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EBV Protein BNLF2a Exploits Host Tail-Anchored Protein Integration Machinery To Inhibit TAP

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EBV, the prototypic human γ-herpesvirus, persists for life in infected individuals, despite the presence of vigorous antiviral immunity. CTLs play an important role in the protection against viral infections, which they detect through recognition of virus-encoded peptides presented in the context of HLA class I molecules at the cell surface. The viral peptides are generated in the cytosol and are transported into the endoplasmic reticulum (ER) by TAP. The EBV-encoded lytic-phase protein BNLF2a acts as a powerful inhibitor of TAP. Consequently, loading of antigenic peptides onto HLA class I molecules is hampered, and recognition of BNLF2a-expressing cells by cytotoxic T cells is avoided. In this study, we characterize BNLF2a as a tail-anchored (TA) protein and elucidate its mode of action. Its hydrophilic N-terminal domain is located in the cytosol, whereas its hydrophobic C-terminal domain is inserted into membranes posttranslationally. TAP has no role in membrane insertion of BNLF2a. Instead, Asn1 (also named TRC40), a cellular protein involved in posttranslational membrane insertion of TA proteins, is responsible for integration of BNLF2a into the ER membrane. Asn1 is thereby required for efficient BNLF2a-mediated HLA class I downregulation. To optimally accomplish immune evasion, BNLF2a is composed of two specialized domains: its C-terminal tail anchor ensures membrane integration and ER retention, whereas its cytosolic N terminus accomplishes inhibition of TAP function. These results illustrate how EBV exploits a cellular pathway for TA protein biogenesis to achieve immune evasion, and they highlight the exquisite adaptation of this virus to its host.

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Abbreviations used in this article: BMH, bis(maleimido)hexane; B/No, BNLF2aOpsin; BN-Ro, BNLF2aRAMP4Opsin; DN, dominant-negative; EnoH, endoglycosidase H₂; ER, endoplasmic reticulum; HA, hemagglutinin; IRES, internal ribosomal entry site; MJS, Mel JuSo; NP-40, Nonidet P-40; PDI, protein disulfide isomerase; PNGaseF, peptide-N-glycosidase F; R-B/No, RAMP4BNLF2aOpsin; RIPA, radiimmunoprecipitation assay; Ro, RAMP4Opsin; TA, tail-anchored; TIR, transmembrane receptor.

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thereof and are transported from the cytosol into the endoplasmic reticulum (ER) by TAP. In the ER, these peptides bind to newly synthesized HLA class I molecules, which subsequently travel to the cell surface for inspection by CTLs (5–7).

Peptide transport by TAP forms a bottleneck in the HLA class I Ag presentation pathway, as substantiated by the identification of TAP-inhibiting proteins in a number of viruses, including EBV. Although these proteins all block TAP-mediated peptide transport, there is no structural or functional homology among them. ICP47, encoded by HSV, is a cytosolic protein that competes for peptide binding to TAP (8–10). Human CMV-encoded US6 is a type I membrane protein blocking ATP binding to TAP (11–13). The UL49.5 proteins of a number of varicelloviruses have been found to block TAP as well (14). These type I membrane proteins cause a conformational arrest of TAP. The UL49.5 protein of bovine herpesvirus-1 additionally targets TAP for degradation. The recently identified cowpox-encoded TAP inhibitor CPXV12 has been classified as a type II membrane protein (15, 16), interfering with peptide transport in an as-yet unresolved way. Finally, the EBV-encoded BNLFL2a protein interferes with both peptide and ATP binding to TAP (17). BNLFL2a is a small membrane-associated protein composed of only 60 aa residues and lacking a bona fide signal sequence. At present, it is unclear whether BNLFL2a is integrated into the ER membrane, and if so, how this might occur. Other important features, such as its mode of interaction with TAP and the mechanism of TAP inhibition, remain to be elucidated. In this study, we characterize the EBV-encoded TAP inhibitor BNLFL2a as a tail-anchored (TA) protein and report on its structural and functional properties that allow this small viral protein to efficiently block T cell recognition.

Materials and Methods

Abs
The following mouse mAbs have been used in this study: W6/32 (18) and B9.12.1 (19) (provided by A. Mulder, Leiden University Medical Center, Leiden, The Netherlands), which both recognize β2-microglobulin–associated HLA class I molecules; 148.3, directed against TAP1 (provided by R. Tampé, Johann Wolfgang Goethe University, Frankfurt/Main, Germany); 435.4, directed against TAP2 (provided by P. van Endert, Hospital Necker-Enfants Malades, Paris, France); 12C5, detecting an influenza virus hemagglutinin (HA) epitope that is used as a protein tag (Roche Diagnostics); H68.4, recognizing transferrin receptor (TfR; Roche Diagnostics); AF8 (20), recognizing calnexin (provided by M. Brenner, Harvard Medical School, Boston, MA); R2-15, recognizing a rhodopsin-derived epitope that is used as a protein tag (21); 9E10, recognizing a cy-Myc–derived epitope that is used as a protein tag (Developmental Studies Hybridoma Bank, Iowa University); DM1A, recognizing tubulin (Sigma-Aldrich); and L2.43, recognizing HLA-DR (American Type Culture Collection). BNLFL2a was detected using the rat mAbs 8E2 and 8E2 (22). The rat mAb 7F6 was used for detection of tapasin (Ref. 23; provided by R. Tampe´). Specific rabbit Abs detected using the rat mAbs 5B9 and 8E2 (22). The rat mAb 7F6 was used for detection of tapasin (Ref. 23; provided by R. Tampe´). Specific rabbit Abs detected using the rat mAbs 5B9 and 8E2 (22).

Cell lines

Mel JuSo (MJS) is a human melanoma cell line (30). T2 cells (31) are TAP-deficient EBV-transformed B lymphoblastoid cells, Reconstiward T2 cells stably expressing TAP1 and TAP2 (T2 TAP1/2) were obtained from P. Lehner (Cambridge Institute for Medical Research, Cambridge, U.K.) (32). HEK 293T cells were used for the production of replication-deficient retroviruses. HEkA is a human cervical cancer cell line (ATCC CCL-2).

