Epstein-Barr Viral BNLF2a Protein Hijacks the Tail-anchored Protein Insertion Machinery to Block Antigen Processing by the Transport Complex TAP*

Received for publication, March 7, 2011, and in revised form, September 19, 2011 Published, JBC Papers in Press, October 7, 2011, DOI 10.1074/jbc.M111.237784

Agnes I. Wycisk‡1, Jiacheng Lin††, Sandra Loch‡, Kathleen Hobohm‡, Jessica Funke†, Ralph Wiencke†, Joachim Koch‡2, William R. Skach†, Peter U. Mayerhofer‡, and Robert Tampé‡3

From the ‡Institute of Biochemistry, Biocenter, Goethe University Frankfurt, Max-von-Laue Str. 9, 60438 Frankfurt, Germany and the †Department of Biochemistry and Molecular Biology, Oregon Health and Science University, Portland, Oregon 97239

Significance:

Virus-infected cells are eliminated by cytotoxic T lymphocytes, which recognize viral epitopes displayed on major histocompatibility complex class I molecules at the cell surface. Herpesviruses have evolved sophisticated strategies to escape this immune surveillance. During the lytic phase of EBV infection, the viral factor BNLF2a interferes with antigen processing by preventing peptide loading of major histocompatibility complex class I molecules. Here we reveal details of the inhibition mechanism of this EBV protein. We demonstrate that BNLF2a acts as a tail-anchored protein, exploiting the mammalian Asna-1/WRB (Get3/Get1) machinery for posttranslational insertion into the endoplasmic reticulum membrane, where it subsequently blocks antigen translocation by the transporter associated with antigen processing (TAP). BNLF2a binds directly to the core TAP complex arresting the ATP-binding cassette transporter in a transport-incompetent conformation. The inhibition mechanism of EBV BNLF2a is distinct and mutually exclusive of other viral TAP inhibitors.

Background: Herpesviruses have evolved sophisticated strategies to escape immune surveillance.

Results: EBV BNLF2a acts as tail-anchored protein and is posttranslationally inserted into the ER membrane, where it arrests core TAP in a transport-incompetent conformation.

Conclusion: BNLF2a exploits the host tail-anchored protein insertion machinery.

Significance: This inhibition mechanism is distinct and mutually exclusive of other viral TAP inhibitors.
crosis and a subset of malignant tumors such as Hodgkin’s disease and nasopharyngeal carcinoma (19). Over 90% of adult human beings worldwide have a latent EBV infection. BNLF2a (60 amino acids, 6.5 kDa) consists of a hydrophilic N-terminal region of 40 residues followed by a predicated C-terminal transmembrane domain of 20 amino acids. Because BNLF2a lacks an N-terminal signal sequence for cotranslational membrane insertion, we anticipated that it belongs to the group of tail-anchored membrane proteins. Thus, BNLF2a and its homologues display a hitherto unique structure and the molecular inhibition mechanism needs to be captured.

Here we established both an in vitro translation and insect cell expression system to study the inhibition mechanism of this viral factor. We demonstrate that BNLF2a is a tail-anchored protein that is posttranslationally inserted into the ER membrane, where it binds directly to the core TAP complex. BNLF2a arrests the TAP heterodimer in a transport-incompetent conformation that excludes binding of the viral immune evasin US6. Thus the TAP inhibition mechanism of EBV BNLF2a is distinct and TAP binding is mutually exclusive from HCMV-US6.

EXPERIMENTAL PROCEDURES

Cloning and Constructs—BNLF2a was synthesized de novo (gene ID 3783720) (20) and was used as a template for PCR amplification. PCR reactions were performed under standard conditions using Phusion DNA polymerase (Finnzymes, Finland) and synthetic oligonucleotide primers (endonuclease cleavage sites are underlined). All constructs were verified by DNA sequencing. For expression in human cells, PCR-generated products were inserted into pIRE2-EGFP (Clontech) via the respective restriction sites upstream of the internal ribosome entry site (IRES) and enhanced GFP. The following primers were used to generate BNLF2aCs-NST: CCGGAATTCCGGATGCTCACGTCG EcoRI forward and GCCGGATCCACATCCGCCGTGCTGTTTTCTTCAATCGCTCTCGGACCCGGTGCAGACAGCAGCGAGCGCAG BamHI reverse. BNLF2aH was created with the following primers: CCGGAAATTCGGATGTTAACATCAATCCGCCGTGCTGTTTTCTTCAATCGCTCTCGGACCCGGTGCAGACAGCAGCGAGCGCAG BamHI reverse. BNLF2a was amplified with the following primers: CGATTACCTGGATCTAGTCATGGTGGCA CGTGTGGG NcoI forward and CGCGGAATTCGATATGGATCGCAGTGCTGCTGTTTTCTTCAATCGCTCTCGGACCCGGTGCAGACAGCAGCGAGCGCAG BamHI reverse and CGCGGATCCACATCCGCCGTGCTGTTTTCTTCAATCGCTCTCGGACCCGGTGCAGACAGCAGCGAGCGCAG BamHI reverse and pIRE2-EGFP-UL49.5-pIRES2-EGFP, respectively. HeLa cells were seeded in 6-well plates with a density of 4 × 10⁵ cells/well and transfected using FuGENE 6 transfection reagent (Roche) with 3.7 µg DNA/well following manufacturer’s protocol. For coimmunoprecipitation experiments, HeLa cells were treated with 2.5 ng/ml interferon-γ (Sigma Aldrich). Start of stimulation was 18 h post-transfection and was continued for 24 h until harvesting of the cells.

