RAFKKQLL), the cells were lysed equally well whether or not BNLF2a was present (Fig. 3 A). These data indicate that BNLF2a blocks presentation of antigens that require endogenous processing before being presented to HLA class I-restricted T cells.

Because HLA class I levels have been found to be markedly reduced during productive EBV infection (12), we...
EBV BNLF2a blocks peptide transport by TAP

Surface HLA class I down-regulation can also result from the reduced supply of peptides to nascent HLA class I molecules in the endoplasmic reticulum. This is due to the efficient generation of peptides by the proteasome and other peptidases and the transport of these peptides into the endoplasmic reticulum by the TAP complex. In EBV-infected B cells that have entered the virus lytic cycle, surface HLA class I down-regulation coincides with reduced peptide transport by TAP (12). To evaluate whether the inhibition of HLA class I expression by BNLF2a involves interference with TAP function, we used an in vitro peptide translocation assay to examine TAP activity in BNLF2aHA-expressing and control MJS cells. In control cells, fluorescent model peptides were efficiently transported across the endoplasmic reticulum membrane in an ATP-dependent manner. In contrast, peptide translocation was strongly impaired in BNLF2aHA-expressing cells (Fig. 4 A).

EBV BNLF2a blocks TAP function by preventing the binding of peptides and ATP

TAP-mediated translocation of peptides into the endoplasmic reticulum is initiated by the interaction of peptides with the cytosolic peptide binding domains of TAP, followed by the binding and hydrolysis of ATP, which facilitates the opening of the transmembrane pore and peptide translocation (14, 15). We therefore evaluated the effect of BNLF2a on these steps in peptide transfer. The ability of peptides to bind to TAP in the presence of BNLF2a was examined by incubating radiolabeled peptides containing a photoactivatable cross-linker with microsomes derived from BNLF2aHA-expressing or control MJS cells. Peptides were covalently linked to their binding partners by UV irradiation and then separated by PAGE. In control MJS cells, radiolabeled peptides covalently linked to TAP were observed (Fig. 5 A; expressed as 100% in the quantitation depicted in Fig. 5 B), whereas such peptides failed to bind TAP in microsomes.
from BNLF2aHA-expressing cells (Fig. 5, A and B). As a specificity control, microsomes were incubated with both the radiolabeled reporter peptide and an unlabeled peptide derived from the HSV-1 ICP47 protein that is known to prevent peptide binding to TAP (16, 17). No binding of the radiolabeled peptide to TAP was observed in the presence of ATP. Translocated peptides that had become glycosylated in the endoplasmic reticulum were recovered by adsorption to concanavalin A–sepharose beads. After elution, the recovered peptide was quantitated by fluorometry in arbitrary units. Error bars represent the SEM of triplicates in a representative experiment. (B) Digitonin lysates of BNLF2aHA-expressing and control MJS-GFP cells were subjected to immunoprecipitation (IP) with antibodies specific for TAP1 (148.3), TAP2 (435.4), tapasin (Rgp46C), HLA class I heavy chains (HC10), and HLA class II DRα chains (DA6-147). Cell lysates and immune complexes were separated by SDS-PAGE, followed by Western blot analysis and staining with anti-HA antibody (12CA5) to detect HA-tagged BNLF2a.

Figure 4. EBV BNLF2a blocks peptide transport by TAP. (A) TAP-dependent peptide transport in BNLF2aHA-expressing and control MJS cells was assessed by permeabilizing the cells with streptolysin O and incubating them with a fluoresceinated peptide in the presence or absence of ATP. Translocated peptides that had become glycosylated in the endoplasmic reticulum were recovered by adsorption to concanavalin A–sepharose beads. After elution, the recovered peptide was quantitated by fluorometry in arbitrary units. Error bars represent the SEM of triplicates in a representative experiment. (B) Digitonin lysates of BNLF2aHA-expressing and control MJS-GFP cells were subjected to immunoprecipitation (IP) with antibodies specific for TAP1 (148.3), TAP2 (435.4), tapasin (Rgp46C), HLA class I heavy chains (HC10), and HLA class II DRα chains (DA6-147). Cell lysates and immune complexes were separated by SDS-PAGE, followed by Western blot analysis and staining with anti-HA antibody (12CA5) to detect HA-tagged BNLF2a.