Production of and transduction with replication-deficient retroviruses

plRZS retroviral vectors were transfected into the Phoenix A packaging cell line to produce replication-deficient recombinant retroviruses, as described previously (17). For the production of replication-deficient recombinant lentiviruses, HEK 293T cells were transfected with a plV-
CMV-IRES-GFP lentiviral vector encoding the gene-of-interest, in addition to pCMV-VSVG, pMDLg-RRE, and pRSV-REV (provided by R. C. Hoeben) using calcium phosphate precipitation, as described previously (33). After 48 and 72 h, lentivirus-containing culture supernatants were collected and passed through a 0.45-μm filter.

Retroviral transductions were performed essentially as described previously (17). In brief, cells were infected with retroviruses and FACS sorted to achieve reporter expression in all cells. Lentiviral transductions were performed according to the same protocol used for retroviral transductions.

**Transient transfection of MJS cells**

Cells were transiently transfected using the Amaxa Nucleofector II, according to the manufacturers’ instructions. For the transfection of MJS cells, program T-020 and solution L were used.

**Immunofluorescence**

MJS cells were grown overnight on coverslips and fixed in 4% paraformaldehyde for 30 min at 37°C. After permeabilization with 0.1% Triton X-100 for 5 min, cells were stained with primary Abs for 60 min. Subsequently, cells were stained with fluorescently labeled secondary Abs (Jackson ImmunoResearch Laboratories) for 30 min and embedded on microscope slides with Mowiol (Sigma-Aldrich). All incubation steps were performed at room temperature unless stated otherwise. Cells were visualized with a confocal microscope (Leica TCS SP2).

**Immunoprecipitation and Western blot analysis**

Immunoprecipitation experiments, SDS-PAGE, and immunoblotting were performed as described previously (22). Cells were lysed using either 0.5% Nonidet P-40 (NP-40) buffer or 1% digitonin buffer and subsequently centrifuged to obtain postnuclear lysates. For transiently transfected cells, total cell lysates were made by boiling the cells in sample buffer [0.05 M Tris (pH 8), 2% SDS, 10% glycerol, 5% 2-ME, and 0.025% bromophenol blue].

For coimmunoprecipitation experiments, 1% digitonin cell lysates were incubated with specific Abs and protein A- and protein G-Sepharose beads (GE Healthcare). Proteins of interest were detected by incubating the membranes with specific Abs, followed by HRP-conjugated secondary Abs (Jackson ImmunoResearch Laboratories or DukOcytometry). Ab binding was visualized using ECL plus (GE Healthcare).

**Endoglycosidase H and peptide-N-glycosidase F digestion**

Postnuclear NP-40 lysates were denatured in sample buffer and treated with Endoglycosidase H2 (EndoH; New England Biolabs) or peptide-N-glycosidase F (PNGaseF; Roche), according to the instructions of the manufacturer.

**Proteinase K digestion**

Cells were either lyzed in radioimmunoprecipitation assay (RIPA) buffer (1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS in PBS) for 45 min and subsequently centrifuged at 16,000 × g for 4°C for 20 min to obtain postnuclear lysates or resuspended in permeabilization buffer [0.04% digitonin, 25 mM HEPES (pH 7.2), 115 mM potassium acetate, 5 mM MgOAc, 2.5 mM MgCl2, and 0.5 mM EDTA] for protein digestions. Samples were incubated with proteinase K at 4°C for 20 min, followed by the addition of 2 mM PMSF to stop proteolysis. The postnuclear RIPA samples were denatured in sample buffer. The digitation-permeabilized cells were first pelleted at 16,000 × g at 4°C for 10 min and subsequently denatured in sample buffer.

**Peptide transport assay**

Peptide transport assays were performed as described previously (22). In brief, 2 × 106 cells were permeabilized with 2 IU/ml Streptolysin O (Murex Diagnostics) at 37°C for 10 min and subsequently incubated with 4.5 μM of the fluorescentated peptide CVNKTERRAY (provided by W. Benchjuijsen and J. W. Drijfhout, Leiden University Medical Center) in the presence of 10 mM ATP or 0.125 M EDTA at 37°C for 10 min. Peptide transport was blocked by the addition of 1 ml cold lysis buffer [1% Triton X-100, 500 mM NaCl, 2 mM MgCl2, and 50 mM Tris HCl (pH 8)]. Glycosylated peptides were isolated from postnuclear lysates by incubation with Con A-Sepharose beads (GE Healthcare) at 4°C for 2 h.

After washing of the beads, glycosylated peptides were eluted from the beads with elution buffer [500 mM mannopyranoside, 10 mM EDTA, and 50 mM Tris HCl (pH 8)] at room temperature during a 1-h incubation step. Fluorescence was measured using a Mithras LB 940 multilabel reader (Berthold Technologies). Values obtained for the samples incubated in the presence of ATP were corrected for the background values obtained in the absence of ATP.

**Flow cytometry**

Surface levels of HLA class I and HLA class II were analyzed by flow cytometry. Cells were stained with specific primary Abs, washed, and stained with secondary goat anti-mouse PE Abs (Jackson ImmunoResearch Laboratories) or goat anti-mouse allophycocyanin Abs (Leinco Technologies) at 4°C. Cells were analyzed on a FACSCalibur flow cytometer using CellQuest Pro software (BD Biosciences).

