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Specific Targeting of the EBV Lytic Phase Protein BNLF2a to the Transporter Associated with Antigen Processing Results in Impairment of HLA Class I-Restricted Antigen Presentation

Daniëlle Horst,* Daphne van Leeuwen,* Nathan P. Croft,‡ Malgorzata A. Garstka,* Andrew D. Hislop,‡ Elisabeth Kremmer,‡ Alan B. Rickinson,‡ Emmanuel J. H. J. Wiertz,* and Maaike E. Ressing2a§

EBV persists for life in the human host while facing vigorous antiviral responses that are induced upon primary infection. This persistence supports the idea that herpesviruses have acquired dedicated functions to avoid immune elimination. The recently identified EBV gene product BNLF2a blocks TAP. As a result, reduced amounts of peptides are transported by TAP from the cytoplasm into the endoplasmic reticulum (ER) lumen for binding to newly synthesized HLA class I molecules. Thus, BNLF2a perturbs detection by cytotoxic T cells. The 60-aa-long BNLF2a protein prevents the binding of both peptides and ATP to TAP, yet further mechanistic insight is, to date, lacking. In this study, we report that EBV BNLF2a represents a membrane-associated protein that colocalizes with its target TAP in subcellular compartments, primarily the ER. In cells devoid of TAP, expression levels of BNLF2a protein are greatly diminished, while ER localization of the remaining BNLF2a is retained. For interactions of BNLF2a with the HLA class I peptide-loading complex, the presence of TAP2 is essential, whereas tapasin is dispensible. Importantly, we now show that in B cells supporting EBV lytic replication, the BNLF2a protein is expressed early in infection, colocalizing and associating with the peptide-loading complex. These results imply that, during productive EBV infection, BNLF2a contributes to TAP inhibition and surface HLA class I down-regulation. In this way, EBV BNLF2a-mediated evasion from HLA class I-restricted T cell immunity contributes to creating a window for undetected virus production. The Journal of Immunology, 2009, 182: 2313–2324.

ytotoxic T lymphocytes play an essential role in the control of many viral infections. Through their TCRs, CD8+ CTLs scrutinize body cells for the presence of intracellular foreign invaders reflected at the cell surface by virus-derived peptides bound to host MHC class I molecules (HLA in humans). In general, these peptides result from protein degradation by cytosolic proteasomes and are then transported into the endoplasmic reticulum (ER) lumen by the transporter associated with Ag processing, TAP. TAP is a heterodimer composed of two multimeric-spanning subunits, TAP1 and TAP2, with cytosolic C-terminal domains for the binding of peptide substrates and ATP. To ensure efficient acquisition of optimal peptide cargo by nascent MHC class I complexes, TAP is part of the MHC class I peptide-loading complex (PLC). Besides TAP1 and TAP2, the PLC comprises MHC class I H chains, β2-microglobulin (β2m), tapasin, ERp57, and the chaperone calreticulin. Tapasin bridges TAP and MHC class I molecules and stabilizes TAP expression (1, 2). The complex of tapasin and the thiol oxidoreductase ERp57 exerts a peptide-editing function (3, 4). Once newly synthesized MHC class I H chain/β2m/peptide trimers are assembled, they are allowed to exit the ER and travel through the Golgi to the cell surface.

Even in the face of functional antiviral immune responses, herpesviruses are capable of establishing lifelong persistent infections in their hosts. This hallmark is shared by all members of the herpesvirus family, classified into α-, β-, and γ-subfamilies. The past decade has provided ample evidence that herpesviruses have acquired gene products that thwart host antiviral responses (5–8). Prominent among these are viral proteins that interfere with MHC class I-restricted Ag presentation at various locations along the Ag processing route. For example, MHC class I molecules are retained intracellularly in cells infected with both human and murine CMV (HCMV and MCMV, respectively; both β-herpesviruses). To achieve this, the HCMV US3 protein prevents the exit of MHC class I molecules from the ER in a tapasin-dependent manner (9, 10), and in MCMV m152/gp40 is responsible for the retention of

NGFR, truncated nerve growth factor receptor; PDI, protein disulfide isomerase; PLC, peptide-loading complex.

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MHC class I in the ER-Golgi intermediate compartment (11). An alternative strategy of MHC class I immune evasion adopted by several viruses is the acceleration of MHC class I turnover. In cells expressing the HCMV US2 or US11 proteins, MHC class I molecules are degraded rapidly upon synthesis and, interestingly, this occurs by proteasomes following the dislocation of MHC class I molecules into the cytosol (12, 13). Increased degradation of MHC class I molecules is also achieved by viral E3 ubiquitin ligases encoded by two members of the γ-herpesvirus subfamily. The murine γ-herpesvirus MHV68 encodes mK3, the expression of which leads to proteasomal degradation of MHC class I as well as other components of the PLC (14–18). The human Kaposi sarcoma-associated herpesvirus expresses two proteins, K3 and K5, that target MHC class I molecules for proteolysis at a different location compared with mK3, i.e., in lysosomes following endocytosis (19–21). Enhanced lysosomal degradation is also effected by two β-herpesvirus gene products; the U21 protein of the human herpesvirus HHV-7 (22, 23) and m06/gp48 of MCMV (24) redirect class I molecules into the cytosol (12, 13). Increased degradation of MHC class I molecules occurs by proteasomes following the dislocation of MHC class I expressing the HCMV US2 or US11 proteins, MHC class I molecules into the cytosol (12, 13). Enhanced lysosomal degradation is also effected by two β-herpesvirus gene products; the U21 protein of the human herpesvirus HHV-7 (22, 23) and m06/gp48 of MCMV (24) redirect newly synthesized MHC class I complexes to endolysosomal compartments.

Finally, TAP appears to be an attractive target for viral inhibition of Ag presentation to MHC class I-restricted CTLs, and TAP is blocked by a growing list of herpesvirus-encoded proteins. Within the α-herpesvirus subfamily, some members of the simplex viruses and the varicelloviruses encode TAP-inhibiting proteins.

Infection with HSV type 1 or 2 leads to immediate early expression of ICP47, blocking peptide transport by TAP. ICP47 binds to the cytosolic domains of both TAP subunits, thereby outcompeting the binding of Ag-derived peptides (25–32). The TAP inhibitor encoded by members of the varicelloviruses, UL49.5, lacks sequence homology to ICP47. UL49.5 codes for a transmembrane protein, US6, which inhibits TAP through its ER lumenal domain by interfering with ATP binding to the cytosolic nucleotide-binding domains of TAP (35–39). Until recently, only the subfamily of γ-herpesviruses lacked a dedicated TAP-inhibiting protein.