Flow Cytometry—MHC I surface expression was analyzed using the phycoerythrin-coupled antibody W6/32 (Sigma Aldrich). Start of stimulation was 18 h post-transfection and was continued for 24 h until harvesting of the cells.
buffer, the corresponding antibody (1:5 in FACS buffer) was added to the cells and incubated for 15 min on ice in the dark. Subsequently, the cells were washed twice with FACS buffer and finally resuspended in 0.5 ml. The cells were analyzed using a FACSAria flow cytometer (BD Biosciences). For each experiment, 3 x 10^5 cells were evaluated.

In Vitro Translation and ER Insertion—Plasmids (pSP64-BNLF2a<sup>CR-NST</sup>) containing a C-terminal C8 tag followed by an N-core glycosylation site and three extra methionines, pSP64-UL49.5 (17), pSP64-Ramp<sup>opsin</sup> (22), and pSP64-BPL (21), 1 µg per 25-µl reaction were transcribed and translated in vitro in rabbit reticulocytes lysate (Promega) in the presence of [35S]Met (Hartmann Analytic, Braunschweig, Germany, 10 µCi per 25-µl reaction). After incubation for 90 min at 30 °C, translation was stopped by addition of puromycin (2 mM final). For cotranslational membrane insertion, dog pancreas rough microsomes (RM, Promega) were added before the transcription/translation reaction. For posttranslational membrane insertion, translation was performed in the absence of microsomes. After puromycin treatment and translation termination, rough microsomes were added, and the samples were incubated for an additional 30 min at 30 °C.

For the generation of truncated mRNAs lacking a stop codon, BNLF2a<sup>CR-NST</sup> was amplified directly from the pSP64-BNLF2a<sup>CR-NST</sup> plasmid as described above using the GATT-TAGGTACACTATAGAATAC SP6-forward and CATCAT-CATGTTGCTGTTC TCTC reverse primers. The purified PCR product was transcribed in vitro using SP6 RNA polymerase (27). In vitro translations of mRNA templates in wheat germ cell-free extract (tRNA Probes, LLC, College Station, TX) were performed for 40 min at 26 °C in the presence of [35S]Met (tRNA Probes) as indicated, and other components as described (28). WRB-67 inhibitory peptide (residue 35–101) was kindly provided by M. Mariappan and R. S. Hegde (MRC, Cambridge, UK).

Rough microsomes were collected by sedimentation through a 0.5 M sucrose cushion in HEPES buffer (10 mM HEPES (pH 7.5), 100 mM KAc, 1 mM MgAc, 1 mM DTT) at 100,000 x g for 20 min at 4 °C. Translation products were analyzed either directly or solubilized for 10 min at 100 °C in denaturation buffer (0.5% SDS, 40 mM DTT) prior to EndoH treatment (New England Biolabs, 25 units/µl for 1 h in 50 mM sodium citrate (pH 5.5), 0.25% SDS, 20 mM DTT). Proteins were then examined by Tricine/SDS-PAGE (10%) and autoradiography (PhosphorImager, GE Healthcare). Intensities were quantified using ImageJ. For carbonate extraction assays, membranes were collected by sedimentation as before. After incubation in carbonate buffer (0.1 M Na<sub>2</sub>CO<sub>3</sub> (pH 11.5)) for 15 min on ice, the membranes were collected by centrifugation (100,000 x g for 20 min at 4 °C), washed, and centrifuged before final resuspension in carbonate buffer. The supernatants and the resuspended pellets were neutralized with glacial acetic acid and further analyzed as above.

Immunoprecipitation and Immunoblotting—Raji microsomes were prepared from human Burkitt’s lymphoma cells (Raji) (29). Proteins were translated in vitro in the presence of Raji microsomes in rabbit reticulocytes lysate as described above. After completion of translation, microsomes were collected by sedimentation through a 0.5 M sucrose cushion in HEPES buffer (10 mM HEPES (pH 7.5), 100 mM KAc, 1 mM MgAc, 1 mM DTT) at 100,000 x g for 10 min at 4 °C. Microsomes were solubilized in 1 ml of lysis buffer L1 (20 mM Tris/HCl (pH 7.5), 150 mM NaCl, 5 mM MgCl<sub>2</sub>, 2% (w/v) digitonin (Carl Roth, Karlsruhe, Germany)).