**Subcellular fractionation**

Fractionation of cells was performed essentially as described previously (34). A total of 107 cells were washed and resuspended in 1 ml heme-nonglycogenization buffer [0.25 M sucrose, 10 mM triethanolamine, 10 mM potassium acetate, and 1 mM EDTA (pH 7.6)]. Cells were homogenized on ice using a Dounce homogenizer with a tight fitting pestle (100–110 strokes). The homogenate was spun at 10,000 × g at 4°C for 10 min. The 1.000 × g pellet was stored on ice, and the supernatant was subsequently centrifuged at 10,000 × g at 4°C for 30 min. The 10,000 × g pellet was also stored on ice, and the supernatant was now centrifuged at 100,000 × g at 4°C for 60 min using a TLA 120.2 fixed-angle rotor and a Beckman Optima TLX ultracentrifuge. The 100,000 × g supernatant was collected, and all pellets (1,000 × g, 10,000 × g, and 100,000 × g) were resuspended in 0.5% NP-40 buffer. After incubation at 4°C for 30 min, lysates were centrifuged at 16,000 × g at 4°C for 15 min. Sample buffer was added to all four supernatants, and the samples were incubated at 95°C for 5 min. Protein expression was analyzed by Western blotting.

**RT-PCR**

RNA was isolated from transiently transfected MJS cells using TRIzol (Invitrogen), according to the instructions of the manufacturer. cDNA was synthesized using Oligo(dT)20 (Invitrogen) and Moloney murine leukemia virus reverse transcriptase (Finnzymes). To verify that no genomic DNA was present, parallel reactions were performed in the absence of reverse transcriptase.

To detect GAPDH, BNLFL2a (full-length), BNLFL2a-phlic, and BNLFL2a-phobic cDNA, the following primers were used: 5′-CAT CAC CAT CTT CCT CCA GGA GCA-3′ (asGAPDH), 5′-CGG TGG TGA TCT GTG GAG CAC-3′ (asGAPDH), sBNFL2a-phlic, sBNFL2a-phlic, sBNFL2a-phobic, and sBNFL2a-phobic.

**Generation of semipermeabilized cells**

Semipermeabilized cells were generated essentially as described by Wilson et al. (35). 6 × 106 cells were washed, resuspended in KH buffer [110 mM KOAc, 20 mM HEPES (pH 7.2), and 2 mM MgOAc] containing 5 μg/ml digitonin and incubated on ice for 5 min. Subsequently, cells were washed with KH buffer, resuspended in HEPES buffer [50 mM HEPES (pH 7.2) and 90 mM KOAc], and incubated on ice for 10 min. After centrifugation, cells were resuspended in KH buffer and incubated with 1 mM CaCl2 and micrococcal nuclease for 12 min at room temperature to remove endogenous mRNA. The nuclease was inhibited by the addition of 4 mM EGTA, and after centrifugation, cells were resuspended in KH buffer and stored at −80°C.

**In vitro transcription and translation**

pDNA3(-IRES-nlsGFP)–derived vectors were linearized and used for in vitro transcription with T7 polymerase (Invitrogen). Transcripts were translated at 30°C for 90 min in the presence of [35S]methionine/cysteine (EasyTag EXPRESS S3 protein labeling mix; PerkinElmer) in rabbit reticulocyte lysate (Promega). To examine membrane insertion, canine pancreatic microsomal membranes (Promega) were added either at the start of translation or after termination of translation by the addition of 1 mM cycloheximide. Alternatively, semipermeabilized cells were added after termination of translation by cycloheximide. These posttranslation membrane insertion reactions were incubated at 30°C for 30 min. Microsomal membranes or semipermeabilized cells were separated from the supernatant by centrifugation and denatured in sample buffer. Samples were separated by SDS-PAGE and visualized by phosphorimaging. Densitometric analysis was done with Quantity One.
**Chemical cross-linking with bismaleimido)hexane**

Chemical cross-linking experiments were performed essentially as described previously (27). In vitro transcription and translation reactions were performed in the absence of microsomal membranes as described above. After translation, samples were filtered using micro bio-spin P-6 columns (Bio-Rad) and equilibrated in 20 mM HEPES-KOH (pH 7.5), 80 mM KOAc, and 0.5 mM Mg(OAc)$_2$ and subsequently cross-linked with 250 μM bismaleimido)hexane (BMH) (Pierce Biotechnology) at room temperature for 20 min. Reactions were quenched by the addition of 10 mM DTT and incubation on ice for 5 min and subsequently denatured in the presence of 1% SDS by incubation at 65°C for 5 min. After addition of non-denaturing buffer [1% Triton X-100, 50 mM Tris (pH 7.4), 300 mM NaCl, 5 mM EDTA, 0.05% sodium azide, 10 μM leupeptin, and 1 mM 4-[2-(aminoethyl)benzenesulfonyl] fluoride], samples were subjected to denaturing immunoprecipitations. Isolated immune complexes were washed with NET buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, and 0.1% SDS] and denatured in sample buffer. Samples were separated by SDS-PAGE and visualized by phosphorimaging.

**Metabolic labeling of cells**

HeLa cells were transfected using calcium phosphate transfection as described previously (36). After 42 h, cells were depleted of methionine and cysteine for 2 h and subsequently metabolically labeled by addition of 100 μCi/ml [35S]methionine/cysteine (EasyTag EXPRESS35S protein labeling mix (PerkinElmer) for 15 min. Cells were lysed using 1% Triton X-100 buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 1.5 mM MgCl$_2$, 10% glycerol, 1% Triton X-100, 1 mM EDTA, 1 mM PMSF, and protease inhibitor mixture complete EDTA free (Roche)] on ice for 10 min. Nonsoluble material was pelleted by centrifugation at 16,000 g at 4°C for 10 min. The lysates were diluted with immunoprecipitation buffer [20 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, and 0.1% Triton X-100] and incubated with specific Abs and protein A-Sepharose beads (Amersham Biosciences) for 3 h. Isolated immune complexes were washed five times with immunoprecipitation buffer. Immune complexes were denatured in sample buffer, separated by SDS-PAGE and visualized by phosphorimaging. Densitometric analysis was done with ImageJ.

**Tertiary structure model of BNLF2a**

BNLF2a three-dimensional structural predictions were made using the I-TASSER server with National Center for Biotechnology Information Reference Sequence: YP_401721.1 without assigning additional restraints (37). The first model predicted by I-TASSER was used as input for the CHARMM-GUI Membrane Builder (38) using the following options: Orientation, Align the First Principle Axis Along Z; System Building Options, Replacement method; and other settings as default. The ribbon presentation was generated using the Pymol program.

