

# Processing of a Multiple Membrane Spanning Epstein-Barr Virus Protein for CD8<sup>+</sup> T Cell Recognition Reveals a Proteasome-dependent, Transporter Associated with Antigen Processing-independent Pathway

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## Abstract

Epstein-Barr virus (EBV) latent membrane protein (LMP)2 is a multiple membrane spanning molecule which lacks ectodomains projecting into the lumen of the endoplasmic reticulum (ER). Human CD8<sup>+</sup> cytotoxic T lymphocytes (CTL)s recognize a number of epitopes within LMP2. Assays with epitope-specific CTLs in two different cell backgrounds lacking the transporter associated with antigen processing (TAP) consistently show that some, but not all, LMP2 epitopes are presented in a TAP-independent manner. However, unlike published examples of TAP-independent processing from endogenously expressed antigens, presentation of TAP-independent LMP2 epitopes was abrogated by inhibition of proteasomal activity. We found a clear correlation between hydrophobicity of the LMP2 epitope sequence and TAP independence, and experiments with vaccinia minigene constructs expressing cytosolic epitope peptides confirmed that these more hydrophobic peptides were selectively able to access the HLA class I pathway in TAP-negative cells. Furthermore, the TAP-independent phenotype of particular epitope sequences did not require membrane location of the source antigen since (i) TAP-independent LMP2 epitopes inserted into an EBV nuclear antigen and (ii) hydrophobic epitope sequences native to EBV nuclear antigens were both presented in TAP-negative cells. We infer that there is a proteasome-dependent, TAP-independent pathway of antigen presentation which hydrophobic epitopes can selectively access.

Key words: MHC class I presentation • CD8<sup>+</sup> epitopes • hydrophobicity • Epstein-Barr virus • TAP independence

## Introduction

CD8<sup>+</sup> CTL recognition of target cells requires the presentation of peptide epitopes on the cell surface in the context of MHC class I molecules (1). In the great majority of cases, these epitopes are generated from endogenously expressed proteins by breakdown in the cytosol via a large multicatalytic protease complex, the proteasome (2). Thereafter, the resultant peptides are pumped from the

cytosol into the endoplasmic reticulum (ER)\* by the transporter associated with antigen processing (TAP), a heterodimeric complex composed of TAP1 and TAP2 subunits whose function is ATP dependent (3–5). Of the many peptides transported in this way, a small number are selected by virtue of their ability to bind with high affinity to the peptide-binding grooves of nascent MHC class I molecules, thereby stabilizing the MHC class I-β2-microglobulin-peptide complex and allowing its subsequent transport to the cell surface (6, 7). Importantly, this is the major route of antigen processing not just for cytosolic proteins but also for membrane/secretory proteins which natu-

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\*Abbreviations used in this paper: EBV, Epstein-Barr virus; ER, endoplasmic reticulum; LCL, lymphoblastoid cell line; LMP, latent membrane protein; moi, multiplicity of infection; TAP, transporter associated with antigen processing.

rally access the ER but can be returned to the cytosol by retrograde transport (8) and there targeted for processing by the conventional TAP-dependent, proteasome-dependent route (9–13).

Nevertheless, in cells lacking a functional TAP transporter, of which the best example is the human T2 cell line where both TAP1 and TAP2 genes are deleted (14), some MHC–peptide complexes are still presented at the cell surface (15, 16). In this context, a number of possible mechanisms have been identified for the TAP-independent presentation of antigenic peptides from endogenously expressed proteins. The best known example involves peptides derived from signal sequences of membrane/secretory proteins (10, 15, 16); signal sequences target such proteins to the Sec61 translocon and, after protein translocation, are naturally liberated in the ER by the action of the signal peptidase enzyme. A second example involves certain epitopes located within the ectodomains of transmembrane proteins which, rather than being generated in the conventional way after retrograde transport to the cytosol, appear to be liberated within the lumen of the ER by as yet poorly defined ER proteases (17–19). Thirdly, there is at least one example of a naturally secreted viral protein from which an epitope is generated by the trans-Golgi-resident protease furin (20). Note that in all the above cases the pathway of epitope presentation is not only TAP independent but also proteasome independent.