Within the γ-herpesvirus subfamily, EBV is the prototype γ-herpesvirus infecting >90% of adult human beings worldwide. Although asymptomatic in the majority of infected individuals, EBV is the causative agent of infectious mononucleosis and a number of malignant tumors, such as Burkitt’s lymphoma and nasopharyngeal carcinoma. The importance of T cells in controlling EBV infection is underscored by the development of EBV-transformed B cell lymphomas in immunocompromised patients that can be cured with adoptively transferred EBV-specific CTLs (40). Following primary infection, EBV resides in memory B cells, mostly as a latent infection. During latency, only a fraction (up to nine) of viral proteins are expressed, thereby reducing the target Ags to be recognized by the host immune system. EBV occasionally reactivates from latency, which leads to the expression of >20 viral proteins and the production of viral particles that can amplify the latent pool or mediate viral transmission to another host. It is now becoming increasingly clear that EBV has also acquired dedicated immune evasion strategies that delay the elimination of virus-producing cells. Using a newly developed culture system for productive EBV infection, we have found that HLA class I expression is down-regulated at the surface of lytically EBV-infected B cells when compared with cells harboring a latent EBV infection, and this coincides with a block in TAP-mediated peptide transport (41). Subsequently, we identified BNLF2a as an EBV lytic cycle protein capable of blocking TAP function by interfering with binding of both peptides and of ATP to TAP (42). Functional homologues of EBV BNLF2a were found in Old World primate γ-herpesviruses. Thus, γ-herpesviruses also appear to encode immune evasion molecules specifically targeting peptide transport by TAP.

In this article, we report novel mechanistic aspects of the EBV-encoded BNLF2a protein, representing the first dedicated inhibitor of TAP in γ-herpesviruses, and we provide evidence for its role during natural EBV infection.

Materials and Methods

Antibodies

The following mouse mAbs have been used in this study: W6/32, which recognizes β2m-associated HLA class I molecules (43); HC10, recognizing non-β2m-associated class I HLA IgG1; and 1D4, recognizing the HCMV US2 or US11 proteins, MHC class I molecules (44). In the current study, all mAbs were used at a constant concentration of 5 μg/ml.

Methods

B cells were isolated from lymph nodes of C57BL/6 mice and co-cultured with an EBV-producer cell line, P3X63-Ag8.653, in the presence of irradiated spleen cells. B cells were cultured in RPMI supplemented with 10% FCS, 50 μg/ml gentamycin, and 100 units/ml penicillin-streptomycin. The medium was changed every 2–3 days. Culture supernatants were collected and stored at −20°C until used. Cultures were maintained for 5–7 days before harvesting for experiments. Supernatants were collected and stored at −20°C until used. Bacteria were grown in LB medium and harvested by centrifugation for 30 min at 16,000 rpm. Bacteria were resuspended in PBS and stored at −20°C until used. Mouse HW9251 and HW9252, rat HW9253, and human HW9254 were used as APCs for the ELISA experiments. Mouse HW9251 and HW9252, rat HW9253, and human HW9254 were used as APCs for the ELISA experiments.

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BNLF2aΔCys product of PCR7 was cloned into the pLZRS IRES GFP vector.

Retroviral vectors were transfected into the Phoenix A packaging cell line to produce replication-deficient recombinant retroviruses, as described (42).

Cell lines and retroviral transduction

Cell lines were maintained in complete culture medium consisting of RPMI 1640 (Invitrogen) supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, with the exception of STF-169, STF-169/TAP2, and BRE-169 cells, which were maintained in complete culture medium consisting of DMEM (Invitrogen) supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Retroviral transductions were performed essentially as described (42). In brief, cells were transduced with BNLF2aHA4M-, BNLF2aΔCys-, or control IRES-GFP-expressing retroviruses. Transduced cells were sorted by FACS to achieve expression in all cells.

MelJuSo (MJS) is a human melanoma cell line (46). Both MJS cells and MJS cells stably transfected with TAP1-GFP (MJS/TAPI-GFP) (47) were transduced with replication-deficient retroviruses encoding BNLF2aHA4M-IRES-NEGR to generate the cell lines MJS/BNLF2a and MJS/TAPI-GFP/BNLF2a, respectively. To generate MJS/BNLF2aΔCys cells, cells were transduced with BNLF2aΔCys-IRES-GFP retroviruses.

MoDo (48), T2 (49), and .220 (50) cells are EBV-transformed B lymphoblastoid cell lines (LCLs). MoDo cells were obtained from E. Goulmy (Leiden University Medical Center, Leiden, The Netherlands). T2 cells stably transduced to express TAP1 only (T2 TAP1) or both TAP1 and TAP2 (T2 TAP1 TAP2) were obtained from J. W. Drijfhout (Leiden University Medical Center, Leiden, The Netherlands). T2 cells stably transduced to express TAP1 only (T2 TAP1) or both TAP1 and TAP2 (T2 TAP1 TAP2) were obtained from P. Lehner (Cambridge Institute for Medical Research, Cambridge, U.K.) (51), and .220 cells were obtained from E. Reits (Academic Medical Center, Amsterdam, The Netherlands). MoDo LCL, T2, T2 TAP1, and T2 TAP1 TAP2 cells were retrovirally transduced to express BNLF2aHA4M (coexpressing GFP as a marker), thereby generating the following cell lines: LCL/BNLF2a, T2/BNLF2a, T2 TAP1/BNLF2a, and T2 TAP1 TAP2/BNLF2a, respectively. T2BNLF2a T2 cells were additionally transduced with a retrovirus encoding BNLF2aHA4M-IRES-NEGR, resulting in the T2BNLF2a (G/N) cell line.

For immunofluorescence microscopy, T2 and T2 TAP1 TAP2 cells were transduced with retroviruses encoding BNLF2aHA4M-IRES-NEGR, resulting in the T2BNLF2a (N) and T2 TAP1 TAP2/BNLF2a (N) cell lines. Transduction of .220 cells with a retrovirus encoding BNLF2aHA4M-IRES-NEGR resulted in the .220 (TPN)/BNLF2a cell line. In parallel, cells were transduced with control IRES-GFP retroviruses.

For peptide transport assays, T2 and AKBM cells were utilized. T2 cells were additionally transduced with a retrovirus encoding BNLF2aHA4M-IRES-NEGR, resulting in the T2BNLF2a (N) and T2 TAP1 TAP2/BNLF2a (N) cell lines. Transduction of .220 cells with a retrovirus encoding BNLF2aHA4M-IRES-NEGR resulted in the .220 (TPN)/BNLF2a cell line. In parallel, cells were transduced with control IRES-GFP retroviruses.

AKBM cells and BNLF2aΔCys were utilized. AKBM cells and BNLF2aΔCys were utilized.

Immunofluorescence

T2 and AKBM cells were utilized. T2 cells were utilized.

T2 and AKBM cells were utilized to detect actin and BNLF2a cDNA, the following primers were used: 5′-TTAGTCTGCTGACGTCTG-3′ (sense); 5′-GGGGTGTTGAAG-3′ (antisense); 5′-GGGGTGTTGAAG-3′ (antisense); and 5′-GGGGTGTTGAAG-3′ (antisense). Actin was used as a housekeeping control.

Flow cytometry

Surface levels of HLA class I, HLA class II, and transferrin receptor were analyzed by flow cytometry. Cells were stained with mAbs W6/32, L243, and CD71, respectively, and, after washing, with secondary goat anti-mouse PE Abs (Jackson ImmunoResearch Laboratories) or goat anti-mouse allophycocyanin Abs (Leinco Technologies) at 4°C. Stained cells were analyzed by FACSCalibur flow cytometer using CellQuest Pro software (BD Biosciences).

Quantitative PCR

Cellular RNA was isolated from cells using TRIzol (Invitrogen) according to the manufacturer’s instructions. cDNA was synthesized using random nanomer primers and Moloney murine leukemia virus reverse transcriptase (Finnzymes). The absence of genomic DNA was verified by parallel reactions performed in the absence of reverse transcriptase.