**Results**

**The C-terminal hydrophobic domain of BNLF2a serves as a membrane anchor**

EBV BNLF2a has an N-terminal hydrophilic domain and a hydrophobic C-terminal domain with a predicted α-helical structure (Fig. 1). These features are reminiscent of TA proteins, a class of proteins that are inserted into membranes via their hydrophobic C termini, leaving their N termini exposed in the cytosol. To evaluate whether the topology of BNLF2a resembles that of TA proteins, two approaches were taken.

First, proteinase K digestions were performed on semi-permeabilized cells. Cells stably expressing BNLF2a were either semipermeabilized with digitonin or lysed in RIPA buffer. In digitonin-permeabilized cells, proteinase K could only reach and degrade cytosolic protein domains, leaving luminal structures intact. Accordingly, an Ab directed against the cytoplasmic domain of TIR could no longer detect TIR when the cells were semipermeabilized and treated with proteinase K (Fig. 2A, compare lanes 1 and 2). Under these conditions, an Ab against the ER-luminal domain of calnexin detected a lower m.w. form of calnexin, representing a molecule from which the cytoplasmic domain had been removed by proteinase K (Fig. 2A, lane 2). After lysis in RIPA buffer, all membranous structures are dissolved, thereby allowing proteinase K to degrade both cytosolic and luminal proteins (Fig. 2A, lane 4).

To study whether and how BNLF2a is orientated in the membrane, an Ab that specifically recognizes the N-terminal domain of BNLF2a and an Ab specific for the HA-tag added to the C terminus of BNLF2a were used. Proteinase K treatment of digitonin-permeabilized cells resulted in a marked reduction in the amount of BNLF2a as detected by the Ab directed against the N-terminal domain of the viral protein (Fig. 2A, lane 2). Staining with the anti-HA Ab revealed a lower m.w. form of BNLF2a in proteinase K-treated, digitonin-permeabilized cells (Fig. 2A, lane 4).

This band was absent from untreated cells (Fig. 2A, lane 1). After

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A. Sequence BNLF2a

| MVHVLERALEQQSSACGLGPSSTRPSPHPCEDPDVSRLLLLVVLCLFLCLLI |

B. Hydrophobic index

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**FIGURE 1.** Protein structure of EBV-encoded BNLF2a. A. Amino acid sequence of BNLF2a with the hydrophobic C terminus highlighted. Secondary structure prediction of BNLF2a as determined by the I-TASSER server. Sheets are indicated with S and helixes with H. B. Hydrophobicity profile of BNLF2a as determined by the method of Kyte and Doolittle (39).
BNLF2a is not easily accessible to proteinase K, possibly because its N-terminal domain is membrane-bound and not exposed in the cytosol. Thereby, BNLF2a exhibits one of the hallmarks of TA proteins.

**FIGURE 2.** Hydrophobic domain of BNLF2a serves as a membrane anchor. A, MJS/BNLF2a cells, either lysed in RIPA buffer or permeabilized with digitonin, were incubated with or without 100 μg/ml proteinase K. Protein samples were separated by SDS-PAGE and analyzed by Western blotting for the presence of the control transmembrane proteins TFR and calnexin. BNLF2a was detected using a mAb specific for BNLF2a (5B9) and the HA-tag added to the C terminus of BNLF2a (BN). B, MJS cells stably expressing GFP (lane 2), BNLF2a (lane 3), or BNo (lane 4) were stained for HLA class I (mAb W6/32) and HLA class II (L243) and analyzed by flow cytometry. Lane 1 represents a negative control (secondary Ab only). C, Postnuclear lysates of MJS cells stably expressing GFP, BNLF2a, or BNo were incubated with EndoH. Protein samples were separated by SDS-PAGE and analyzed by Western blotting for the presence of TFR (mAb H68.4) and calnexin (mAb AF8). BNLF2a was detected using a mAb specific for BNLF2a (5B9) and the HA-tag added to the C terminus of BNLF2a (12CA5). B, MJS cells stably expressing BNLF2a (5B9) and subsequently subjected to Western blotting. Treatment of BNo with EndoH resulted in a protein band migrating at a lower m.w. (Fig. 2C, compare lanes 4 and 5), demonstrating that the opsin-tag was indeed glycosylated and thus present in the ER lumen. The glycosylated form of BNo can be communoprecipitated with TAP1 (Fig. 2D, lane 4), indicating that this form of BNo is capable of interacting with TAP.

Cell lysates of BNLF2a- and BNo-expressing cells were treated with EndoH to remove N-linked glycans from ER-resident proteins and subsequently subjected to Western blotting. Treatment of BNo with EndoH resulted in a protein band migrating at a lower m.w. (Fig. 2C, compare lanes 4 and 5), demonstrating the presence of the N-terminal domain.

**BNLF2a is inserted into membranes posttranslationally**

Another characteristic of TA proteins is their posttranslational membrane insertion. Because of the localization of the hydrophobic domain at the C terminus, this domain is released from ribosomes only after completion of protein synthesis (40, 41). To explore whether BNLF2a is inserted into membranes posttranslationally, in vitro translation reactions were performed for BNo. As controls, RAND4 and HLA-A2 were taken along; RAND4 is a known TA protein that is inserted into membranes posttranslationally (27), whereas integration of the type I membrane protein HLA-A2 occurs strictly cotranslationally. As a source of ER, microsomal membranes were added either during the translation reaction or after protein synthesis was blocked, allowing proteins to be inserted into the membranes both co- and posttranslationally or exclusively posttranslationally, respectively. As expected, Ro was membrane-inserted irrespective of the moment of membrane addition (Fig. 3A, lanes 5 and 6), whereas membrane integration of HLA-A2 was entirely dependent on the presence of microsomes during translation (Fig. 3A, lanes 3 and 4).