In earlier work, we (21) and others (22) have reported TAP-independent processing of an Epstein-Barr virus (EBV)-coded antigen, the latent membrane protein (LMP)2, in the T2 cell background in vitro. Furthermore, CD8<sup>+</sup> CTL responses to LMP2 have been identified within a TAP-deficient individual (23), and, if such responses are indeed induced by direct priming, their existence would suggest functionality of this TAP-independent pathway in vivo. LMP2 is an integral membrane protein with short cytosolic NH<sub>2</sub>- and COOH-terminal domains flanking 12 tandemly arranged transmembrane domains that are joined by tight loops showing minimal projection into the ER (24, 25). Of four CTL epitopes identified within LMP2, three lying within transmembrane sequences were presented in the TAP-negative T2 cell line, whereas a fourth epitope located on a cytosolic loop was not (21, 22). Extending this work to other epitopes within LMP2 and then to epitopes in EBV nuclear antigens, we present evidence for a novel pathway of TAP-independent presentation which does not require membrane location of the source antigen, is proteasome-dependent and selectively involves hydrophobic epitope sequences.

## Materials and Methods

**Cell Lines.** Standard EBV-transformed lymphoblastoid cell lines (LCLs), the EMO LCL derived from a patient with a homozygous mutation in the TAP2 allele (23), the TAP1/TAP2-negative T2 cell line (14), and T2 cells stably transfected either with rat TAP1 and TAP2 alleles (designated T3 cells; reference 26) or with the HLA B\*2704 (T2:B27.04) or HLA B\*3501 (T2:

B35.01) alleles (27, 28) were maintained in RPMI 1640 containing 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (growth medium). A human papilloma virus negative cervical carcinoma cell line, C-33 A (ATCC HBT-31; HLA type A2, B7, B44), and a SV40-immortalized human keratinocyte (SVK) line (HLA type A2, A29, B8, B40) were maintained in DME medium supplemented as above.

**Peptides.** Synthetic peptides were synthesized by standard fluorenyl-methoxycarbonyl chemistry (Alta Bioscience) and dissolved in DMSO, and their concentration determined by biuret assay.

**CTL Clones.** EBV-specific CTL clones were generated from virus-immune donors by autologous LCL stimulation as described previously (29, 30), and mapped to the following epitopes: HLA A\*0201-restricted epitopes, CLGGLLTMV (CLG) and LLWTLVLL (LLW); the HLA B\*4001-restricted epitope, IEDPPFNSL (IED); the HLA B\*2704-restricted epitope, RRRWRRLTV (RRR); and the HLA A\*2402-restricted epitope, TYGPVFMCL (TYG) all from LMP2; the HLA-B\*3501-restricted epitope, HPVGEADYFEY (HPV) from EBNA1; the HLA B\*3501-restricted epitope, YPLHEQHGM (YPL) from EBNA3A; the HLA B\*2705-restricted epitope, HRCQAIRKK (HRC) from EBNA3B; the HLA B\*2705-restricted epitope, FRKAQIQGL (FRK) from EBNA3C; the HLA A\*0201-restricted epitopes, GLCTLVAML (GLC) from BMLF1, TLDYKPLSV (TLD) from BMRF1; and YVLDHLIVV (YVL) from BRLF1 (references 31 and 32, data not shown). The LMP2-specific HLA-B63 restricted CTL clone EFA c8.24 has been described previously (23).

**Cytotoxicity Assays.** Chromium release assays on target cells infected overnight with recombinant vaccinia viruses (multiplicity of infection [moi] of 10 unless otherwise indicated) were carried out as described previously (29). Peptide-loaded targets were exposed immediately before the assay (during the last hour of Chromium labeling) to the epitope peptide at a final concentration of  $2 \times 10^{-8}$  M unless otherwise stated or to an equivalent amount of DMSO solvent as a control. For assays of proteasome dependence, C-33 A or SVK cells were first incubated for 1 h in the presence of 100 µM lactacystin (provided by E.J. Corey, Harvard University, Boston, MA; reference 33) or of 1 µM epoxomicin (34) before recombinant vaccinia virus infection and the infected target cells incubated overnight in fresh medium before being used in a standard 5-h chromium release assay.