To detect actin and BNLF2a cDNA, the following primers were used: 5′-GGCGTCTCCTACCGTTGAGATGAGTGT-3′ (sense); 5′-GGGGTGTTGAAG-3′ (antisense); 5′-GGGGTGTTGAAG-3′ (antisense); and 5′-GGGGTGTTGAAG-3′ (antisense). Actin was used as a housekeeping control.

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The hydrophobic C terminus of BNLF2a is highlighted in dark gray, the cysteine residues are highlighted in light gray, and the HA4M tag is shown in italics. The epitopes recognized by the anti-BNLF2a (α-BNLF2a) and anti-HA (α-HA) mAbs are underlined. B. Immunofluorescence analysis of MJS/TAP1-GFP cells stably expressing BNLF2a and control MJS/TAP1-GFP cells. After fixing and permeabilizing the cells, intracellular localization of the BNLF2a protein was visualized using the BNLF2a-specific Ab 8E2. Nuclei were visualized using Hoechst stain (Sigma-Aldrich).

EBV BNLF2a represents a membrane-associated protein

Immunofluorescence analysis suggested that BNLF2a was absent from the cytosol despite the fact that the protein lacks an N-terminal signal sequence. BNLF2a contains a hydrophobic domain at its C terminus that could serve as a tail anchor for membrane insertion (Fig. 1A). To investigate whether BNLF2a is a cytosolic or a membrane-associated protein, we performed subcellular fractionation experiments.

Cell homogenates of control GFP-expressing cells and BNLF2a-expressing cells were sedimented by differential centrifugation to separate membranous organelles (1,000, 10,000, and 100,000 × g pellet fractions) from cytosol (100,000 × g supernatant fraction). As expected, the recycling membrane protein transferrin receptor is detected by Western blotting in all pellet fractions, whereas the cytosolic GFP is only present in the supernatant fraction of GFP-expressing control cells (Fig. 2, lane 4). Calnexin-staining in the 1,000 and 10,000 × g pellets suggests these to contain ER-derived microsomes (Fig. 2). The fact that the 100,000 × g supernatant was completely devoid of BNLF2a, combined with the detection of BNLF2a in the 1,000 and 10,000 × g pellets of MJS/BNLF2a cells (Fig. 2, lanes 5 and 6), indicates that BNLF2a is membrane associated rather than cytosolic. A similar distribution pattern was observed for components of the PLC, including TAP1, TAP2, tapasin, and HLA class I heavy chains (Fig. 2). Thus, BNLF2a occurs as a membrane-associated protein in subcellular compartments, most likely ER, where it colocalizes with the PLC.

EBV BNLF2a does not drastically alter the composition of the PLC

Because BNLF2a colocalizes and coprecipitates with various components of the PLC, we next addressed the question whether binding of the viral TAP inhibitor would alter the composition of the PLC. By Western blotting, we investigated the proteins that coprecipitated with major constituents of the PLC from cells lysed in the presence of the mild detergent digitonin (Fig. 3). Substantial amounts of BNLF2a associated with TAP1, TAP2, and tapasin in BNLF2a-expressing MJS cells (Fig. 3A), in agreement with our previous data (42). TAP1, TAP2, tapasin, and HLA class I heavy chains were all detected in immune complexes precipitated with Abs specific for TAP1, TAP2, or tapasin. The amounts of coprecipitated proteins were similar for BNLF2a-expressing and control
cells (Fig. 3A). From these experiments, we conclude that expression of BNLF2a does not expel individual components from the PLC, nor does it prevent the binding of HLA class I to the PLC.

We additionally performed immunoprecipitations with two Abs against HLA class I that, under native immunoprecipitation conditions, recognize different pools of class I molecules; mAb HC10 is directed against free, immature HLA class I H chains (59), whereas mAb W6/32 recognizes a conformational epitope on β2m-associated, peptide-loaded, mature HLA class I molecules (43). Please note that under denaturing conditions, as in Western blots, probing with HC10 reveals the total pool of HLA class I. BNLF2a only coprecipitates with immature (HC10-reactive) HLA class I (Fig. 3B), substantial amounts of which reside in the PLC as visualized by the presence of TAP1, TAP2, and tapasin in these HC10-reactive immune complexes. Interestingly, BNLF2a expression appears to lead to increased amounts of HC10-reactive HLA class I (Fig. 3B), although the total amounts of HLA class I co-precipitated with TAP1, TAP2, or tapasin were not drastically altered (Fig. 3A). This paradox could indicate that the total amounts of HLA class I H chains within the PLC are similar but that their HC10-reactivity is increased, for instance as a consequence of reduced stability of the HLA class I chain-β2m complex. BNLF2a does not interact with mature, W6/32-reactive HLA class I complexes that have probably left the PLC (Fig. 3B), no coprecipitation of TAP1, TAP2, or tapasin with mAb W6/32). In BNLF2a-expressing cells, the W6/32-reactive HLA class I complexes appear not fully matured, because they display a slightly faster mobility in SDS-PAGE compared with the HLA class I heavy chains from control cells, reminiscent of the compromised glycan modifications normally accompanying maturation.

In conclusion, the above results indicate that, although BNLF2a appears to retard the formation of stable HLA class I complexes, the overall composition of the PLC is not affected by the expression of BNLF2a.

Cysteine residues of BNLF2a are dispensable for TAP inhibition

The amino acid sequence of EBV BNLF2a contains four cysteine residues (Fig. 1A), three of which are conserved in BNLF2a homologues of Old World primate γ-herpesviruses (42). To investigate whether these cysteine residues are critical to BNLF2a function and, thereby, to HLA class I immune evasion, we created a mutant with all of the cysteines replaced by serines (BNLF2aΔcys). Through retroviral transduction, MJS cells were generated stably expressing the cysteine-less BNLF2a at levels comparable to those of wild-type HA4M-tagged BNLF2a, as detected in Western blotting (Fig. 4A).

First, the contribution of the cysteine residues to the association of BNLF2a with the PLC was evaluated. Postnuclear digitonin lysates of control, BNLF2a-, and BNLF2aΔcys-expressing MJS cells were subjected to immunoprecipitations with Abs specific for TAP1 (148.3), TAP2 (435.4), and tapasin (7F6) to assess association of BNLF2a with these PLC-components (A) or HC10 and W6/32. Abs recognizing immature and mature HLA class I molecules, respectively, to investigate the effect of BNLF2a expression on HLA class I maturation (B). Denatured immunoprecipitates were separated by SDS-PAGE and stained for TAP1 (148.3 mAb), TAP2 (435.4 mAb), tapasin (7F6 mAb), HLA class I H chains (HC10 mAb), and BNLF2a (5B9 mAb) in Western blot (WB) analysis.

FIGURE 2. EBV BNLF2a is a membrane-associated protein. Subcellular fractionation of MJS/GFP and MJS/BNLF2a cells was performed by differential centrifugation, as described in the Materials and Methods. 1,000 × g, 10,000 × g, and 100,000 × g pellets and 100,000 × g supernatant (sup) fractions were separated by SDS-PAGE and analyzed by Western blotting (WB) for the presence of the control transmembrane protein transferrin receptor (TR; H68.4 mAb), the control cytosolic protein GFP (anti-GFP rabbit sera), as well as calnexin (anti-calnexin rabbit sera), BNLF2a (5B9 mAb), TAP1 (148.3 mAb), TAP2 (435.4 mAb), tapasin (7F6 mAb), and HLA class I H chains (HC) (HC10 mAb).