BNo was integrated into microsomal membranes that were added after termination of translation (Fig. 3A, lane 2), indicating that BNLF2a can be inserted into membranes posttranslationally. The amounts of glycosylated proteins were higher when membranes were added either during the translation reaction or after protein synthesis was blocked, allowing proteins to be inserted into the membranes both co- and posttranslationally or exclusively posttranslationally, respectively. As expected, Ro was membrane-inserted irrespective of the moment of membrane addition (Fig. 3A, lanes 5 and 6), whereas membrane integration of HLA-A2 was entirely dependent on the presence of microsomes during translation (Fig. 3A, lanes 3 and 4).

In conclusion, the hydrophobic C-terminal domain of BNLF2a is inserted into the ER membrane, whereas the N-terminal domain is exposed in the cytosol. Thereby, BNLF2a exhibits one of the hallmarks of TA proteins.
BNLF2a or the control protein GFP were homogenized and performed using T2 and T2 TAP1/2 cells. Cells expressing additionally investigated by subcellular fractionation experiments insignificant increase in the percentage of glycosylated, membrane-A TAP (Fig. 4).

Translation was stopped by addition of cycloheximide to ascertain that protein insertion occurred post-translationally. No differences were seen for the membrane in-cycloheximide to ascertain that protein insertion occurred post-translation by cycloheximide. Microsomes were pelleted, denatured, and separated by SDS-PAGE. Translation products were visualized by phosphorimaging. B. Quantification of A. The amount of protein that was inserted into microsomes addition microsomes as deduced from its presence in the 1,000 g 1,000 g pellet fractions (Fig. 4C). This is consistent with previous results (22) and the proteinase K digestion experiments (Fig. 2A). A similar distribution was observed for BNLF2a in TAP-deficient cells (Fig. 4D).

In conclusion, TAP is dispensable for membrane insertion of the BNLF2a protein.

Asna1 facilitates membrane insertion of BNLF2a

The cellular protein Asna1 is involved in membrane insertion of TA proteins that have a highly hydrophobic tail (44). Because BNLF2a contains an extremely hydrophobic C terminus [predicted ΔG = −3.249 (45)], we investigated whether this viral protein requires Asna1 for membrane insertion. To examine the interaction of Asna1 with BNLF2a, radioactively labeled BNo was synthesized in vitro and subsequently incubated with the cross-linker BMH. Proteins and cross-linked protein products were immunoprecipitated using Abs specific for the C-terminal opsin-tag and Asna1. In the presence of BMH, a band was observed at the position corresponding to the size of a BNo-Asna1 heterodimer, which was absent from the control samples incubated without cross-linker (Fig. 5A, lanes 2 and 4, 1 and 3, respectively). This band was also not detected when preimmune serum or a control anti–c-Myc Ab was used for precipitation (Fig. 5A, lanes 6 and 8), demonstrating specificity of the association between BNLF2a and Asna1. The results of these experiments are comparable to those performed with Ro (Ref. 27 and data not shown). Thus, we conclude that the viral TAP inhibitor BNLF2a interacts with Asna1.

Next, living cells were used to investigate functional involvement of Asna1 in membrane insertion of BNLF2a using a dominant-negative (DN) mutant of Asna1 (SW1). This protein has the aspartic acid residue at position 74, located in the nucleotide binding site, replaced by an alanine residue (29). Cells were transiently transfected with BNo and no, wild-type, or DN Asna1. Western blot analysis of cell lysates confirmed similar transfection efficiencies in cells expressing wild-type or DN Asna1 (Supplemental Fig. 2). After pulse-labeling of the cells, Asna1 and BNo were precipitated using Abs against Asna1 and against the opsin-tag, respectively. Although some endogenous Asna1 was present in the cells, there was clear overexpression of the transfected Asna1 proteins (Fig. 5B). Upon expression of wild-type Asna1, an increase in the amount of glycosylated BNo was observed. In contrast, the amount of glycosylated BNo was decreased in cells expressing the DN form of Asna1 (Fig. 5B). These data demonstrate that membrane insertion of BNLF2a is aided by Asna1.

Finally, we investigated whether Asna1 contributes to the function of BNLF2a as a TAP inhibitor. To this end, MJS cells were transiently transfected to express either wild-type or DN Asna1 in combination with BNLF2a, the latter using a BNLF2a-IRES-GFP vector, resulting in simultaneous expression of the viral protein and GFP. After 48 h, cell surface expression of HLA class I molecules was determined by flow cytometry. For GFP-expressing control cells, no alterations in HLA class I cell surface expression were observed as compared with untransfected, GFP-negative cells (Fig. 5C, left panel). Coexpression of BNLF2a and wild-type Asna1 sedimented by differential centrifugation, thereby separating membranous organelles (pellet fractions) from cytosol (supernatant fraction). Protein content of the different fractions was analyzed by Western blotting. Whereas the membrane protein TIR was detected in the pellet fractions, cytosolic GFP was only detected in the supernatant fraction (Fig. 4C, 4D), showing separation of membrane and cytosolic proteins using this assay.

TAP1, TAP2, and tapasin are detected in the pellet fractions (Fig. 4C, 4D). In TAP-expressing cells, BNLF2a was also associated with membranes as deduced from its presence in the 1,000 g and 10,000 g pellet fractions (Fig. 4C). This is consistent with previous results (22) and the proteinase K digestion experiments (Fig. 2A). A similar distribution was observed for BNLF2a in TAP-deficient cells (Fig. 4D).

In conclusion, TAP is dispensable for membrane insertion of the BNLF2a protein.
BNLF2a were fractionated and analyzed as described in Supplemental Fig. 4). Therefore, chimeric constructs were generated encompassing either the hydrophilic domain or the hydrophobic tail anchor. However, these mutant proteins were unstable (Supplemental Fig. 3). To dissect the functions of the hydrophilic and hydrophobic domains of BNLF2a, mutants of the viral protein were generated. First, an attempt was made to express deletion mutants of BNLF2a in the presence of \[^{35}S\]methionine/cysteine using mRNA encoding BNo or Ro. Semipermeabilized T2 or T2 TAP1/2 cells were added after termination of translation by cycloheximide. Total lysates of these cells were separated by SDS-PAGE. Translation products were visualized by phosphorimaging.