Membranes isolated from S9 insect cells (1 mg of total protein) or 2 x 10<sup>6</sup> transfected HeLa cells, respectively, were resuspended in 0.5 ml lysis buffer as described above. After solubilization for 60 min on ice, non-solubilized proteins were removed by centrifugation at 100,000 x g for 30 min at 4 °C. The supernatant was incubated with Dynabeads (M-280 sheep anti-mouse IgG, Dynal Biotech, Hamburg, Germany), which had been preloaded with antibodies. Dynabeads loaded with antibodies either directed against the unrelated ER-resident protein Sec61α or directed against other unrelated proteins (anti-myc or anti-HC10, respectively) were used as adequate negative controls. Beads were washed three times with 1 ml of washing buffer (20 mM Tris/HCl, 150 mM NaCl, 2 mM EDTA, 0.2% (w/v) digitonin (pH 7.5)). Proteins were eluted in SDS sample buffer (2% SDS, 50 mM Tris/HCl, 200 mM DTT, 10% glycerol, 0.05% bromphenol blue (pH 8.0)) for 3 min at 65 °C. Samples were denatured for 20 min at 65 °C and separated by Tricine/SDS-PAGE (10%). After electrotransfer onto polyvinylidene difluoride membranes, proteins were either detected by phosphoimaging or by incubating the membranes with specific antibodies as indicated. Horseradish peroxidase-conjugated secondary antibodies were detected with Lumi-Imager F1 (Roche).

Cross-linking of TAP—Membranes isolated from S9 insect cells (0.5 mg of total protein) were resuspended in 100 µl of ice-cold PBS buffer. The homo-bifunctional cross-linker ethyleneglycol bis(succinimidyl succinate) (Thermo Scientific, Rockford, IL) was added to a final concentration of 0.5 mM. After incubation for 30 min at 4 °C, the reaction was stopped by adding Tris/HCl buffer (pH 7.5) (50 mM final concentration). Membranes were collected by centrifugation (20,000 x g for 8 min at 4 °C) and analyzed by SDS-PAGE (6%) and immunoblotting using TAP1- or TAP2-specific antibodies. As specifically indicated, membranes were preincubated with 10 µM peptide RRYQKSTEL or ICP47 at 4 °C for 1 h prior to cross-linking. ICP47 was expressed and purified as described (9).

Peptide Transport—Membranes isolated from S9 cells (100 µg of protein) were resuspended in 50 µl of AP buffer (5 mM MgCl<sub>2</sub> in PBS (pH 7.4)) in the presence of 3 mM ATP. The transport reaction was started by adding 1 µM of the peptide RRYQNSTOL (O’, fluorescein-labeled cysteine) for 3 min at 32 °C and terminated with 1 ml of ice-cold stop buffer (10 mM EDTA in PBS (pH 7.0)). After centrifugation (20,000 x g for 8 min), the pellet was solubilized in 0.5 ml of lysis buffer L2 (50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 5 mM KCl, 1 mM CaCl<sub>2</sub>, 1 mM MnCl<sub>2</sub>, 1% Nonidet P-40) for 30 min at 4 °C. Non-solubilized proteins were removed by centrifugation, and the supernatant was incubated with 60 µl of ConA-Sepharose (50% w/v, Sigma-Aldrich) for 1 h at 4 °C. After three washing steps with 0.5 ml of lysis buffer each, ConA-bound peptides were specifically eluted with methyl-α-D-mannopyranoside (200 mM) and...
quantified by a fluorescence plate reader (λ_{ex/em} 485/520 nm, Polarstar Galaxy, BMG Labtech, Offenburg, Germany). Background transport was determined in the presence of apyrase (1 units/sample). All measurements were performed in triplicate.

**Peptide Binding**—Membranes (100 μg of total protein) prepared from S9 cells were incubated with 0.5 μM of the peptide R9LFlu (RRYØKSTEL; Ø, fluorescein-labeled cysteine) in 50 μl of AP buffer for 15 min at 4 °C. Free peptides were removed by washing the membranes twice with 100 μl of ice-cold AP buffer using a vacuum manifold with 96-well filter plates (0.65 μm of polyvinylidene difluoride membranes, MultiScreen, Millipore). After elution with AP buffer containing 1% SDS, peptides were quantified by a fluorescence plate reader. All measurements were performed in triplicate. Background binding was determined in 100-fold excess of unlabeled peptide R9L (RRYQKSTEL). The soluble domain of BNLF2a (amino acids 2–42, BNLF2a2–42) was prepared by solid-phase synthesis using the Fmoc/t-Bu-strategy. BNLF2a2–42 was purified by reverse-phase C_{18} HPLC and labeled with 5(6)-carboxyfluorescein succinimidyl ester (Invitrogen) at the N terminus. The identity of the unlabeled and labeled BNLF2a2–42 was confirmed by mass spectrometry.