FIGURE 3. EBV BNLF2a interacts with intact HLA class I PLCs. Postnuclear digitonin lysates of MJS cells stably expressing BNLF2a (BN) and control MJS cells (c) were subjected to immunoprecipitations (IP) using Ab specific for TAP1 (148.3), TAP2 (435.4), and tapasin (7F6) to assess association of BNLF2a with these PLC-components (A) or HC10 and W6/32. Abs recognizing immature and mature HLA class I molecules, respectively, to investigate the effect of BNLF2a expression on HLA class I maturation (B). Denatured immunoprecipitates were separated by SDS-PAGE and stained for TAP1 (148.3 mAb), TAP2 (435.4 mAb), tapasin (7F6 mAb), HLA class I H chains (HC10 mAb), and BNLF2a (5B9 mAb) in Western blot (WB) analysis.

First, the contribution of the cysteine residues to the association of BNLF2a with the PLC was evaluated. Postnuclear digitonin lysates of control, BNLF2a-, and BNLF2aΔcys-expressing MJS cells were subjected to immunoprecipitations with mAbs specific for components of the PLC, and these immune complexes were analyzed for coprecipitation of BNLF2a by Western blotting (Fig. 4B). Both wild-type BNLF2a and the BNLF2aΔcys mutant were found to associate with TAP1, TAP2, tapasin, and HC10-reactive HLA class I H chains (Fig. 4B), but not with control transferrin receptors. Thus, the cysteine residues of BNLF2a are dispensable for interactions of BNLF2a with the PLC.

Second, we compared TAP-inhibiting properties of wild-type and cysteine-less BNLF2a in an in vitro peptide translocation assay. In permeabilized control cells, fluorescent peptides were efficiently transported across the ER membrane in an ATP-dependent manner, whereas peptide translocation was significantly impaired both in wild-type and BNLF2aΔcys-expressing cells.
C terminus of the viral protein.

BNLF2a expression using mAb 12CA5 specific for the HA tag added to the H68.4). Precipitates were separated by SDS-PAGE and analyzed for (R.gp48C), HLA class I H chains (HC10), and transferrin receptor (TfR; (IP) using Abs specific for TAP1 (148.3), TAP2 (435.4), tapasin/H9004 (cys lysates of MJS cells stably expressing BNLF2a (wt) or BNLF2a/H9004 BNLF2a/H9004 in which the cysteine residues have been replaced by serines (cys) are derived cell lines. Immune evasion by BNLF2a was examined functionally using mutant, a marked down-regulation of HLA class I was observed (Fig. 4D), although this down-regulation was slightly less pronounced than that seen for wild-type BNLF2a-expressing cells. Surface down-regulation was selective for HLA class I molecules, because levels of HLA class II and transferrin receptor remained unaffected (Figs. 4D and 5C) (42).

In summary, BNLF2aΔcys interacts with the PLC, blocks TAP-mediated peptide transport, and down-regulates surface HLA class I levels, indicating that the cysteine residues within BNLF2a are not essential for immune evasion from HLA class I-restricted Ag presentation.

Interaction of EBV BNLF2a with the PLC requires the presence of TAP2 but not tapasin

To investigate the contribution of different members of the PLC to the inhibition of TAP function by BNLF2a, we made use of mutant B cell lines lacking individual components of the PLC. In comparison to the MJS cells used up to this point, B cells represent more professional APCs displaying, amongst others, elevated levels of TAP and HLA class I molecules. We therefore first verified that BNLF2a expression also yielded an HLA class I immune evasion phenotype in B cells. A wild-type B LCL, MoDo, was retrovirally transduced to stably express BNLF2a; for comparison, control cells were transduced with GFP-encoding retroviruses (Fig. 5A, lanes 1 and 2). As anticipated, BNLF2a-expressing B cells displayed impaired TAP-dependent peptide translocation (Fig. 5B, bars labeled “LCL”), surface HLA class I down-regulation (Fig. 5C, left panel), and association of BNLF2a with the PLC (Fig. 5D, lanes 1 and 2) like that previously found for MJS/BNLF2a cells. These results show that BNLF2a functions as an immune evasion protein in B cells, a natural host cell for EBV infection, and indicate that LCLs can be used to delineate the contributors to BNLF2a-induced TAP inhibition.

Mutagenesis and complement selection of LCL 721 resulted in several derivatives displaying low levels of HLA class I surface expression that were subsequently attributed to various genetic defects. Among these mutant LCLs are T2 cells (49) lacking expression of both TAP1 and TAP2 and .220 cells (50) lacking expression of full-length tapasin (TPN). Additionally, T2-derived cell lines have been obtained with reconstituted expression of human TAP1 alone (T2 TAP1) or of both TAP1 and TAP2 (T2 TAP1 TAP2) (51). It was not possible to generate T2 cells expressing TAP2 only due to the instability of TAP2 when expressed in isolation (60). These mutant LCLs were transduced to express BNLF2a (Fig. 5A) and used to evaluate the contribution of TAP2 and tapasin to the viral inhibition of peptide transport.

First, the involvement of TAP was evaluated using the T2-derived cell lines. Immune evasion by BNLF2a was examined functionally using TAP-dependent peptide translocation assays (Fig. 4C). Therefore, the cysteine residues of BNLF2a do not appear critical to the inhibition of TAP function.

Third, flow cytometry was used to analyze cell surface HLA class I expression. On cells stably expressing the BNLF2aΔcys mutant, a marked down-regulation of HLA class I was observed (Fig. 4D), although this down-regulation was slightly less pronounced than that seen for wild-type BNLF2a-expressing cells. Surface down-regulation was selective for HLA class I molecules, because levels of HLA class II and transferrin receptor remained unaffected (Figs. 4D and 5C) (42).

In summary, BNLF2aΔcys interacts with the PLC, blocks TAP-mediated peptide transport, and down-regulates surface HLA class I levels, indicating that the cysteine residues within BNLF2a are not essential for immune evasion from HLA class I-restricted Ag presentation.