Quantification of A. Samples were corrected for the amount of protein inserted into membranes of semipermeabilized T2 cells (set at 100%). Data are represented as mean ± SD. C, T2 TAP1/2 cells (reconstituted with TAP1 and TAP2) stably expressing GFP or BNLF2a were subjected to subcellular fractionation by differential centrifugation, as described in the Materials and Methods. The 1,000 × g, 10,000 × g, and 100,000 × g pellets and 100,000 × g supernatant (sup) were separated by SDS-PAGE and analyzed by Western blotting for the presence of TfR (mAb H68.4), cytosolic GFP (anti-GFP rabbit serum), BNLF2a (mAb 8E2/5B9), TAP1 (mAb 148.3), TAP2 (mAb 435.4), and tapasin (mAb 7F6).

In the corresponding GFP-negative control cells, 75 and 77% of the GFP-positive BNLF2a-expressing cells that coexpressed wild-type Asna1; of the GFP-positive BNLF2a-expressing cells that coexpressed DN Asna1, 43% of the cells had high HLA class I levels. In the corresponding GFP-negative control cells, 75 and 77% of the cells had high HLA class I levels (Fig. 5C). These results demonstrate that expression of a DN mutant of Asna1 hampers BNLF2a-mediated HLA class I downregulation. In contrast, the function of another viral TAP inhibitor, the type I membrane protein BHV-1 UL49.5, is unaffected by expression of DN Asna1 (Supplemental Fig. 3).

Taken together, these results show that membrane insertion of BNLF2a is facilitated by Asna1 and that the ability of the EBV TAP inhibitor BNLF2a to downregulate HLA class I at the cell surface is diminished in the absence of functional Asna1.

The tail anchor of BNLF2a mediates ER retention

To dissect the functions of the hydrophilic and hydrophobic domains of BNLF2a, mutants of the viral protein were generated. First, an attempt was made to express deletion mutants of BNLF2a encompassing either the hydrophilic domain or the hydrophobic tail anchor. However, these mutant proteins were unstable (Supplemental Fig. 4). Therefore, chimeric constructs were generated containing either the N-terminal hydrophilic domain of BNLF2a fused to the C-terminal hydrophobic tail anchor domain of RAMP4 (BN-Ro) or vice versa (R-BNo) (Fig. 6A). MJS cells were transduced to stably express BNo, the BNLF2a chimeras, or GFP as a negative control. Cell lysates were analyzed by Western blotting to ensure proper expression of the constructs. Visualization using a BNLF2a-specific Ab revealed multiple bands for BNo and BN-Ro, representing glycosylated and nonglycosylated proteins (Fig. 6B, middle panel). R-BNo could only be detected using the opsin-specific Ab because this protein lacks the N-terminal BNLF2a epitope recognized by the BNLF2a-specific Ab (Fig. 6B, lane 4, lower panel). Both BN-Ro and R-BNo proteins appeared to be expressed at higher levels than BNo (Fig. 6B, lanes 3, 4, and 2, respectively). Interestingly, in addition to the two bands representing nonglycosylated and glycosylated protein, a third, broad, high m.w. band was detected for BN-Ro using both BNLF2a- and opsin-specific Abs (Fig. 6B, lane 3). This might represent BN-Ro that has passed through the Golgi compartments where the glycans have been modified further to become EndoH-resistant.

To investigate the nature of the protein species observed, cell lysates were treated with EndoH or PNGaseF. Whereas EndoH can only remove immature glycans of ER-resident proteins, PNGaseF can additionally cleave modified, complex glycans of proteins that have traveled to the Golgi and beyond. For BNo, treatment with either EndoH or PNGaseF resulted in reduction of its m.w. to the size of the unglycosylated protein (Fig. 6C, upper left panel, lanes 2 and 3). The same pattern is observed for Ro and R-BNo (Fig. 6C, right panels), showing that BNo, Ro, and R-BNo are all...
methionine/cysteine using mRNA encoding BNo. After cross-linking with 250 μM BMH, the reactions were subjected to immunoprecipitations under denaturing conditions using Abs specific for the ops-in-tag (mAb R2-15) and Asna1 (anti-Asna1 rabbit serum), preimmune rabbit serum, and the control anti–c-Myc Ab (mAb 9E10). Immune complexes were separated by SDS-PAGE and visualized by phosphorimaging. B, HeLa cells were transiently transfected to express BNo and to coexpress the empty vector as a control, Asna1-myc wild-type (Asna1 WT), or the dominant negative Asna1-myc SW1 mutant (Asna1 DN) in a ratio of 3:1. After 42 h, cells were pulsed with [35S]methionine/cysteine for 15 min. Postnuclear lysates were subjected to immunoprecipitations using Abs specific for Asna1 (anti-Asna1 rabbit serum) and the ops-in-tag added to the C terminus of BNLF2a (mAb R2-15). Immune complexes were separated by SDS-PAGE and visualized by phosphorimaging. The amount of glycosylated BNo protein was quantified and compared with the amount of glycosylated BNo protein in empty vector-transfected control cells (set at 100%). C, MJS cells were transiently transfected to express BNLF2a and to coexpress either Asna1 wild-type (Asna1 WT) or the dominant negative Asna1 SW1 mutant (Asna1 DN) in a ratio of 1:3. The BNLF2a-expression vector contains an IRES immediately downstream of the BNLF2a gene to allow coexpression of the viral protein and GFP. As a control, cells were transfected to express GFP. After 48 h, cells were stained for cell surface expression of HLA class I (mAb B9.12.1) and analyzed by flow cytometry.