We subsequently extended these studies by transfecting other available Old World primate cell lines with the different
BNLF2a genes and measuring the surface MHC class I levels of the transfected cells as before. Fig. S1 (available at http://www.jem.org/cgi/content/full/jem.20070256/DC1) shows histograms of MHC class I staining of transfected LLC-MK2 cells (Rhesus macaque), orangutan B cells transformed with the endogenous orangutan herpesvirus (orangutan LCL), and Cos cells (African green monkey). All BNLF2a expression constructs showed down-regulation of surface MHC class I in LLC-MK2 cells and Cos cells, with a more subtle down-regulation observed in transformed orangutan LCLs. The inhibitory effects of the various LCV BNLF2a proteins therefore appear to be conserved and operate in various cells of Old World primate origin.

**DISCUSSION**

This study was prompted by recent work (13) looking into target cell recognition by CD8+ T cells specific for EBV–lytic cycle antigens. This had shown that recognition was most efficient using clones against immediate early antigens, less so for clones against early antigens, and much less so for late antigen-specific effectors, despite the fact that the latter had the highest avidity in epitope peptide titration assays (13). Therefore, antigen processing function appeared to be progressively impaired as cells moved through the lytic cycle. Two observations argued against this being a secondary effect of general cytopathology associated with virus replication. Thus, in B LCLs in vitro where a small percentage of cells enter the lytic cycle, cells in the late phase of virus replication appear to remain viable for several days. More importantly, surface HLA class I levels on lytically infected cells begin to fall in the early phase of the cycle before other surface proteins, including HLA class II, are affected (12). This implied that EBV was actively impeding lytic cycle antigen presentation to CD8+ T cells.

Noting that immune evasion strategies within the herpesvirus family tend to be virus subfamily or genus specific, we focused on the six lytic cycle genes that were unique to the γ 1 genus and of unknown function. This identified one such gene, BNLF2a, as encoding a CD8 immune evasion protein. When expressed from a vaccinia vector in target cells, the BNLF2a protein reproducibly inhibited recognition of various indicator antigens (EBV latent and lytic cycle proteins) by CD8+ T cell clones restricted through several HLA-A, -B, and -C alleles. In contrast, class II antigen presentation in BNLF2a-expressing cells remained intact, and only presentation by the HLA class I pathway was impeded. The BNLF2a protein was found to function by blocking both peptide and ATP binding to the TAP complex, thereby impairing peptide loading of HLA class I molecules and their expression at the cell surface.

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**Figure 5. EBV BNLF2a inhibits peptide and ATP binding to TAP.** (A) Microsomes prepared from BNLF2aHA-expressing and control MJS cells were incubated with a radiolabeled model peptide; where indicated, an excess of ICP47 competitor peptide was included in this incubation. After UV cross-linking, microsomes were lysed, and the proteins were separated by SDS-PAGE and exposed to a phosphoimaging screen. The position where TAP1 migrated in parallel immunoprecipitation experiments is shown by the arrowhead. The asterisk denotes a nonspecific background band. (B) Quantification of triplicate bands representing TAP-bound peptide. Results are shown as the percentage of peptide binding relative to peptide binding in control MJS cells (set as 100%). Error bars represent means ± SD. (C) Digitonin and NP-40 lysates of MJS-BNLF2aHA and control MJS-GFP cells were incubated with ATP-agarose beads. ATP-agarose–bound (pellet) and unbound (supernatant) protein fractions were separated by SDS-PAGE and immunoblotted. Membranes were probed with antibodies specific for TAP1 (148.3), TAP2 (435.4), tapasin (7F6), HLA class I heavy chain (HC10), and the HA tag (12CA5).
cells in selectively amplifying responses to epitopes that are well presented on those target cells. BNLF2a is one of a set of genes, including two others with immunomodulatory function, BCRF1 (viral IL-10 homologue) and BARF1 (viral GCSF-receptor), that have been acquired by the Old World primate viruses of Old World primates but are absent from the New World virus analyzed thus far (27, 28). This implies acquisition, in the last 50 million years or so, after the Old World/New World fork in primate evolution. The Old World primate viruses have also acquired a more complex set of latent growth-transforming genes over this same period. Thus, it may be the combination of these more sophisticated immune-evasion and growth-transforming strategies that underlies the apparently greater prevalence that the Old World viruses achieve in their host population compared with their New World counterpart (29).