**FIGURE 4.** Cysteine residues within BNLF2a are not essential for TAP inhibition. A. BNLF2aHA4M (wt, wild type) and BNLF2aHA4M proteins in which the cysteine residues have been replaced by serines (Δcys) are detectable in Western blots (WB) of total postnuclear Nonidet P-40 lysates of MJS cells stably expressing BNLF2a or BNLF2aΔcys, but not in control MJS cells (c). Western blots were stained for BNLF2a using mAb 5B9. B. BNLF2aΔcys interacts with components of the HLA class I PLC. Digitonin lysates of MJS cells stably expressing BNLF2a (wt) or BNLF2aΔcys (Δcys) and control MJS cells (c) were subjected to immunoprecipitations (IP) using Abs specific for TAP1 (148.3), TAP2 (435.4), tapasin (R.gp48C), HLA class I H chains (HC10), and transferrin receptor (TfR; H68.4). Precipitates were separated by SDS-PAGE and analyzed for BNLF2a expression using mAb 12CA5 specific for the HA tag added to the C terminus of the viral protein. C. BNLF2aΔcys still inhibits TAP-dependent peptide transport. MJS cells stably expressing BNLF2a (wt) or BNLF2aΔcys (Δcys) and control MJS cells (c) were analyzed for their ability to transport peptides via TAP using a dedicated peptide transport assay. Cells were permeabilized with streptolysin O and incubated with flouresceinated peptides in the presence or absence of ATP. Upon cell lysis, Con A-Sepharose beads were used to precipitate translocated peptides that were glycosylated in the ER. After elution from the beads, the amount of precipitated fluorescent peptide was quantified in arbitrary counts using a multilabel reader. Counts obtained for the samples incubated in the absence of ATP never exceeded 5% of the counts obtained for the control samples incubated in the presence of ATP (data not shown). D. HLA class I surface expression is down-regulated on MJS cells stably expressing either wild-type BNLF2a (line 3) or BNLF2aΔcys (line 4) compared with MJS cells expressing GFP (line 2) as detected in flow cytometry using W6/32 as the primary Ab followed by allophycocyanin-conjugated anti-mouse secondary mAbs. Line 1 represents a negative control (no first Ab). HLA class II surface expression, as detected by the mAb L243, is unaffected by the expression of BNLF2aΔcys.
elles were used to further delineate which TAP subunits are required for BNLF2a to interact with the PLC. In immune complexes precipitated from digitonin lysates of BNLF2a-expressing T2 TAP1 TAP2 cells, BNLF2a is detected in association with TAP1, TAP2, and tapasin (Fig. 5D, lane 4), as for LCL cells (Fig. 5D, lane 2). In contrast, no coprecipitation of BNLF2a with TAP1 or tapasin was observed for T2 TAP1 cells (Fig. 5D, lane 6), whereas, as a control, the association of tapasin with HLA class I was detected under these conditions. Because T2 TAP1 TAP2 and T2 TAP1 cells only differ in the expression of the TAP2 subunit, these data demonstrate that the presence of TAP2 molecules is required for the interaction of BNLF2a with the PLC.

Second, we addressed the role of tapasin in BNLF2a-mediated TAP inhibition by exploiting the .220 cells. In these cells, a deletion in exon 2 results in an extensive truncation and severe instability of the tapasin protein (61). Despite the absence of full-length tapasin, BNLF2a induces the inhibition of TAP-mediated peptide transport and surface HLA class I down-regulation (Fig. 5, B and C, sections marked .220 (TPN−) cells). In line with this, full-length tapasin does not need to be present for the association of BNLF2a with the PLC to occur, as shown by coprecipitations of BNLF2a with both TAP1 and TAP2 in .220 (TPN−) cells (Fig. 5D).

In summary, we conclude that expression of tapasin is not essential for the functionality of BNLF2a, whereas TAP2 is required for BNLF2a to associate with the PLC.

**FIGURE 5.** TAP2 is required for association of BNLF2a with the peptide-loading complex, whereas full-length tapasin is not. A, BNLF2a is expressed in EBV-transformed wild-type and PLC-mutant LCLs. Total postnuclear Nonidet P-40 lysates of wild-type LCLs, TAP1 and TAP2 reconstituted T2 cells (T2 TAP1 TAP2), TAP2-deficient B cells (T2 TAP1), and B cells with mutant tapasin (.220 (TPN−)) expressing BNLF2a (BN) or control GFP (c) were separated by SDS-PAGE and Western blots (WB) were analyzed for BNLF2a expression using BNLF2a-specific Abs. B, Effect of BNLF2a on TAP transport activity in PLC-mutant LCLs. LCL, T2 TAP1 TAP2, T2 TAP1, and .220 (TPN−) cells stably expressing BNLF2a (BN) or GFP (c) were analyzed for their ability to transport peptides via TAP using the peptide transport assay described in Fig. 4C. C, HLA class I expression at the surface of BNLF2a-expressing PLC-mutant LCLs. LCL, T2 TAP1 TAP2, T2 TAP1, and .220 (TPN−) cells stably expressing BNLF2a (line 3) or GFP (line 2) were stained for HLA class I (W6/32 mAb) or transferrin receptor (CD71 mAb) and analyzed by flow cytometry. Line 1 represents a negative control (no first Ab). D, Interactions of BNLF2a with components of the PLC in mutant LCLs. Digitonin lysates of LCL, T2 TAP1 TAP2, T2 TAP1, and .220 (TPN−) cells expressing BNLF2a (BN) or GFP (c) were subjected to immunoprecipitation (IP) using Abs specific for TAP1 (148.3), TAP2 (435.4), and tapasin (R gp48C). Precipitates were separated by SDS-PAGE and stained for TAP1 (148.3), TAP2 (435.4), tapasin (7F6), BNLF2a (5B9), and HLA class I heavy chains (HC10) using Western blot (WB) analysis. NA, not applicable (target Ags detected by the TAP2- and tapasin-specific Abs were not expressed in T2 TAP1 and .220 (TPN−) cell lines, respectively).

peptides (Fig. 5B) and high levels of surface HLA class I expression (Fig. 5C). Expression of BNLF2a caused a dramatic inhibition of peptide translocation in T2 TAP1 TAP2 cells and, consequently, a reduction in surface HLA class I (as in LCL cells; Fig. 5, B and C). Surface levels of a control protein, transferrin receptor (TIR), remained unaffected (Fig. 5C). In contrast to the restoration of both TAP subunits, the reconstitution of TAP1 alone was not sufficient to complement the Ag processing defect in T2 cells; accordingly, upon the introduction of BNLF2a into T2 TAP1 cells, no reduction in peptide translocation or surface HLA class I expression was apparent (Fig. 5, B and C). This observation indicates that the block of BNLF2a to HLA class I-restricted Ag presentation occurs entirely at the level of TAP function. Coimmunoprecipitation experiments were used to further delineate which TAP subunits are required for BNLF2a to interact with the PLC. In immune complexes precipitated from digitonin lysates of BNLF2a-expressing T2 TAP1 TAP2 cells, BNLF2a is detected in association with TAP1, TAP2, and tapasin (Fig. 5D, lane 4), as for LCL cells (Fig. 5D, lane 2). In contrast, no coprecipitation of BNLF2a with TAP1 or tapasin was observed for T2 TAP1 cells (Fig. 5D, lane 6), whereas, as a control, the association of tapasin with HLA class I was detected under these conditions. Because T2 TAP1 TAP2 and T2 TAP1 cells only differ in the expression of the TAP2 subunit, these data demonstrate that the presence of TAP2 molecules is required for the interaction of BNLF2a with the PLC.

5B) and flow cytometric analysis of HLA class I surface expression (Fig. 5C). In T2 cells with reconstituted TAP1 and TAP2, TAP was functional as reflected by the transport of fluoresceinlabeled

**TAP is required for stable expression of the BNLF2a protein, but not for its ER localization**

To address whether the TAP complex is essential for ER localization of BNLF2a, we expressed the viral protein in T2 cells that lack both TAP subunits (49). Surprisingly, Western blot analysis of Nonidet P-40 cell lysates revealed that the protein levels of BNLF2a were strongly diminished in the absence of TAP1 and TAP2 (Fig. 6A, compare lane 2 with lane 4). Additional transduction of T2/BNLF2a (Fig. 6A, lanes labeled “G”) cells with a BNLF2a-IRES-NGFR retrovirus did not result in greatly increased BNLF2a protein expression (Fig. 6A, lane 3). For comparison, the control protein transferrin receptor (TIR) was expressed in BNLF2a−/− T2 cells to levels that at least equaled those in T2 TAP1 TAP2 cells (Fig. 6A). These results were confirmed in other TAP-deficient cells, namely immortalized fibroblasts from individuals with TAP defects. BRE cells lack both expression of full-length TAP1 due to a deletion in the coding sequence (53) and that of TAP2, which is unstable in the absence of TAP1 (60). As controls, STF1 cells expressing TAP1 but lacking TAP2 due to a mutation in the TAP2 gene were analyzed in parallel, as well as STF1 cells reconstituted with the human TAP2 gene (STF1/TAP2 cells) (52). Upon transduction of these cell lines with BNLF2a-IRES-NGFR retroviruses, all cells became NGFR− to a comparable degree (data not shown). Whereas abundant BNLF2a expression was
achieved in the TAP\(^+\) cell lines (Fig. 6B, lanes 2 and 4), virtually no BNLF2a protein was detected in lysates from TAP\(^-\) cells (Fig. 6B, lane 6), confirming that the absence of TAP precludes proper expression of this EBV protein.