A

B

C

FIGURE 5. Asna1 is involved in the membrane insertion and functionality of BNLF2a. A. In vitro translation reactions were performed in the presence of [35S]methionine/cysteine using mRNA encoding BNo. After cross-linking with 250 μM BMH, the reactions were subjected to immunoprecipitations under denaturing conditions using Abs specific for the ops-in-tag (mAb R2-15) and Asna1 (anti-Asna1 rabbit serum), preimmune rabbit serum, and the control anti–c-Myc Ab (mAb 9E10). Immune complexes were separated by SDS-PAGE and visualized by phosphorimaging. B, HeLa cells were transiently transfected to express BNo and to coexpress the empty vector as a control, Asna1-myc wild-type (Asna1 WT), or the dominant negative Asna1-myc SW1 mutant (Asna1 DN) in a ratio of 3:1. After 42 h, cells were pulsed with [35S]methionine/cysteine for 15 min. Postnuclear lysates were subjected to immunoprecipitations using Abs specific for Asna1 (anti-Asna1 rabbit serum) and the ops-in-tag added to the C terminus of BNLF2a (mAb R2-15). Immune complexes were separated by SDS-PAGE and visualized by phosphorimaging. The amount of glycosylated BNo protein was quantified and compared with the amount of glycosylated BNo protein in empty vector-transfected control cells (set at 100%). C, MJS cells were transiently transfected to express BNLF2a and to coexpress either Asna1 wild-type (Asna1 WT) or the dominant negative Asna1 SW1 mutant (Asna1 DN) in a ratio of 1:3. The BNLF2a-expression vector contains an IRES immediately downstream of the BNLF2a gene to allow coexpression of the viral protein and GFP. As a control, cells were transfected to express GFP. After 48 h, cells were stained for cell surface expression of HLA class I (mAb B9.12.1) and analyzed by flow cytometry.

The hydrophilic domain of BNLF2a is essential for immune evasion

To examine functionality of the BNLF2a chimeras, cells stably expressing BNo or the chimeras were analyzed for HLA class I downregulation by flow cytometry. Whereas GFP-expressing control cells had high levels of HLA class I at their surface, virtually no HLA class I was detected on cells expressing BNo (Fig. 7A, upper left panel). Cells expressing BN-Ro also had a diminished surface expression of HLA class I molecules when compared with GFP-expressing cells, although this downregulation was not as strong as that observed for BNo-expressing cells (Fig. 7A, compare middle left and upper left panels). In contrast, R-BNo had virtually no effect on HLA class I surface display. No alteration in HLA class II levels was seen for any of the cell lines (Fig. 7A, right panels).

Functionality of the BNLF2a chimeras was further tested using an in vitro peptide translocation assay. In GFP-expressing control cells, peptides were efficiently translocated across the ER membrane. BNo blocked TAP-mediated peptide transport almost completely, whereas BN-Ro had a weaker but still significant effect. No inhibition was seen upon expression of R-BNo (Fig. 7B). These results are in agreement with the extent of (mutant) BNLF2a-mediated HLA class I downregulation observed by flow cytometry.

Interactions between the BNLF2a chimeras and the TAP complex were examined in communoprecipitation experiments. BN-Ro coprecipitated with TAP, as did full-length BNo (Fig. 7C, upper panel, lanes 2 and 3), implying that the N-terminal domain of BNLF2a is sufficient to mediate association with TAP complex. No coprecipitation of R-BNo with TAP was observed (Fig. 7C, lower panel, lane 3), indicating that interactions with TAP do not occur in the absence of the N terminus of BNLF2a.

Taken together, these results indicate that the hydrophilic domain of BNLF2a is capable of binding to the TAP complex and inhibiting TAP function. The tail anchor of BNLF2a mediates insertion into the membrane and retains the viral protein in the ER.
Discussion

In this study, we have identified the EBV-encoded TAP inhibitor BNLF2a as a TA protein. TAP is not required for integration of BNLF2a into membranes. Instead, membrane insertion of BNLF2a is facilitated by Asna1. Accordingly, Asna1 is required for efficient HLA class I downregulation mediated by the viral protein. BNLF2a exerts its function as an immune evasion protein through the combined action of its two subdomains. The hydrophobic C-terminus of BNLF2a is inserted into membranes and mediates ER retention, whereas the hydrophilic N-terminal domain of BNLF2a resides in the cytosol and blocks TAP function.

As a characteristic of TA proteins, BNLF2a is inserted into membranes posttranslationally. In the absence of membranes, the viral TAP inhibitor was found to associate with Asna1. This protein is part of a larger complex, the cytosolic transmembrane domain recognition complex, that facilitates insertion of TA proteins into membranes.
membranes (27, 46). Expression of a DN mutant of Asna1 resulted in reduced insertion of BNLF2a into membranes and impaired BNLF2a-mediated HLA class I downregulation. These results indicate that the cellular Asna1 pathway for TA protein biogenesis has been adopted by EBV to elude the host immune system.

The effect of DN Asna1 appears not absolute: membrane insertion of BNLF2a is partially reduced and reversal of BNLF2a-mediated HLA class I downregulation is also incomplete. Recently, another immune evasion protein, the human herpesvirus 6-encoded protein U24, was found to rely on Asna1 for downregulation of CD3ε and TFR. Also in those cases, no complete reversion of human herpesvirus 6 U24 function was observed for cells expressing DN Asna1 (47). In view of the important role of TA proteins in many cellular processes, it is likely that alternative pathways facilitating membrane insertion of TA proteins exist that may partially compensate for Asna1 deficiency.

Using chimeras of the viral TAP inhibitor and another TA protein, RAMP4, dedicated functions could be assigned to different domains of BNLF2a. Although a RAMP4-BNLF2a fusion protein lost all immune evasive properties, the BNLF2a-RAMP4 chimera retained most of its TAP-inhibiting capacity. This indicates that the cytosolic hydrophilic N-terminal domain of BNLF2a is essential for TAP inhibition and HLA class I downregulation. The reduced activity of BNLF2a-RAMP4 compared with wild-type BNLF2a suggests that the tail anchor adds to the efficiency of TAP inhibition. The cytosolic domain and the tail anchor of BNLF2a are both required for stable protein expression. Unexpectedly, although BNLF2a and RAMP4 reside in the ER, the BNLF2a-RAMP4 fusion protein is expressed both at the ER membrane and in post-ER compartments. These findings indicate that the hydrophobic C terminus of BNLF2a plays a role in ER targeting and retention.