**RESULTS**

**EBV BNLF2a Has a Tail-anchored Topology**—BNLF2a is composed of a hydrophilic N-terminal region (40 amino acids) followed by a hydrophobic segment (20 amino acids) with a predicted α-helical structure (Fig. 1). Notably, BNLF2a does not harbor an N-terminal signal sequence. These characteristic traits are indicative for tail-anchored (TA) proteins, which are targeted to subcellular membranes by their C-terminal transmembrane segment, hereby exposing their N-terminal domain toward the cytosol (30). TA proteins are released from free ribosomes and are posttranslationally inserted into membranes (31). To examine whether BNLF2a has indeed a tail-anchored topology, we extended its C terminus by an N-glycosylation site (NST) placed sufficiently far from the transmembrane segment by an intervening C8 epitope tag (BNLF2aC8-NST). To ensure that these extra residues do not affect BNLF2a activity, we expressed BNLF2aC8-NST in HeLa cells and analyzed the effect on MHC I surface expression by flow cytometry (Fig. 2A). Similar to UL49.5 (17, 18), expression of BNLF2aC8-NST causes a down-regulation of MHC I surface expression, thereby confirming the activity of C-terminally extended constructs. Similar results were obtained for wild-type and differently tagged versions, e.g. BNLF2aHA. It is worth mentioning that the total amount of MHC I was not altered in BNLF2a transiently transfected HeLa cells (Fig. 2B). BNLF2aHA coimmunoprecipitated with TAP1 and TAP2 in IFN-γ stimulated HeLa cells (Fig. 2C, supplemental Fig. S1A), demonstrating that C-terminally tagged BNLF2a binds to the PLC (18, 32). Notably, the slight difference in migration behavior of directly solubilized or coimmunoprecipitated BNLF2a is explained by different lipid-to-detergent ratios associated with the small hydrophobic peptide. BNLF2aC8-NST-transfected cells were analyzed by immunoblotting (supplemental Fig. S1B). Apart from non-glycosylated BNLF2a, the glycosylated form was detected by a 10-kDa upshift (supplemental Fig. S1B). Removal of N-linked oligosaccharides with endoglycosidase H (EndoH) resulted in the disappearance of the 16-kDa protein and an increase in non-gly-
cosylated BNLF2a, indicating that the viral factor was inserted into the ER with tail-anchored topology.

**BNLF2a Inserts Posttranslationally into the ER Membrane**—We next established an *in vitro* translation system for BNFL2a⁷⁸⁰⁰ NST (Fig. 3). As the single N-terminal methionine of BNLF2a is posttranslationally removed, we added three methionines at the C terminus for [35S]Met labeling. After *in vitro* translation in the presence of dog pancreas microsomes, two BNLF2a species were detected, corresponding to the non-glycosylated and glycosylated species (Fig. 3B, lanes 2 and 4). Glycosylated BNLF2a was EndoH-sensitive.

To determine whether BNLF2a is posttranslationally inserted into the ER membrane, the translation reaction was performed in the absence of microsomes. After translation termination by puromycin and addition of microsomes, BNLF2a still inserted into ER membranes as demonstrated by N-glycosylation (Fig. 3B, lanes 4 and 5). In contrast, the viral TAP inhibitor UL49.5, as a type I membrane protein and a model secretory protein, bovine prolactin, both with cleavable leader sequences, were only processed and translocated into the ER membrane cotranslationally. In contrast, the tail-anchored model protein Ramp4 (22), harboring a C-terminal opsin tag (including an N-glycosylation site), inserts posttranslationally into microsomes (Fig. 3B).

In contrast, the tail-anchored protein Ramp4 (22), harboring a C-terminal opsin tag (including an N-glycosylation site), inserts posttranslationally into microsomes (Fig. 3B). In contrast to the signal recognition particle protein SRP54, both glycosylated and unglycosylated BNLF2a are resistant to carbonate extraction to a similar extent as calnexin, an ER-resident integral membrane protein (Fig. 3C). Protease K treatment results in a slight downshift of both glycosylated and non-glycosylated BNLF2a, demonstrating that the C terminus remained intact (supplemental Fig. S2). Taken together, these results demonstrate the ER insertion and the tail-anchored topology of BNLF2a.

Because tail-anchored insertion requires that the hydrophobic substrate and any associated factors must be kept soluble until ER membranes are added, membrane targeting *in vitro* is typically less efficient than *in vivo*, which may explain why not all BNLF2a was glycosylated postranslationally. The important finding here is that BNLF2a⁷⁸⁰⁰ NST does undergo postranslational targeting, glycosylation, and membrane integration to a significant extent.

Another strategy to uncouple translation and membrane insertion is to translate, *in vitro*, BNFL2a⁷⁸⁰⁰ NST in wheat germ extract using truncated mRNAs lacking a stop codon. During translation, BNFL2a nascent chains are therefore not released and remain tethered to the ribosome. Such BNFL2a-containing ribosome-nascent chain complexes were then collected by centrifugation and subsequently incubated with mammalian microsomes and cell lysate. After release from the ribosomes, BNFL2a⁷⁸⁰⁰ NST was still inserted into the ER membranes as demonstrated by N-glycosylation and carbonate extraction (supplemental Fig. S3). In the ribosome-nascent chain com-

**FIGURE 2.** BNLF2a inhibits the MHC I antigen presentation by targeting the PLC. A, down-regulation of MHC I surface expression. HeLa cells were transiently transfected with BNLF2a⁷⁸⁰⁰ NST-pIRES2-EGFP, BNLF2aHA-pIRES2-EGFP, or UL49.5-pIRES2-EGFP, respectively (black solid histogram). Peptide-loaded MHC I molecules at the cell surface were stained with PE-coupled W6/32 antibodies. During flow cytometry, only transfected cells (EGFP-positive) were analyzed. As controls, cells were transfected with empty pIRES2-EGFP vector and stained either with W6/32 (green dashed histogram) or the isotype control (blue dotted histogram). B, transfection of BNLF2a does not affect the expression level of the MHC I and TAP. Equal amounts of MHC I and TAP1 were confirmed by SDS-PAGE (10%) and immunoblotting with the corresponding antibodies. C, BNFL2a⁷⁸⁰⁰ NST associates with the PLC. γ-IFN-stimulated HeLa cells were solubilized with 2% digitonin. Solubilized proteins were immunoprecipitated (IP) using TAP1/2 specific antibodies. As negative controls, either myc-antibody (mock) or an antibody specific for the ER translocon protein Sec61α (shown in supplemental Fig. S1A) were used. Samples were analyzed by Tricine/SDS-PAGE (10%) and immunoblotting with the corresponding antibodies. An aliquot (1/20) of the solubilized input (S) is shown. Asterisk, immunoglobulin heavy chain.
Inhibition Mechanism of EBV BNFL2a