**Construction of Recombinant Vaccinia Viruses.** Unless otherwise stated, recombinant vaccinia viruses were generated using established protocols based on homologous recombination and selection of TK-recombinant viruses using the pSC11 transfer plasmids (35). The HLA A\*2402, B\*2704, and B\*4001 cDNAs were blunt-end cloned into the Sma1 cloning site pSC11, before construction of the recombinant vaccinia viruses vA24, vB27, and vB40. The vaccinia viruses expressing the EBV antigens LMP2 (vLMP2), EBNA1 lacking the glycine-alanine repeat (vE1dGA), EBNA3A (vEBNA3A), EBNA3B (vEBNA3B), EBNA3C (vEBNA3C), BMLF1 (vBMLF1), BMRF1 (vBMRF1), BRLF1 (vBRLF1), the HLA B\*3501 heavy chain (vB35), and control virus vTK-have been described previously (29, 36)

**Chimaeric Constructs.** Construction of BMLF1 mutants containing LMP2-derived peptide epitopes was carried out using the plasmid pBMLF1 (36) as follows. The LMP2 peptide epitopes CLG and TYG were inserted separately into the coding sequence of BMLF1 at the Nco1 site (corresponding to amino acid position 93) generating pBMLF1-CLG-N and pBMLF1-TYG-N, or at the EcoR1 site (corresponding to BMLF1 amino acid position

379) generating pBMLF1-CLG-E and pBMLF1-TYG-E. Insertion of LMP2 epitopes was achieved by annealing overlapping oligonucleotides encoding each epitope, followed by ligation of the annealed products with pBMLF1 linearized with either Nco1 or EcoR1. For insertion of epitopes into the Nco1 site the following oligonucleotides were used: for the CLG epitope, 5'-CATGCATGCCTCGGTGGCCTGCTCACCATGGTA-3' and 5'-CATGTACCATCGTGAGCAGGCCACCGAGGCATG-3'; and for the TYG epitope, 5'-CATGCAACATACGGTCCAGTTTTTATGTGCCTC-3' and 5'-CATGGAGGCACATAAA-AACTGGACCGTATGTTG-3'. For insertion of epitopes into the EcoR1 site the following oligonucleotides were used: for the CLG epitope, 5'-AATTTATGTGCCTCGGTGGCCTGCTC-ACCATGGTAGCCC-3' and 5'-AATTGGGCTACCATGGTGAGCAGGCCACCGAGGAACATA-3'; and for the TYG epitope, 5'-AATTAAGAACATACGGTCCAGTTTTTATGTGCCTCGCTC-3' and 5'-AATTGACCGAGGCACATAAAA-ACAGGTCCTGATGTTCTT-3'.

All constructs were sequenced to confirm correct in frame insertion of the LMP2 epitopes into BMLF1. The coding sequences for BMLF1 and BMLF1 epitope chimaeras were then excised by digestion with Xba1 and HindIII, and ligated into the vaccinia transfer vector pRB21 (37) which had been digested with Nhe1 and HindIII. The recombinant vaccinia viruses vBMLF1, vBMLF1-CLG-N, vBMLF1-CLG-E, vBMLF1-TYG-N, and vBMLF1-TYG-E were generated using standard methods based on restoration of wild-type plaque phenotype.