Next, we determined BNLF2a mRNA levels in the T2-derived cell lines. Using semiquantitative RT-PCR, no major differences in BNLF2a transcripts became apparent when comparing TAP\(^-\) and TAP\(^+\) cells (data not shown). To obtain quantitative information, quantitative PCR was performed on cDNA isolated from T2/BNLF2a (Fig. 6C, left bar marked “G”) cells, additionally transduced T2/BNLF2a (middle bar marked “G/N”) cells, and T2 TAP1 TAP2/BNLF2a cells (right bar marked “G”). Additional transduction of the TAP\(^-\) cells with BNLF2a retroviruses did yield enhanced BNLF2a mRNA expression to levels exceeding those in TAP-reconstituted T2 cells (Fig. 6C), but this was not accompanied by markedly higher BNLF2a protein levels (Fig. 6A). Thus, the combined results indicate that TAP is required for stable expression of BNLF2a at the protein level.

Despite the low levels of BNLF2a protein in TAP-deficient cells, immunofluorescence stainings were performed to determine the intracellular localization of BNLF2a in the absence of TAP. The signal obtained with the BNLF2a-specific mAb 8E2 was much weaker in T2 cells compared with T2 TAP1 TAP2 cells (Fig. 6D), which is in agreement with the Western blotting results. Yet, irrespective of coexpression of TAP, BNLF2a colocalized with the ER marker PDI (Fig. 6D). In conclusion, the EBV-encoded TAP inhibitor BNLF2a is unstable when expressed in cells lacking TAP complexes but still localizes to the ER, implying that ER localization of BNLF2a is not dependent on the interaction of BNLF2a with TAP.

During productive EBV infection, BNLF2a colocalizes and interacts with the HLA class I PLC

Up to this point, we have shown that the EBV lytic phase protein BNLF2a, when expressed in isolation, interacts with the PLC, inhibits peptide transport by TAP, and induces surface HLA class I down-regulation. As a result, BNLF2a prevents recognition by HLA class I-restricted T cells (above data and Ref. 42). Having observed this immune-evasive property of BNLF2a, we aimed at investigating whether BNLF2a fulfills a similar function in the context of natural EBV infection. To this end, we exploited a strategy to identify and isolate populations of productively EBV-infected Burkitt’s lymphoma cells (AKBM cells) on the basis of expression of a reporter fusion protein, rat CD2-GFP, placed under the control of an early EBV promoter (41). This lytic cycle culture

lysates of STF1, STF1/TAP2, and BRE cells expressing BNLF2a (BN) or corresponding control cells (c) were separated by SDS-PAGE and Western blots were analyzed for actin and BNLF2a expression using the mAbs AC-74 and 5B9, respectively. C, Quantification of BNLF2a mRNA levels in T2 cells in the presence or absence of TAP. RNA isolated from T2 and T2 TAP1 TAP2 cells expressing BNLF2a (BN) was used for the synthesis of cDNA. PCRs were performed in the presence of SYBR Green to allow real-time detection of actin mRNA as a control and BNLF2a mRNA. Cycle threshold (Ct) values were calculated and BNLF2a mRNA expression in the various cell lines was corrected for actin expression using the formula \(2^{-\Delta\Delta Ct}\). For comparison, relative BNLF2a mRNA levels were expressed as a percentage of the levels in T2 TAP1 TAP2/BNLF2a cells set at 100%. D, Localization of BNLF2a is similar in T2 and T2 TAP1 TAP2 cells. After fixing and permeabilizing T2/BNLF2a (N) and T2 TAP1 TAP2/BNLF2a (N) cells, intracellular localization of BNLF2a and the ER marker PDI was visualized by confocal microscopy using mAb 8E2 and anti-PDI rabbit serum, respectively. The arrows point to the cells that have been magnified in the pictures displayed in the panels shown below. Scale bar is 10 μm.
system, in combination with the newly generated mAbs specific for BNLF2a, permitted us now to examine BNLF2a during replicative EBV infection.

By Western blot analysis of cells stably transduced to express BNLF2a, the viral protein was detectable as a 7-kDa band that was absent from control cells (Figs. 2–6). Postnuclear Nonidet P-40 lysates of EBV+ B cells (LCL and AKBM) were chosen as a source of naturally expressed BNLF2a. Approximately 1–5% of B cells latently infected with EBV spontaneously enters the lytic cycle. The EBV-encoded, immediately early transactivator BZLF1 is critical to viral reactivation, and cells transformed with an EBV mutant lacking the BZLF1 gene (BZ1KO) fail to enter the lytic cycle (55). Because a specific 7 kDa-protein band was detected only in wild-type EBV-transformed B cells and not in reactivation-deficient BZ1KO cells (Fig. 7A), BNLF2a appears expressed strictly during lytic infection. AKBM cells provide the opportunity to efficiently induce and synchronize EBV reactivation by crosslinking surface IgG. In lytic AKBM cells, isolated for 18 h postinduction, BNLF2a protein expression was observed, but not in latently infected cells (Fig. 7A). Compared with LCLs, BNLF2a expression levels appeared largely elevated in lytic AKBM cells, reflecting the amounts of cells in the lytic cycle ranging from ≈5% of LCLs to >90% of induced and sorted AKBM cells (Fig. 7A).

Next, we examined the intracellular localization of BNLF2a in productively infected cells using fluorescence microscopy. At 6 h postinduction, ~30–40% of cells have entered the lytic phase of infection as marked by the expression of rat CD2-GFP (Fig. 7B, row 1). Interestingly, BNLF2a is expressed in some cells that are not (yet) GFP positive (Fig. 7B, row 1), implying that BNLF2a is expressed early in infection, likely before rat CD2-GFP is localized to the cell surface, whereas BNLF2a is expressed intracellularly (Fig. 7B, rows 1 and 2). In AKBM cells stained for BNLF2a expression, a typical perinuclear staining is observed that mostly coincides with the expression of calnexin and TAP1 (Fig. 7B, rows 3–6). This colocalization of BNLF2a with calnexin and TAP1 in lytically EBV-infected AKBM cells resembles the situation observed in MJS cells stably expressing BNLF2a (Fig. 1), suggesting that the intracellular localization of BNLF2a is not influenced by the coexpression of other viral proteins. From these experiments, we conclude that BNLF2a colocalizes with TAP in the ER compartment in the context of a lytic EBV infection.