BNFL2a inserts posttranslationally into ER membranes where it binds to TAP. A, schematic illustration of the in vitro-translated proteins BNFL2a\textsubscript{C\textsubscript{R}-NST}, UL49.5, and RAMP4\textsubscript{opsin}. Predicted transmembrane segments (TM) are shown as gray boxes. An N-glycosylation site was added to epitope-tagged BNFL2a and RAMP4. UL49.5 has a signal sequence (SP) that is cleaved by the signal peptidase. B, BNFL2a inserts posttranslationally into the ER membrane. In vitro translation reactions were performed in rabbit reticulocyte lysate in the presence of \textsuperscript{35}S-Met using templates encoding BNFL2a\textsubscript{C\textsubscript{R}-NST}, RAMP4\textsubscript{opsin}, UL49.5, or bovine prolactin (BPL), respectively. Microsomal membranes were added either at the start of translation (during, lanes 2 and 3) or after termination of translation by puromycin (post, lanes 4 and 5). Translation products were analyzed either directly (lane 1, without microsomes) or solubilized (lanes 2-5) and treated with EndoH (lanes 3 and 5). Proteins were separated by Tricine/SDS-PAGE (10%) and visualized by phosphoimaging. glyc glycosylated protein; pre, preprotein with an uncleaved signal sequence. C, glycosylated BNFL2a is an integral membrane protein. BNFL2a\textsubscript{C\textsubscript{R}-NST} was posttranslationally inserted into ER membranes and then subjected to extraction at pH 11.5. Supernatant (Sn), membrane pellet (Pe), and an aliquot before centrifugation (T) were analyzed by SDS-PAGE and visualized by phosphoimaging (BNFL2a\textsubscript{C\textsubscript{R}-NST} or immunoblotting for SRP54 (peripheral membrane protein) and calnexin (CNX; integral membrane protein) as controls. D, posttranslationally inserted BNFL2a interacts with TAP. BNFL2a\textsubscript{C\textsubscript{R}-NST} and UL49.5 were in vitro-translated and inserted into TAP-containing Raji microsomes post- or cotranslationally, respectively. After translation, microsomes were collected by sedimentation through a sucrose cushion, solubilized with 2% digitonin, and subjected to immunoprecipitations using antibodies specific for TAP2 or anti-myc (mock). Immune complexes (IP) and 1/20 aliquot of the translation reaction (T) and of the solubilate (S) were separated by Tricine/SDS-PAGE (10%) and visualized by phosphoimaging. pre-BPL, preprotein with signal sequence.

BNFL2a Exploits the Asna-1/WRB Machinery for ER Insertion—Recently, the structural basis and mechanism for membrane-associated steps in tail-anchored protein insertion by the yeast Get3/receptor complex composed of the ER membrane proteins Get1 and Get2 (34) have been deciphered (35–37). The large cytosolic loop of Get1 (amino acids 21–104, including a coiled-coil region) inhibited tail-anchored protein insertion into yeast microsomes (35). The mammalian tryptophan-rich basic protein (WRB) shows sequence similarity to yeast Get1 and acts as the ER membrane receptor for TRC40/Asna-1, the mammalian homologue of yeast Get3 (38). In analogy to Get1, the coiled-coil domain of WRB interfered with Asna-1 mediated insertion of TA proteins (38), and a similar recombinant inhibitory peptide (WRB-67) reduced glycosylation and, hence, membrane insertion of in vitro-translated proteins, the hydrophobic transmembrane segment of BNFL2a remains hidden within the ribosomal exit tunnel until ribosomal release. Therefore, less BNFL2a aggregates during translation, as shown by increased carbonate resistance and, hence, membrane insertion (supplemental Fig. S3). However, a fraction of membrane-inserted BNFL2aC8-NST molecules remained non-glycosylated. These unmodified viral proteins are either inaccessible for the oligosaccharyltransferase or insufficiently processed by the glycosylation machinery. However, carbonate extraction indicates clearly that BNFL2a, even if translated in a plant cell-free system, efficiently inserts posttranslationally into ER membranes if mammalian cytosolic factors are present (supplemental Fig. S3).

In Vitro-translated BNFL2a Binds to TAP—Because translated proteins typically comprise less than 0.1% of the microsomal protein in cell-free translation (33), we determined whether in vitro-translated BNFL2a would still target to TAP. After in vitro translation of BNFL2a\textsubscript{C\textsubscript{R}-NST} in the presence of Raji isolated from B lymphoblastic Raji cells, the TAP complex was solubilized and immunoprecipitated using a TAP2-specific antibody. As control, the type I membrane protein UL49.5 was translated cotranslationally. Our results show that both viral factors coimmunoprecipitate with TAP (Fig. 3D). Notably, glycosylated and non-glycosylated BNFL2a interact with TAP. We demonstrate that in vitro-translated BNFL2a and UL49.5 are not only inserted properly into the ER membrane but also bind to their physiological target.