**Minigene Constructs.** Expression of minimal epitopes from vaccinia viruses was achieved by cloning synthetic minigenes coding for the LMP2 epitopes CLG, TYG, IED, RRR, the EBNA3A epitope YPL, the BMLF1 epitope GLC, and the BMRF1 epitope TLD into the vaccinia transfer vector pSC11.30R.2 (19). Peptide epitopes were inserted into the Nco1/BglII cut vector by annealing overlapping oligonucleotides coding for the appropriate epitope sequences as follows: CLG sense 5'-CATGTGCCTCGGTGGCCTGCTCACCATGGT-ATAATTAATTAAG-3' and antisense 5'-GATCCTTAATTAATTATACCATGGTGAGCAGGCCACCGAGGCA-3'; TYG sense 5'-CATGACATACGGTCCAGTTTTTATGTGCCTC-TAATTAATTAAG-3' and antisense 5'-GATCCTTAATTAATTAGAGGCACATAAAAACTGGACCGTATGT-3'; IED sense 5'-CATGATTGAGGACCCACCTTTTAATTCTCTT-TAATTAATTAAG-3' and antisense 5'-GATCCTTAATTAATTAAAGAGAATTAAGGTGGGTCCTCAAT-3'; RRRW sense 5'-CATGAGAAGATGGCGCCGTTTGACTGTTTAA-TTAATTAAG-3' and antisense 5'-GATCCTTAATTAATTAACAGTCAAACGGCGCCATCTCCTTCT-3'; YPL sense 5'-CATGTACCCATTACATGAACAACACGGCATGTAA-TTAATTAAG-3' and antisense 5'-GATCCTTAATTAATTAATCATGCCGTGTTGTTTCATGTAATGGGTA-3'; GLC sense 5'-CATGGGGCTCTGCACCCTGGTGGCCATGCTATA-ATTAATTAAG-3' and antisense 5'-GATCCTTAATTAATTATAGCATGGCCACCGAGGTGCAGAGCCC-3'; TLD sense 5'-CATGACCCTAGACTACAAGCCTCTGAGTGTGTAA-TTAATTAAG-3'; and antisense 5'-GATCCTTAATTAATTAACACTCAGAGGCTGTAGTCTAGGGT-3'.

In addition, another vaccinia recombinant vL+CLG expressing an ER-targeted form of the CLG epitope was constructed by cloning the above CLG oligonucleotide minigene sequence into the vaccinia transfer vector pSC11L<sup>+</sup> (19). This recombinant encodes a 26-mer peptide composed of the influenza haemagglutinin HA-1 leader sequence MKANLLVLLCALAAADA followed by the CLG epitope. The integrity of all minigene constructs was

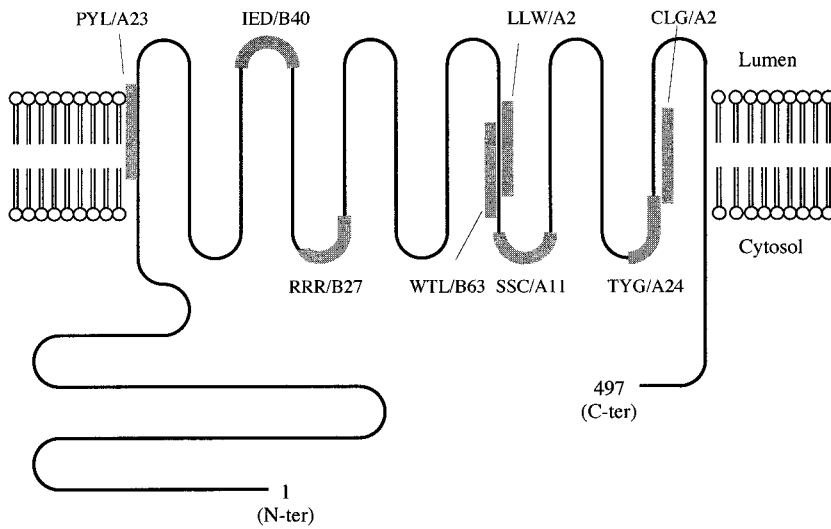
checked by sequencing before construction of recombinant vaccinia viruses as described previously (35).

**Immunoblotting and Immunofluorescence.** For immunoblotting, protein extracts were resolved by SDS-PAGE (10% gel), transferred to nitrocellulose, and probed with specific antibodies followed by detection using a chemiluminescence protocol (Amersham Pharmacia Biotech). LMP2 was detected using the rat monoclonal antibody 14B7 (38), human TAP1/TAP2 using rabbit antisera (39), actin using a monoclonal antibody (Sigma-Aldrich), and BMLF1 using rabbit antiserum  $\alpha$ SM53 (40). For immunofluorescence assays of LMP2 and BMLF1 expression, cell smears were fixed in 1% paraformaldehyde and stained using the above reagents followed by an appropriate second step antibody.