BNLF2a is the first TAP-specific inhibitor identified in γ1-herpesviruses, and like the other three herpesvirus TAP inhibitors identified so far, BNLF2a has its own unique structure and mechanism of action. Simplexviruses and varicelloviruses, both members of the α subfamily, use different strategies...
to inactivate TAP. The simplexviruses HSV1 and 2 encode a small cytosolic protein, ICP47, which acts as a high affinity competitor for peptide binding to TAP yet does not affect ATP binding to the transporter complex (5, 6, 17, 30, 31). In contrast, the varicellovirus bovine herpesvirus 1 and several of its relatives encode the UL49.5 gene product, which render TAP translocation incompetent without affecting its ability to bind peptide or ATP (32, 33). Ultimately, bovine herpesvirus 1 UL49.5 targets TAP1 and TAP2 for degradation by proteasomes (32, 33). In the case of β-herpesviruses, the prototypic virus of this subfamily, human CMV, inhibits TAP function via the endoplasmic reticulum luminal domain of the transmembrane protein US6. This protein reduces association of ATP to TAP1 and promotes binding to TAP2 but does not interfere with peptide binding, nor does it influence the stability of the transporter complex (34–38). In the case of the γ2-herpesvirus, mouse γ-herpesvirus 68, TAP is targeted secondarily for slow degradation by the virus-encoded ubiquitin ligase mK3, whose primary function is to rapidly target MHC class I heavy chains for proteasomal degradation (39–46).

In contrast to the described TAP inhibitors in the other herpesviruses, BNLF2a disables TAP function by blocking both the association of peptides with the peptide binding site and the interaction of ATP with the nucleotide binding domains of the transporter. How this small 60–amino acid protein, which consists of a hydrophilic N-terminal domain of 42 residues and a hydrophobic domain of 18 residues, achieves both of these functions is currently unclear. Despite the absence of an obvious N-terminal signal sequence, preliminary data suggest that BNLF2a is membrane-associated (unpublished data). This may be a consequence of binding to TAP or may result from post-translational insertion of the hydrophobic C terminus into the endoplasmic reticulum membrane, as has been reported for other proteins (47). The small size of BNLF2a and the relative distance between the peptide and ATP binding sites of TAP may preclude BNLF2a from acting directly on both of these sites. The expected exposure of BNLF2a’s N terminus in the cytosol would, however, suggest that this part interacts with cytosolic domains of the TAP complex. BNLF2a may then interfere directly with peptide or ATP binding while simultaneously disrupting subsequent steps of the translocation cycle through an indirect mechanism, e.g., by inhibiting conformational transitions required for proper function of the TAP complex.

The ability of an infected cell to escape CD8+ T cell recognition is likely to be most efficient when multiple points of the antigen processing pathway are targeted by viral immune evasion proteins. In the case of HSV2-infected cells, two viral proteins, the TAP inhibitor ICP47 and the host shutoff protein encoded by UL−41, have been shown to act synergistically to prevent CD8+ T cell lysis (48). In such an infection model, the long-lived TAP complexes are unlikely to be affected in the short term by the virus host shutoff function. Thus, cells infected with mutant HSV viruses lacking either of these two genes show partial inhibition of CD8+ T cell function, whereas cells infected with viruses expressing both genes show a dramatic reduction in their ability to be lysed by CD8+ T cells. Interesting in this context is the recent identification of the EBV-encoded gene BGLF5, which is at least one gene responsible for host protein synthesis shutoff (49). Expression of BGLF5 is sufficient to inhibit synthesis of host proteins, including HLA class I molecules, causing a decrease in surface class I levels. Coexpression of BNLF2a and BGLF5 by EBV could then represent an effective strategy for inhibiting the HLA class I antigen processing pathway (12).

In summary, the prototype γ1–herpesvirus, EBV, evades CD8+ T cell detection in the lytic cycle through a strategy that is unique in its molecular detail yet targets antigen presentation at the same point, TAP-mediated peptide transport, that is targeted by several other herpesviruses. The fact that the TAP inhibitors encoded by the different herpesvirus genera share no obvious homology presents a striking example of convergent evolution. Their existence testifies to the strength of the evolutionary pressure exerted on many herpesviruses by CD8+ T cell responses against virus replicative infections.

The present work indicates that, despite their ability to amplify viral loads in vivo through nonreplicative latent infection, EBV and Old World γ1–herpesvirus relatives have indeed been subjected to the same immune pressure.