Finally, we investigated whether BNLF2a interacts with components of the HLA class I PLC in the context of the full repertoire of lytic phase proteins. Sorted, productively EBV-infected AKBM cells (lytic) and control EBV+ cells in latency (latent) were lysed using 1% digitonin to preserve protein-protein interactions. Lysates were subjected to immunoprecipitation with Abs against TAP1, TAP2, and tapasin. Western blots of the immunoprecipitates were probed with TAPI- or BNLF2a-specific mAbs. In both latently and lyrically EBV-infected cells, TAP1 was found to coprecipitate with TAP2 and tapasin (Fig. 7C), indicating PLCs were present. In lyrically EBV-infected cells, BNLF2a associated with TAP1, TAP2, and tapasin (Fig. 7C) as evidence of BNLF2a interacting with the PLC in productively EBV-infected cells.

In conclusion, these data demonstrate that in EBV+ B cells, BNLF2a is expressed during the lytic cycle, colocalizes with TAP, and occurs in association with the HLA class I PLC, thereby allowing interference with TAP function during the productive phase of EBV infection.

**FIGURE 7.** During productive EBV infection, BNLF2a colocalizes with TAP and interacts with the PLC. A, BNLF2a expression in EBV-transformed B cells and productively infected EBV+ Burkitt’s lymphoma cells. BNLF2a expression in EBV-transformed B cells was examined using Western blot (WB) analysis (5B9 mAb) of postnuclear Nonidet P-40 lysates of B cells transformed with either wild-type (wt) EBV or BZLF1 KO (BZ1KO) virus. Deletion of BZLF1 from EBV precludes entry from latency into the lytic cycle for BZ1KO LCL, whereas 1–5% of wild-type EBV-transformed LCLs spontaneously enters the lytic cycle. EBV+ AKBM Burkitt’s lymphoma cells were incubated with anti-IgG for 18 h to reactivates EBV and were subjected to magnetic cell sorting to obtain an almost pure population of lytically infected AKBM cells. Postnuclear Nonidet P-40 lysates of uninduced (latent) and lytic AKBM cells and control LCLs (MoDo) were separated by SDS-PAGE and analyzed by Western blotting using BNLF2a-specific rat mAbs. B, BNLF2a colocalizes with TAP1 and calnexin during lyric EBV infection. AKBM cells were incubated with anti-IgG for 6 h to induce productive EBV infection and, after fixing and permeabilizing the cells, the intracellular localization of BNLF2a, calnexin, and TAP1 was visualized by confocal microscopy using the Abs 8E2, AF8, and 148.3, respectively. Rat CD2-GFP was used as a marker for lytic phase induction. The arrows point to the cells that have been magnified in the pictures displayed in the panels shown below. Scale bar is 10 μm. C, In the context of productive EBV infection, BNLF2a associates with the HLA class I PLC. Uninduced (latent) and sorted lyrically infected AKBM cells (as in A) were lysed using 1% digitonin and subjected to immunoprecipitation (IP) using Abs specific for TAPI (148.3), TAP2 (435.4), and tapasin (R gp48C). Precipitates were separated by SDS-PAGE and analyzed for TAPI and BNLF2a expression on Western blots (WB) probed with Abs specific to these proteins.
BNLF2a lacks an obvious N-terminal signal sequence (Fig. 1) at their N termini for cotranslational membrane insertion, as integral type I membrane proteins with cleavable signal sequences US6 (35, 36) and BHV-1 UL49.5 (33). The latter two represent), reminiscent of two other TAP inhibitors, HCMV D

Discussion
In this study, we have acquired the following novel information on the EBV-encoded TAP inhibitor BNLF2a: it localizes to the ER membrane together with TAP; it does not drastically alter the composition of the PLC; and it inhibits TAP function even in the absence of tapasin. BNLF2a cannot interact with the PLC in cells devoid of TAP2, and the presence of TAP is required for BNLF2a protein stability. Importantly, BNLF2a expression results in HLA class I down-regulation at the surface of B cells, representing natural host cells for EBV. Finally, the lytic phase protein BNLF2a is abundantly present during EBV replication in B cells and interacts with PLC components, allowing blockade of TAP function.

Despite the absence of apparent sequence homology, mechanistically, BNLF2a shares inhibition of peptide binding with the HSV-encoded ICP47 protein (25, 30) and obstruction of ATP binding with both the HCMV-encoded US6 protein (37, 38) and the equine herpesvirus-encoded UL49.5 protein (34). In the following, we discuss features of BNLF2a in comparison with other viral TAP inhibitors (Table I).

The ER-resident MHC class I PLC forms a key target for viral immune evasion. When analyzing the subcellular distribution of BNLF2a, we observed strict membrane association and colocalization with TAP and the ER markers calnexin and PDI (Figs. 1, 2, 6D, and 7B), reminiscent of two other TAP inhibitors, HCMV US6 (35, 36) and BHV-1 UL49.5 (33). The latter two represent integral type I membrane proteins with cleavable signal sequences at their N termini for cotranslational membrane insertion, as well as transmembrane domains toward their C termini. In contrast, BNLF2a lacks an obvious N-terminal signal sequence (Fig. 1A). Still, the EBV-encoded TAP inhibitor is membrane associated and, even in the absence of TAP, localizes to the ER (Fig. 6D). In this respect, BNLF2a differs from HSV ICP47, which also lacks a signal sequence but has been detected primarily as a cytosolic protein with small amounts associating with membranes (26, 31). ICP47 is unstructured in aqueous solutions but adopts an α-helical structure upon association with membranes (62, 63), thereby rendering the protein functionally active as a competitor for the binding of peptides at the cytosolic domains of TAP. No structural information is available for BNLF2a, but the amino acid composition of this EBV protein is remarkably hydrophilic at its C-terminal end. This hydrophilic domain creates the possibility of anchoring BNLF2a in the ER membrane while leaving the N terminus exposed in the cytosol. This way of membrane anchoring BNLF2a could contribute to the observed membrane localization.

In addition to colocalizing in the ER, BNLF2a also appears to associate with PLCs, as deduced from coimmunoprecipitation experiments (Figs. 3–5 and 7 and Ref. 42). Incorporation of BNLF2a did not drastically alter the overall composition of the PLC. Still, PLC-associated HLA class I H chains from BNLF2a-expressing cells displayed increased reactivity with mAb HC10 under native conditions (Fig. 3), indicating these H chains to be devoid of peptide and β2m. Instability of HLA class I molecules might be caused by a block or delay in HLA class I maturation due to impaired peptide supply in the ER following BNLF2a-mediated TAP inhibition. This is in line with results showing retarded acquisition of endoglycosidase H-resistant glycans on HLA class I heavy chains in BNLF2a-expressing cells (data not shown). Taken together, BNLF2a interacts with intact PLCs and, by preventing TAP-mediated peptide transport, effects retardation of HLA class I maturation, ultimately leading to reduced HLA class I display at the cell surface.

Substitution of the four cysteines within BNLF2a did not abolish its interaction with PLC components and, accordingly, the cysteine-less BNLF2a mutant retained its TAP-inhibiting properties (Fig. 4). Two of four cysteines in BNLF2a are anticipated to be exposed in the cytosol, a reducing environment where disulfide bond formation is less likely; the other two cysteines are in the presumed membrane-spanning domain. The situation is different for US6; its eight cysteine residues reside ER luminal and are likely to be involved in the formation of an intramolecular and possibly also an intermolecular network of S=S bridges. This oligomer formation of US6 supposedly contributes to an aberrant conformation of the TAP transporter, thereby preventing acquisition of ATP for energizing peptide transport (64).