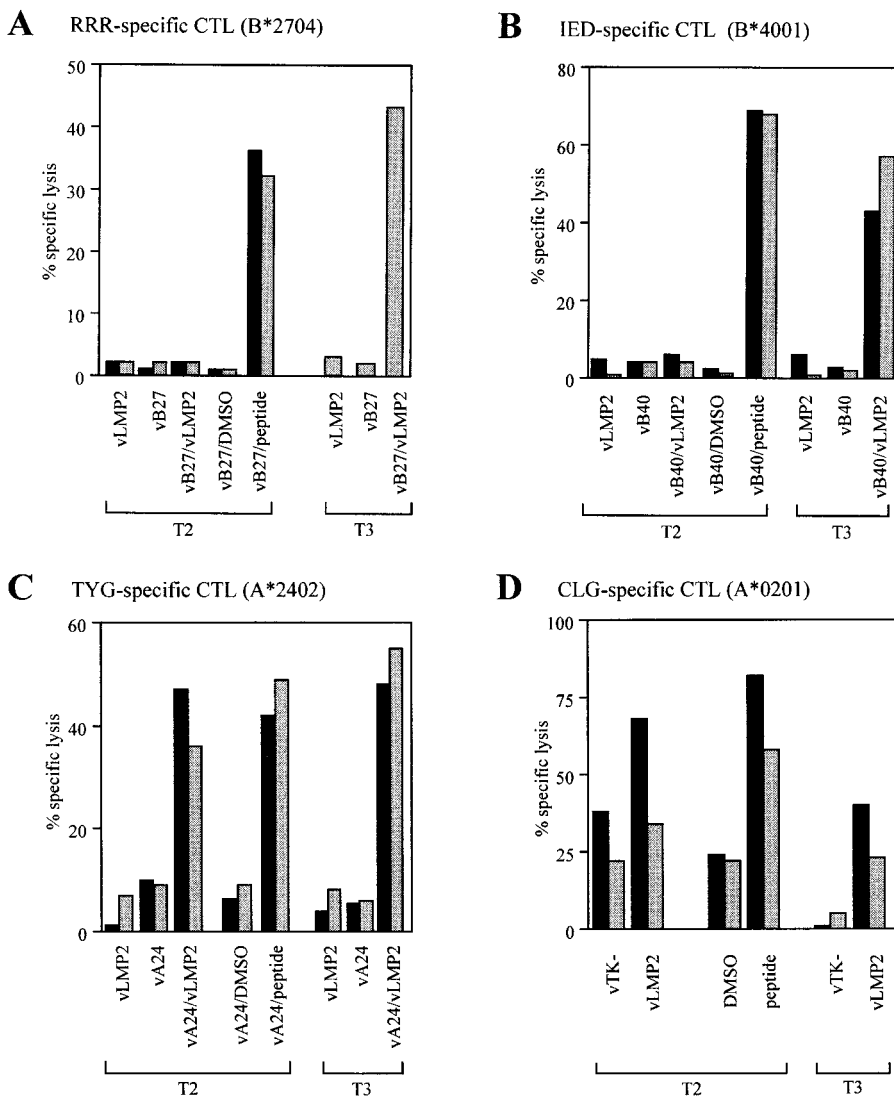
## Results

**Presentation of B\*4001- and B\*2704-restricted Epitopes from LMP2 Is TAP Dependent.** Fig. 1 illustrates the topology of the multiple membrane spanning LMP2 protein and identifies eight CD8<sup>+</sup> CTL epitopes, either previously known or defined in this study, plus their HLA restriction alleles. Earlier work has classified three epitopes lying within transmembrane sequences (CLG/A\*0201, LLW/A\*0201, and PYL/A\*23) as TAP independent and one epitope situated on a cytosolic loop (SSC/A\*1101) as TAP dependent (21, 22). To extend this analysis to other epitopes, we first carried out chromium release assays in the TAP-negative T2 cell line, expressing LMP2 in these cells via a recombinant vaccinia virus and where necessary providing the appropriate HLA allele through a second vaccinia construct.