Previously, we have shown that the TAP complex is required for stable expression of the BNLF2a protein (22). We now demonstrate that TAP confers protection from proteolysis to the viral protein. This was inferred from experiments in TAP-deficient cells, where the hydrophilic domain of BNLF2a is freely exposed in the cytosol and readily accessible for degradation by the ubiquitin proteasome system and/or other proteases. Further evidence for a role of TAP in BNLF2a’s association with or insertion into membranes was not found.

On the basis of these combined results, we propose the following model for the biogenesis and function of BNLF2a. After translation, the EBV-encoded TA protein interacts with Asna1, facilitating insertion of the hydrophobic C terminus of BNLF2a into the ER membrane and leaving the hydrophilic N-terminal domain exposed in the cytosol (Fig. 8). The hydrophobic C-terminal domain is required for retention of BNLF2a in the ER and increases the efficiency of TAP inhibition. The N-terminal domain interacts with the TAP complex. This arrangement ensures...
effectiveness with the binding of both peptide and ATP to TAP, thereby blocking TAP-mediated peptide transport.

In conclusion, this study reveals how an EBV-encoded protein uses a cellular pathway for TA protein biogenesis to inhibit TAP-mediated peptide transport, allowing the virus to effectively impair HLA class I-restricted Ag presentation and escape from CTL recognition. Homologs of EBV-encoded BNLF2a have been found in several related lymphocryptoviruses occurring in Old World primates. These BNLF2a proteins all impair HLA class I-restricted Ag presentation, most likely through a similar strategy as described for EBV BNLF2a in the current study. These findings highlight the importance of CTL immunity in protection against lymphocryptoviruses and illustrate how millions of years of coevolution have resulted in an excellent adaptation of EBV and related lymphocryptoviruses to their respective host species.

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Disclosures
The authors have no financial conflicts of interest.

References
Figure S1. TAP protects BNLF2a from cleavage by proteinase K.

T2 cells (lacking TAP) and T2 TAP1/2 cells (reconstituted with TAP1 and TAP2), both stably expressing BNLF2a, were permeabilized with digitonin and incubated with or without proteinase K. Protein samples were separated by SDS-PAGE and analysed by Western blotting for the presence of BNLF2a (mAb 8E2).
Figure S2. Protein expression levels in transiently transfected HeLa cells.

HeLa cells were transiently transfected to express BNo and to co-express either Asna1-myc wild-type (Asna1 WT) or the dominant negative Asna1-myc SW1 mutant (Asna1 SW1) in a ratio of 3:1. Untransfected cells were taken along as a control. After 42 hours, cell lysates were prepared, separated by SDS-PAGE and analysed by Western blotting for protein expression using antibodies against the control protein tubulin (mAb DM1A), the c-Myc tag added to Asna1 (mAb 9E10) and GFP (anti-GFP rabbit serum). GFP is expressed from the same mRNA as BNLF2a using an internal ribosomal entry site (IRES), thereby correlating GFP protein levels with BNLF2a expression.
Asna1 is involved in BNLF2a-mediated HLA class I downregulation.

A  MJS cells were transiently transfected to co-express viral TAP inhibitors and either Asna1-myc wild-type (Asna1 WT) or the dominant negative Asna1-myc SW1 mutant (Asna1 DN) in a ratio of 1:3 to evaluate the effect of the TA protein insertion machinery on HLA class I immune evasion. The TAP inhibitors were: EBV BNLF2a (TA protein) and bovine herpesvirus-1 UL49.5 (type I membrane protein). The empty pcDNA3 vector and GFP were used as controls. After 48 hours, cells were stained for surface expression of HLA class I (mAb B9.12.1) and the control protein HLA class II (mAb L243) and analysed by flow cytometry. These results show that BNLF2a-mediated HLA class I downregulation is dependent on functional Asna1, whereas UL49.5-mediated HLA class I downregulation is unaffected by DN Asna1.

Figure S3.
B Quantification of A. To allow easier comparison of the data in A, we quantified the mean fluorescence intensities of HLA class I and HLA class II for the range of GFP expression. The latter reflects the expression of the viral TAP inhibitors. Samples were normalized for HLA expression on their GFP negative cells (set at 100%). At the same time, samples were normalized for HLA expression at the surface of the corresponding empty vector control samples (set at 100%). Data are represented as mean ± SD.
Figure S4. The hydrophilic and hydrophobic domains of BNLF2a are incapable of HLA class I downregulation when expressed individually.

A  MJS cells were transiently transfected to express GFP, BNLF2a (BN), the N-terminal hydrophilic domain of BNLF2a (philic) or the C-terminal hydrophobic domain of BNLF2a (phobic). After 48 hours, cells were stained for HLA class I (mAb B9.12.1) or HLA class II (mAb L243) and analysed by flow cytometry. Untransfected cells were taken along as a control.

B  Total protein lysates of the transiently transfected cells (see S2A) were separated by SDS-PAGE and analysed by Western blotting for the expression of transferrin receptor (TfR; mAb H68.4) and BNLF2a (mutants) using the mAbs 5B9, recognizing the N-terminus of BNLF2a, and 12CA5, recognizing the HA-tag added to the C-terminus of full length BNLF2a and the hydrophobic mutant. Untransfected cells were taken along as a control.
RNA of the transiently transfected MJS cells (see S2A) was isolated and used for the synthesis of cDNA. PCR reactions were performed to detect *GAPDH* as a control and either full length *BNLF2a* (BN) or the N-terminal hydrophilic (philic) and C-terminal hydrophobic (phobic) domains of *BNLF2a*. Untransfected cells were taken along as a control.