To delineate the role of individual PLC components for BNLF2a-induced TAP inhibition, mutant B cell lines were used. A critical role for TAP2 is implicated by the observation that BNLF2a no longer coprecipitates with the PLC in TAP2-deficient cells (T2 TAP1 cells; Fig. 5). At this stage, we cannot discriminate whether this is due to TAP2 being essential in the formation of proper transporter complexes to which BNLF2a binds, or whether TAP2 by itself represents the direct interaction partner targeted by BNLF2a. For comparison, assembly of TAP subunits into a preformed heterodimer is absolutely required for US6 (37, 64), whereas BHV-1 UL49.5 can interact weakly with single TAP subunits, albeit interactions with the TAP1-TAP2 complex are much stronger (54, 65). In BNLF2a-expressing T2 TAP1 cells, tapasin can still interact with HLA class I H chains (Fig. 5) despite the absence of TAP heterodimers. Because tapasin immunoprecipitates from T2 TAP1 cells do not contain detectable amounts of BNLF2a, direct interactions between tapasin and the EBV protein are unlikely. Accordingly, BNLF2a expression induced HLA class I down-regulation in tapasin-deficient 220 cells. In this respect, BNLF2a resembles US6, for which tapasin is not required to inhibit TAP function (36).

Coexpression of TAP is required for BNLF2a protein stability (Fig. 6). This unexpected observation is in line with TAP being the direct interaction partner for BNLF2a. Interestingly, the viral protein appears stable in cells in which only the TAP1 subunit is present (Fig. 5), suggesting that a (transient) association may occur between TAP1 and BNLF2a. It has not been possible to visualize such a direct interaction by coimmunoprecipitation experiments

### Table I. Comparison of the mechanisms by which herpesvirus-encoded proteins specifically interfere with TAP-mediated peptide transport

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<th>Virus</th>
<th>Inhibitor</th>
<th>Degradation of TAP1/2</th>
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<th>Interference with ATP Binding</th>
<th>Conformational Alterations</th>
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a Equine herpesvirus 1 and 4.
b Pseudorabies virus.

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2m. Instability of HLA class I molecules might be caused by a block or delay in HLA class I maturation due to impaired peptide supply in the ER following BNLF2a-mediated TAP inhibition. This is in line with results showing delayed acquisition of endoglycosidase H-resistant glycans on HLA class I heavy chains in BNLF2a-expressing cells (data not shown). Taken together, BNLF2a interacts with intact PLCs and, by preventing TAP-mediated peptide transport, effects retardation of HLA class I maturation, ultimately leading to reduced HLA class I display at the cell surface.

Substitution of the four cysteines within BNLF2a did not abolish its interaction with PLC components and, accordingly, the cysteine-less BNLF2a mutant retained its TAP-inhibiting properties (Fig. 4). Two of four cysteines in BNLF2a are anticipated to be exposed in the cytosol, a reducing environment where disulfide bond formation is less likely; the other two cysteines are in the presumed membrane-spanning domain. The situation is different for US6; its eight cysteine residues reside ER luminal and are likely to be involved in the formation of an intramolecular and possibly also an intermolecular network of S=S bridges. This oligomer formation of US6 supposedly contributes to an aberrant conformation of the TAP transporter, thereby preventing acquisition of ATP for energizing peptide transport (64).

To delineate the role of individual PLC components for BNLF2a-induced TAP inhibition, mutant B cell lines were used. A critical role for TAP2 is implicated by the observation that BNLF2a no longer coprecipitates with the PLC in TAP2-deficient cells (T2 TAP1 cells; Fig. 5). At this stage, we cannot discriminate whether this is due to TAP2 being essential in the formation of proper transporter complexes to which BNLF2a binds, or whether TAP2 by itself represents the direct interaction partner targeted by BNLF2a. For comparison, assembly of TAP subunits into a preformed heterodimer is absolutely required for US6 (37, 64), whereas BHV-1 UL49.5 can interact weakly with single TAP subunits, albeit interactions with the TAP1-TAP2 complex are much stronger (54, 65). In BNLF2a-expressing T2 TAP1 cells, tapasin can still interact with HLA class I H chains (Fig. 5) despite the absence of TAP heterodimers. Because tapasin immunoprecipitates from T2 TAP1 cells do not contain detectable amounts of BNLF2a, direct interactions between tapasin and the EBV protein are unlikely. Accordingly, BNLF2a expression induced HLA class I down-regulation in tapasin-deficient 220 cells. In this respect, BNLF2a resembles US6, for which tapasin is not required to inhibit TAP function (36).

Coexpression of TAP is required for BNLF2a protein stability (Fig. 6). This unexpected observation is in line with TAP being the direct interaction partner for BNLF2a. Interestingly, the viral protein appears stable in cells in which only the TAP1 subunit is present (Fig. 5), suggesting that a (transient) association may occur between TAP1 and BNLF2a. It has not been possible to visualize such a direct interaction by coimmunoprecipitation experiments
performed on lysates from T2 TAP1/BNLF2a cells (Fig. 5). To our knowledge, there is no precedent of a role for TAP in stabilizing protein expression for other known herpesvirus-encoded TAP inhibitors.

For T2 cells reconstituted with TAP1 and TAP2 (T2 TAP1 TAP2 cells), expression of EBV BNLF2a reduced HLA class I levels at the cell surface to those observed for T2 cells lacking a functional TAP transporter (Fig. 5). This observation implies that the only step in the Ag processing pathway affected by BNLF2a is the function of TAP. This, in turn, means that only the display of (viral) antigenic peptides critically depending on TAP transport for HLA class I presentation will be affected by the BNLF2a-imposed block.

Based on and further extrapolating the combined results obtained for BNLF2a expressed in isolation in various cell lines, we propose the following model for EBV-induced immune evasion through the inhibition of TAP-mediated peptide transport. Upon translation, BNLF2a is inserted into the ER membrane, most likely through its C-terminal hydrophobic domain. BNLF2a then interacts with the HLA class I PLC, with TAP being the direct interaction partner and tapasin being dispensable. The interaction of BNLF2a with TAP stabilizes the viral protein. Exposure of the N-terminal domain of BNLF2a in the cytosol could then interfere with the binding of peptides and ATP to the cytosolic domains of TAP, either directly or through the induction of conformational changes of TAP. This culminates in a block of the peptide-transporting function of TAP, ultimately leading to reduced levels of HLA class I molecules at the cell surface and loss of cytotoxic T cell recognition.

In this study, we obtained for the first time information on the EBV-encoded TAP inhibitor in the context of natural viral infection. This was facilitated by the availability of an EBV lytic cycle culture system in B cells and the generation of BNLF2a-specific Abs allowing detection of untagged BNLF2a as it occurs in productively virus-infected cells. Using the in vitro culture system for EBV, a block in TAP-mediated peptide transport and down-regulation of HLA class I cell surface levels have been observed upon initiation of the EBV lytic cycle (41). We have now demonstrated that BNLF2a is expressed in productively EBV-infected cells in which it is found to colocalize with TAP and associate with the HLA class I PLC (Fig. 7). Interestingly, BNLF2a protein expression appeared early in infection, probably before rat CD2-GFP was expressed from the early BMRF1 promoter (Fig. 7). Similarly, other viral BNLF2a TAP inhibitor would prevent transport of these viral peptides into the ER for presentation to CTLs. Similarly, other viral peptides would prevent transport of these viral peptides into the ER for presentation to CTLs.

References