In the first set of assays, we analyzed processing of the B\*2704-restricted epitope RRRWRRLTV (LMP2 aa 236–244) which lies on a cytosolic loop of the protein in its native orientation in the membrane. As shown in Fig. 2 A, there was clearly no specific recognition of T2 target cells coinfecting with a vaccinia virus expressing HLA B\*2704 (vB27) and a vaccinia virus expressing LMP2 (vLMP2), whereas T2 cells infected with vB27 and pulsed with the RRR epitope peptide were efficiently lysed. As a control target in these assays we included the T3 cell line which was derived from T2 by stable transfection with the rat TAP1 and TAP2 cDNAs (26). Importantly, when T3 cells were coinfecting with vB27 and vLMP2, there was significant recognition of LMP2 by RRR-specific CTLs, confirming that presentation of the RRR epitope was indeed TAP dependent. In this case we were also able to test T2: B27.04 cells (a HLA B\*2704 stable transfectant of T2; reference 27) and again found that these cells were unable to present the RRR epitope when infected with vLMP2 but were well recognized after peptide loading (data not shown). Then we carried out a similar set of assays using CTL clones specific for the HLA B\*4001-restricted epitope IEDPPFNSL (LMP2 amino acid [aa] 200–208), which in contrast to the RRR epitope, lies on a luminal loop of the protein (Fig. 1). Interestingly, this epitope also proved to be TAP dependent. Thus, as shown in Fig. 2 B, there was no specific lysis of T2 cells coinfecting with vB40 and



**Figure 1.** Diagrammatic representation of the LMP2 molecule in its native orientation in the membrane. The positions of CD8<sup>+</sup> T cell epitope sequences are shown; epitopes are identified by the first three letters of their amino acid sequence and by their HLA class I restricting alleles.



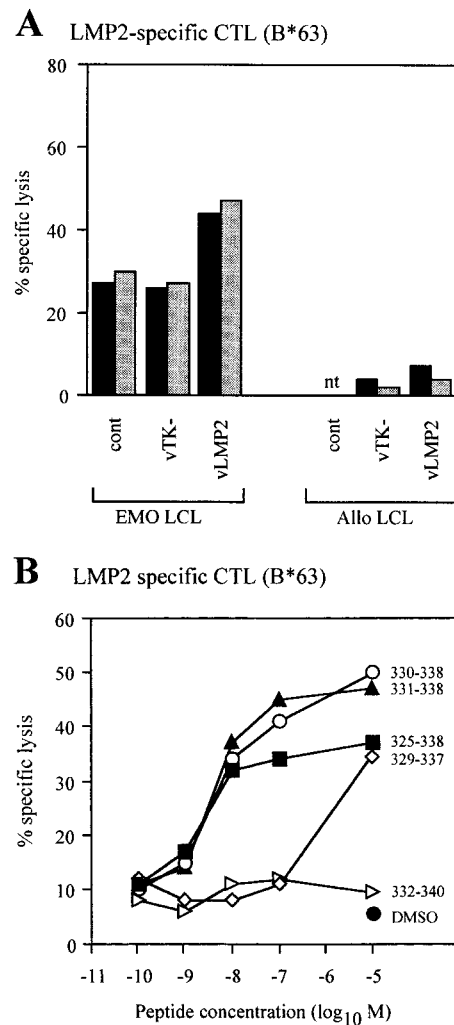
**Figure 2.** Definition of LMP2 epitopes as TAP dependent or TAP independent. Data from cytotoxicity assays using CTL clones specific for (A) RRR/B27, (B) IED/B40, (C) TYG/A24, and (D) CLG/A2 as effectors and either T2 cells (TAP-negative) or T3 cells (TAP1/TAP2-transfectant of T2) as targets. Target cells were infected with a vaccinia recombinant-expressing LMP2 (vLMP2) and, where necessary, with a recombinant expressing the relevant HLA class I restriction element (vB27, vB40, vA24) either alone or in combination with vLMP2. In the case of T2 cell assays, target cells (where necessary infected with vB27, vB40, or vA24) were preexposed to the epitope peptide as a positive control or to an equivalent concentration of DMSO solvent as a negative control. vTK- is a control vaccinia recombinant. Results are shown as percentage of specific lysis in standard 5-h chromium release assays using effector:target ratios of 5:1 (black bar) and 2:1 (shaded bar).

vLMP2 (vB40/vLMP2), whereas T3 cells coinfecting with both viruses or T2 cells infected with vB40 and pulsed with the IED epitope peptide were well recognized.