BNFL2a Exploits the Asna-1/WRB Machinery for ER Insertion—Recently, the structural basis and mechanism for membrane-associated steps in tail-anchored protein insertion by the yeast Get3/receptor complex composed of the ER membrane proteins Get1 and Get2 (34) have been deciphered (35–37). The large cytosolic loop of Get1 (amino acids 21–104, including a coiled-coil region) inhibited tail-anchored protein insertion into yeast microsomes (35). The mammalian tryptophan-rich basic protein (WRB) shows sequence similarity to yeast Get1 and acts as the ER membrane receptor for TRC40/Asna-1, the mammalian homologue of yeast Get3 (38). In analogy to Get1, the coiled-coil domain of WRB interfered with Asna-1 mediated insertion of TA proteins (38), and a similar recombinant inhibitory peptide (WRB-67) reduced glycosylation and, hence, membrane insertion of in vitro-translated proteins.
BNLF2a

FIGURE 4. Asna-1 and WRB are involved in the posttranslational ER membrane insertion of BNLF2a. In vitro translation reactions were performed in wheat germ extract in the presence of [35S]Met using truncated BNLF2a<sup>CR-NST</sup> mRNA templates lacking a stop codon. After translation, BNLF2a<sup>CR-NST</sup>-containing ribosome-nascent chain complexes were collected by centrifugation through a sucrose cushion and resuspended in microsomal membrane containing rabbit reticulocyte lysate in the presence of either 200 μg/ml R9L peptide as mock control (−) or WRB-67 inhibitory peptide (A, 200 μg/ml; B, concentrations as indicated). After ribosomal release by puromycin and RNase treatment, samples were further incubated for 30 min at 32 °C. Translation products were analyzed by Tricine/SDS-PAGE (10%) and visualized by phosphoimaging. Histograms show the average amount of glycosylated BNLF2a protein. The error bars indicate the mean ± S.D. of three independent experiments. *p = 0.05 (Student’s t test).

Ramp<sup>Q91408</sup>in from 65 ± 8% to 40 ± 5% in the presence of 200 μg/ml WRB-67 (data not shown). Strikingly, WRB-67 reduced the tail-anchored membrane insertion of BNLF2a<sup>CR-NST</sup> in a dose-dependent manner (Fig. 4). In conclusion, BNLF2a inserts into the ER membrane posttranslationally, exploiting the WRB/Asna-1 (Get3/Get1) machinery, and thus represents the first viral TAP inhibitor with a tail-anchored targeting mechanism.

Posttranslational ER Targeting of BNLF2a Is Independent of TAP—It still remains unclear whether BNLF2a binds directly or indirectly to TAP and whether BNLF2a requires or not the assistance of TAP for a proper ER insertion. We therefore expressed BNLF2a in insect cells, which achieve high-level expression of membrane proteins, including core TAP subunits, but lack any other factors of the adaptive immune system (39). Despite the fact that these cells do not contain endogenous TAP, BNLF2a was properly inserted into ER membranes and N-core glycosylated (supplemental Fig. S4). Thus, targeting of BNLF2a to the ER membrane is independent of TAP.

Interaction of BNLF2a and TAP—We next examined whether BNLF2a binds to TAPI/2 in the absence of components of the adaptive immune system and peptide-loading complex. Insect cells were coinfected with a baculovirus encoding both TAP subunits and with an additional virus encoding BNLF2a<sup>CR-NST</sup>. As shown by coimmunoprecipitation using TAP2-specific antibodies, both non-glycosylated and glycosylated BNLF2a are associated with the TAP complex (Fig. 5A). EndoH treatment further indicated that the higher molecular band corresponds to N-core glycosylated and ER-resident BNLF2a. These results demonstrate that the interaction between BNLF2a and TAPI/2 does not require other components of the peptide-loading complex. Because BNLF2a consists of a rather small hydrophilic N-terminal region of approximately 40 residues, it might be possible that this region is capable to bind to TAP in analogy to a TAP-substrate peptide. We therefore examined whether a BNLF2a-soluble domain (amino acids 2–42, BNLF2<sup>2–42</sup>), if provided in 100-fold molar excess, is able to outcompete the binding of a fluorescein-labeled peptide (R9L<sup>Flu</sup>) to TAP. However, neither did BNLF2<sup>2–42</sup> compete with R9L<sup>Flu</sup> for binding to TAPI/2, nor did fluorescein-labeled BNLF2<sup>2–42</sup> bind directly to TAP with detectable affinity (supplemental Fig. S5). Therefore, these results suggest that the interaction between TAPI/2 and BNLF2a requires the full-length protein.