MATERIALS AND METHODS

CD8+ T cell clones, vaccinia viruses, and cytotoxicity assays. EBV-specific CD8+ T cell clones were generated from PBMCs of infectious mononucleous patients by limiting dilution cloning, as previously described (11). CD8+ T cell clones used in this study were specific for the following epitopes derived from the respective EBV gene products: RAKFKQLL from BZLF1 and QAKWRLQTL from EBNA3A (both presented by HLA-B*0801 [50, 51]), GLCTLVAML from BMLF1 (presented by HLA-A*0201 [11]), CYDHAQTHL from BMRF1 (presented by HLA A*2402 [13], YHLIVDTDSL from EBNA2 (presented by HLA-B*0838 [52]), HRCQAIRKK from EBNA3B (presented by HLA-B*2705 [53]), and an undefined epitope derived from BGLF4 (presented by HLA-C*0101 [13]). CD4+ T cell clones specific for the HLA DQ5–presented epitope SDDLEPYIDPNMEP from EBNA 3C were produced by the stimulation of PBMCs from healthy donors, with their autologous EBV-transformed B cell line and limiting dilution cloning these cells, as previously described (54). All experiments were approved by the South Birmingham Health Authority Local Research Ethics Committee. Recombinant vaccinia viruses were used to express individual EBV B95.8 strain genes, as previously described, and a vaccinia virus lacking an insertion (vTk−) used for control infections (13, 54). Target cells infected with the vaccinia virus for cytotoxicity assays were either SV40-transformed keratinocytes, HLA-matched EBV-transformed B LCLs, or the melanoma cell line MJ5 (HLA-A*01, -B*08, and -Cw*0701) (55). Target cells were infected with vaccinia viruses at a multiplicity of infection of 10, incubated for 16 h, and used as targets in standard 5-h chromium release assays. Where indicated in the figures, target cells were sensitized with synthetic CTL epitope peptide (Alta Biosciences) at a concentration of 5 μM for 90 min before use in cytotoxicity assays.

Antibodies. The antibodies used in this study have been described elsewhere (32, 55). Mouse mAbs used to detect human cellular proteins were as follows: W6/32 or B9.12.1, which recognizes β2-microglobulin–associated HLA class I complexes (Immunotech); HC10 (provided by H. Ploegh, Whitehead Institute for Biomedical Research, Cambridge, MA), specific for HLA class I heavy chains; anti–HLA-DR mAb L243 (American Type Culture Collection); DA6-147, specific for HLA-DRα chains (provided by P. Creswell, Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, CT); and 148.3 directed to TAP1 and 435.4 directed to TAP2.
Retroviral transduction of cell lines. Retroviral constructs were engineered by cloning the BNLF2α gene into the pLZRα retroviral vector. Immediately downstream from this gene was an IRES, which allowed co-expression of a marker gene, truncated nerve growth factor. The BNLF2α sequence was modified at the C terminus to encode four methionines and the influenza HA epitope to allow detection (denoted as BNLF2αHA). Virus was produced using the Phoenix packaging cell line and used to transduce MJS cells, as previously described (55). As a control, MJS cells were transduced with an IRES–GFP–expressing retrovirus.

Flow cytometry analysis of surface HLA class I and II. Surface levels of HLA class I and II molecules were measured by flow cytometric analysis after staining with the antibodies W6/32 or B9.12.1 for class I molecules or L243 for class II molecules. In all cases, aliquots of cells were stained with the appropriate isotype controls, and bound antibodies were detected using a goat anti-mouse phycoerythrin antibody. Stained cells were analyzed on a flow cytometer (Epics; Beckman Coulter), and data was processed using WinMDI software (Scripps Research Institute).

Peptide transport assay. TAP-mediated peptide transport assays were conducted by permeabilizing aliquots of 3 × 10^6 MJS cells with 2.5 IU/ml of streptolysin O (Murex Diagnostics) and incubating these cells with 200 nM of the fluorescent amine peptide CVNKTERAY (provided by W. Benckhuijen and J. W. Drijfhout, Leiden University Medical Center, Leiden, Netherlands) in the presence or absence of 10 mM ATP at 37°C for 10 min. Translocation was terminated by lysing cells with ice-cold lys buffer (1% Triton X-100, 500 mM NaCl, 2 mM MgCl₂, 50 mM Tris HCl, pH 8), and the nuclei were removed by centrifugation. Peptides that had been glycosylated in the endoplasmic reticulum were isolated from lysates by incubation with concanavalin A–sepharose beads (GE Healthcare). These peptides were eluted from the beads with 500 mM mannopyranoside, 10 mM EDTA, 50 mM Tris HCl, pH 8, and fluorescence was detected using a fluorescence plate reader (Cytofluor; PerSeptive Biosystems).

Western blot analysis and immunoprecipitations. SDS-PAGE and immunoblotting were performed as previously reported (32, 36). MJS cells transfected with retrovirus expressing either BNLF2αHA or GFP were lysed using either 1% NP-40 or 1% digitonin made in 50 mM Tris HCl, pH 7.5, 5 mM MgCl₂, 150 mM NaCl, 1 mM leupeptin, and 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride. Lysates of solubilized proteins, equivalent to 2 × 10⁷ cells, were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (GE Healthcare). Proteins of interest were detected by incubating the membranes with specific antibodies followed by horseradish peroxidase (HRP)–conjugated secondary antispecies antibodies (Jackson ImmunoResearch Laboratories). Bound HRP-labeled antibodies were visualized using ECL Plus (GE Healthcare).

For immunoprecipitation experiments, cell lysates prepared with 1% digitonin solution were incubated with the different specific antibodies for at least 2 h at 4°C. Protein G–sepharose beads (GE Healthcare) were used to isolate immune complexes, which were washed with 0.1% digitonin and subjected to Western blot analysis. Antibodies that bound the blotted proteins were detected using ExactaCruz (Santa Cruz Biotechnology, Inc.), according to the manufacturer’s instructions.

Peptide binding assay. TAP–containing microsomal membranes were prepared from MJS cells transfected with BNLF2αHA or GFP by homogenization of the cells with a cell cracker (EMBL) and removal of the nuclei by centrifugation. The ability of peptides to bind to TAP complexes in the microsomes was assessed by incubation with an 125I-labeled peptide 5PS2 (ERYDKSE[BPA]-L [57]) containing a photoactivatable cross-linker in the absence or presence of 2.5 μM of unlabeled HSV-1 ICP47 competitor peptide (16). After incubation for 15 min on ice, microsomes were washed with PBS, and peptide–protein interactions were covalently linked by UV exposure for 10 min on ice at a distance of 2 cm using a UVP lamp (B-100A; Blak-Ray) with a mercury lamp (Par 38; Sylvania). Samples were analyzed by SDS-PAGE, and bound peptide was quantitated by phosphoimaging. Migration of peptide–bound TAP1 was confirmed by immunoprecipitation.

ATP-agarose binding assay. MJS cells transfected with BNLF2αHA or GFP were lysed with 1% digitonin or 1% NP-40, as described in Western blot analysis and immunoprecipitations. Nuclei were removed, and lysates were incubated with hydrated C-8 ATP-agarose beads (13-μM; final concentration; Sigma-Aldrich) at 4°C for 2 h. The beads were centrifuged to provide a supernatant fraction and a pellet fraction. Proteins bound to the ATP-agarose pellet fraction were eluted with 500 mM EDTA, and these as well as the supernatant fraction were analyzed by Western blot.

Cloning and transient expression of BNLF2α genes. BNLF2α homologues were cloned from the following LCVs: papio herpesvirus, rhesus herpesvirus, pan herpesvirus, orangutan herpesvirus, and gorilla herpesvirus. PCR primers were designed to hybridize to a sequence flanking the BNLF2α region, which was conserved between EBV and the rhesus herpesvirus. PCR products were amplified from either a genomic DNA fragment of rhesus herpesvirus (provided by F. Wang, Harvard Medical School, Boston, MA) or from DNA extracted from B cell lines spontaneously transformed with the endogenous LCV of each host species. This amplified BNLF2α region of each virus was cloned into the plasmid pCR2.1TOPO (Invitrogen), and inserts were sequenced using standard techniques. Sequences are available from GenBank/EMBL/DDJB under accession nos. EF207711 (papio herpesvirus), EF207712 (pan herpesvirus), EF207713 (orangutan herpesvirus), and EF207714 (gorilla herpesvirus).

For functional studies, the BNLF2α genes were subcloned into a modified pCDNA3 vector (provided by E. Reits) and expressed under the control of the CMV immediate early promoter. Immediately downstream from BNLF2α was an IRES element followed by the GFP gene, allowing co-expression of this marker. Plasmids were transfected into either MJS cells, the Rhesus macaque kidney cell line LLC-MK2, or the African green monkey kidney fibroblast-like cell line Cos using Lipofectamine 2000 (Invitrogen), or transfected by electroporation into orangutan B cells transformed with their endogenous LCV. Transfected cells were cultured for 48 h before staining for surface marker expression and flow cytometric analysis, as described in Flow cytometry analysis of surface HLA class I and II.

Online supplemental material. Fig. S1 shows histograms of the fluorescence intensity of MHC class I staining on Old World primates cells transfected with plasmids expressing BNLF2α homologues. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20070256/DC1.

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