BNLF2a and US6 Are Mutually Exclusive in TAP Inhibition—We next examined how two viral proteins compete for TAP inhibition. The ER-luminal domain of US6 of HCMV specifically blocks ATP binding to and peptide translocation by TAP (12, 13). After coexpression of TAPI/2, BNLF2a<sup>CR-NST</sup> and US6<sup>myc</sup> in S9 cells, coimmunoprecipitations were performed using antibodies against C8-tagged BNLF2a (anti-C8) or myc-tagged US6 (anti-myc), respectively. TAPI and TAP2 coprecipitated with either BNLF2a<sup>CR-NST</sup> or US6<sup>myc</sup>, confirming the specific interaction of each viral inhibitor with the TAP complex (Fig. 5B). Strikingly, US6 was not coprecipitated with BNLF2a-TAP complexes. Vice versa, BNLF2a was not detected in coprecipitated US6-TAP complexes. Because both BNLF2a and US6 are expressed in high excess, as demonstrated by almost complete inhibition of peptide transport by TAP (see below), the total amount of both viral factors is sufficient to occupy all TAP complexes. In conclusion, TAP complexes cannot interact with US6 and BNLF2a at the same time.

BNLF2a Prevents a Peptide-induced Conformational Change of TAP—Interestingly, the ER-luminal domain of US6 arrests TAP in a conformation that inhibits ATP binding to the cytosolic NBDs (13). Because both factors exclude each other from binding to TAP, BNLF2a and US6 may arrest distinct conformations of the TAP complex. Moreover, it has been reported that viral inhibitors have a direct influence on TAP conformation. The presence of either ICP47 or US6 prevents peptide-induced conformational rearrangements of the transporter (12, 13, 40). To investigate the influence of BNLF2a on the conformational change following peptide binding, S9 membranes containing TAPI/2 were incubated with the lysine-specific bivalent chemical cross-linker ethylene glycol bis(succinimidyl succinate). In the presence of peptides, cross-linking of the TAPI/2 heterodimer, most likely via the NBDs (12, 40), was detected at 200 kDa (Fig. 6A). ICP47 abolished the chemical
cross-linking of the TAP heterodimer. The weak cross-linking of TAP, even without addition of peptides, was explained by the presence of endogenous peptides. Strikingly, when BNLF2a and TAP1/2 were coexpressed, we could not detect a cross-linked TAP heterodimer, even after the addition of peptides. It is worth mentioning that BNLF2a did not interfere with TAP heterodimerization per se, as BNLF2a was coprecipitated with fully assembled TAP1/2 complexes (Fig. 6B). The total amount of BNLF2a was sufficient to occupy all TAP1/2-heterodimers, as shown by almost complete inhibition of TAP-mediated peptide transport (see below). In the absence and presence of BNLF2a, comparable amounts of TAPI were coimmunoprecipitated with TAP2 and vice versa (Fig. 6B). Thus, BNLF2a prevents a conformational change of TAP induced by peptide binding.

BNLF2a Targets the Core TAP and Inhibits Peptide Binding and Translocation—The core TAP complex has been demonstrated to be essential and sufficient for peptide translocation into the ER lumen (39). We therefore compared the inhibitory effect of BNLF2a on peptide translocation of the full-length and core TAP complex (Fig. 7A, right and left panel, respectively). BNLF2aCR-NST blocks peptide translocation into the ER lumen by full-length and core TAP. In contrast to herpesviral US6 (13, 41, 42), BNLF2aCR-NST blocks peptide binding to full-length and core TAP, indicating that the extra four N-terminal transmembrane helices of each TAP subunit are dispensable for BNLF2a-mediated TAP inhibition (Fig. 7B). Similar amounts of TAP were present in each binding and translocation assay (Fig. 7C). Taken together, our data illustrate that tail-anchored BNLF2a posttranslationally targets the core TAP complex, preventing peptide binding and conformation switch.

DISCUSSION

Members of the herpesviridae family have evolved a number of strategies to evade immune recognition (8, 43). For example, ICP47 of HSV-1 binds with high affinity to TAP from the cytosol, where it blocks peptide binding (9, 10). In contrast, US6 of HCMV targets TAP via its ER-lumenal domain and prevents...
ATP binding on the opposite side of the ER membrane (12, 13). However, only limited information is available regarding the recently described EBV BNLF2a (18, 32).

As demonstrated by in vitro translation, BNLF2a exhibits three distinct hallmarks of TA proteins: First, the lack of an N-terminal signal sequence; second, a C-terminal membrane anchor, which exposes its C terminus toward the ER lumen; and third, posttranslational insertion into the ER membrane. Thus, BNLF2a is unique among the viral TAP inhibitors with regard to its membrane insertion and inhibition mechanism. Although viral factors such as UL49.5 and US6 are inserted cotranslationally in the ER membrane via their cleavable N-terminal signal sequence, BNLF2a is targeted posttranslationally (Fig. 3B), most likely utilizing the cellular pathways used by endogenous TA proteins. The tail-anchored topology of BNLF2a was also confirmed by expression in insect cells, which lack the components of an adaptive immune system and components of the PLC.

Four main routes of TA protein targeting have been proposed (30, 44). Following release from the ribosome, TA proteins are either inserted into membranes without assistance of other factors or rely on one of three distinct chaperone-mediated pathways. One involves the chaperone machinery Hsp40/70 (45, 46), another is mediated by SRP (47), and a third pathway depends on Asna-1 (48) or its yeast homologue Get3 and their membrane receptors WRB (38) or Get1/Get2 (34), respectively. According to a recently proposed model of tail-anchored protein insertion (35–37), binding of the rigid coiled-coil domain of Get1 to the Get3/tail-anchored protein cargo complex mediates substrate release and membrane insertion. Soluble fragments containing the coiled-coil region of WRB or Get1 are known to inhibit tail-anchored protein insertion (35, 38), and we observed a similar effect for BNLF2a insertion into the ER membrane (Fig. 4A and B). Therefore, we conclude that BNLF2a can exploit the host Asna-1/WRB (Get3/Get1) pathway for ER membrane targeting and insertion.

By adding an N-glycosylation site to the C terminus of BNLF2a, we demonstrate by in vitro translation (Fig. 3B) as well as expression in human and insect cells (supplemental Fig. S1BB and Fig. 5) that the C terminus of BNLF2a faces the ER lumen. The hydrophilic N-terminal region of the viral factor faces the peptide and ATP-binding site of TAP. Because BNLF2a, but not its soluble cytosolic domain, inhibits both peptide and ATP binding to TAP (18), the BNLF2a topology
might be a prerequisite for its functionality. In contrast, US6 targets TAP from the ER lumen and blocks ATP binding to the cytotoxic NBDs of TAP (13). It was surprising that BNLF2a and US6, when expressed together, exclude each other from binding to the TAP complex (Fig. 5B). Steric competition between US6 and BNLF2a is unlikely because their active domains interact with TAP at different sites of the ER membrane.

It has been reported that viral inhibitors arrest TAP in distinct conformations. For example, binding of HSV-ICP47 to TAP inhibits peptide-induced conformational rearrangements of the transporter (12, 40). Remarkably, coexpression of BNLF2a and TAP blocks peptide-induced TAP1/2 cross-linking (Fig. 6A). Combined with the fact that the soluble part of BNLF2a (lacking its transmembrane domain) does not directly compete with substrate peptide binding to TAP (supplemental Fig. S5), we propose that BNLF2a prevents the conformational rearrangement of TAP that is normally induced by peptide binding (40). However, because of limitations of our assay, we are unable to distinguish whether BNLF2a indirectly prevents peptide binding to TAP, hence preventing conformational changes, or whether BNLF2a binding to TAP arrests the transporter in a conformation that is incompatible with peptide binding. In contrast, ICP47 acts directly as a competitive inhibitor of peptide binding to TAP (9, 10).

The PLC is composed of several proteins, all of them necessary to ascertain efficient peptide loading of MHC I, which ultimately results in the presentation of dominant epitopes. Within the PLC, the TAP heterodimer fulfills the important task of peptide translocation from the cytosol into the ER lumen. In approximately providing the WRB-67 inhibitory peptide.

In conclusion, we propose the following inhibition mechanism of BNLF2a (Fig. 8). After release from free ribosomes, BNLF2a is posttranslationally inserted into the ER membrane using the host Asna-1/WRB (Get3/Get1) insertion machinery for TA-anchored targeting of TA proteins. After insertion into the ER membrane, BNLF2a interacts directly with the core TAP complex, arresting a conformation that is incompatible with peptide binding. It will be important to precisely map the BNLF2a interaction sites of the core TAP subunits to reveal details of how BNLF2a prevents TAP-mediated peptide transport. This may aid the development of small therapeutic compounds that modulate immune surveillance.

Acknowledgments—We thank R. Abele (Goethe University Frankfurt) for helpful discussions on the manuscript and M. Mariappan and R. S. Hegde (Medical Research Council, Cambridge, UK) for generously providing the WRB-67 inhibitory peptide.

REFERENCES

1. Abele, R., and Tampé, R. (2009) Curr. Opin. Cell Biol. 21, 508–515
2. Cresswell, P., Ackerman, A. L., Giodini, A., Peaper, D. R., and Wearsch, P. A. (2005) Immunol. Rev. 207, 145–157
3. Rock, K. L., and Goldberg, A. L. (1999) Annu. Rev. Immunol. 17, 739–779
4. Yewdell, J. W. (2005) Curr. Opin. Immunol. 19, 79–86
5. Koch, J., and Tampé, R. (2006) Cell Mol. Life Sci. 63, 653–662
6. Wearsch, P. A., and Cresswell, P. (2008) Curr. Opin. Cell Biol. 20, 624–631
7. Yewdell, J. W., and Hill, A. B. (2002) Nat. Immunol. 3, 1019–1025
8. Parcej, D., and Tampé, R. (2010) Nat. Chem. Biol. 6, 572–580
9. Ahn, K., Meyer, T. H., Uebel, S., Sempé, P., Djaballah, H., Yang, Y., Peterson, P. A., Früh, K., and Tampé, R. (1996) EMBO J. 15, 3247–3255
10. Tomazin, R., Hill, A. B., Jugovic, P., York, I., van Endert, P., Ploegh, H. L., Andrews, D. W., and Johnson, D. C. (1996) EMBO J. 15, 3256–3266
11. Aisenbrey, C., Sizun, C., Koch, J., Herget, M., Abele, R., Bechinger, B., and Tampé, R. (2006) J. Biol. Chem. 281, 30365–30372
12. Hewitt, E. W., Gupta, S. S., and Lehner, P. J. (2001) EMBO J. 20, 387–396
13. Kyritsis, C., Gorbulev, S., Hutschenreiter, S., Pawlitschko, K., Abele, R.