**Presentation of the A\*2402-restricted Epitope from LMP2 Is TAP Independent.** Next, we extended this analysis to look at the HLA A\*2402-restricted epitope, TYG-PVFMCL (LMP2 aa 419–427), predicted to be located partially within a cytosolic loop and a transmembrane section (Fig. 1). Fig. 2 C shows a representative result from chromium release assays using CTL clones specific for this epitope, again using a vaccinia vector vA24 to express the HLA restriction allele in T2 cells and coinfecting with vLMP2 (vA24/vLMP2). In this case, we did observe significant recognition of coinfecting target cells by epitope-specific CTLs, at levels equivalent to that seen with epitope-peptide loading (vA24/peptide). Indeed, levels of lysis of these target cells were similar to those seen following vA24/vLMP2 coinfection of the TAP-positive T3 cell line. For reference, Fig. 2 D shows the corresponding set of results obtained using CTLs specific for the A\*0201-restricted epitope CLGLLTMV (LMP2 aa 426–434), previously shown to be TAP independent (21). Here infection of T2 cells (naturally expressing HLA A\*0201) with vLMP2 was sufficient to induce lysis to levels approaching those seen for CLG epitope-loaded target cells. Note that CLG-specific clones also show significant baseline lysis of the T2 cell line itself, reflecting the fact that these cells are naturally EBV-positive and expressing low levels of LMP2 endogenously.

**Mapping of a TAP-independent, HLA B63-restricted Epitope in LMP2.** In a previous report studying individuals with deficiencies in peptide transport due to a TAP2 mutation, de la Salle et al. identified a CD8<sup>+</sup> CTL clone, restricted through HLA B63, and specific for the EBV LMP2 protein (23). Here we confirmed that this clone recognizes a TAP-independent epitope and went on to identify the minimal epitope sequence. As illustrated in Fig. 3 A the CTL clone from the TAP2-deficient donor EFA (EFA c8.24) shows strong lysis of EMO LCL target cells (derived from a HLA-identical sibling with the same TAP2 mutation) when LMP2 is overexpressed in these cells from a recombinant vaccinia virus (vLMP2). Here again, there is lower but still significant baseline recognition of the EMO LCL itself, reflecting low level expression of LMP2 from the endogenous EBV genome, whereas there was no recognition of a HLA-mismatched LCL target (allo LCL) with or without vLMP2 infection (Fig. 3 A). Parallel assays using CTLs specific for known TAP-dependent epitopes confirmed that the EMO-LCL was indeed operationally TAP-deficient (data not shown).

Subsequently, we mapped the minimal epitope recognized by CTL clone EFA c8.24. Initial screening of LMP2 peptide panels (30) identified one 14-mer peptide (FLLM-LLWTLVLLI, aa 325–338) which mediated rapid T cell-T cell lysis in a visual assay (data not shown). Limiting dilutions of this 14-mer peptide, and of smaller peptides from within this region of LMP2, were then assayed for their ability to enhance lysis of the EMO LCL by EFA c8.24 ef-



**Figure 3.** Mapping of the HLA B\*63-restricted, TAP-independent WTL epitope within LMP2. (A) CTL clone EFA c8.24 derived from a patient with a TAP2-inactivating mutation was assayed against the EMO-LCL derived from an HLA-identical sibling with the same TAP2 mutation and against an HLA-mismatched standard LCL (Allo LCL). Targets were used without vaccinia infection (cont) or after infection with vTK- or with vLMP2. Results are expressed as in Fig. 2 using effector:target ratios of 5:1 (black bar) and 1:1 (shaded bar). (B) Results of a cytotoxicity assay using CTL clone EF c8.24 as effectors and the EMO-LCL as a target. Target cells were preexposed to 10-fold dilutions of peptides ( $10^{-5}$ – $10^{-11}$ M) representing the indicated residues within the LMP2 325–340 region (330–338, ▲ 331–338, ■ 325–338, ◇ 329–337, ▽ 332–340) or to DMSO as a control (●). The minimal epitope mediating optimal recognition was WTLVLLI, LMP2 331–338.

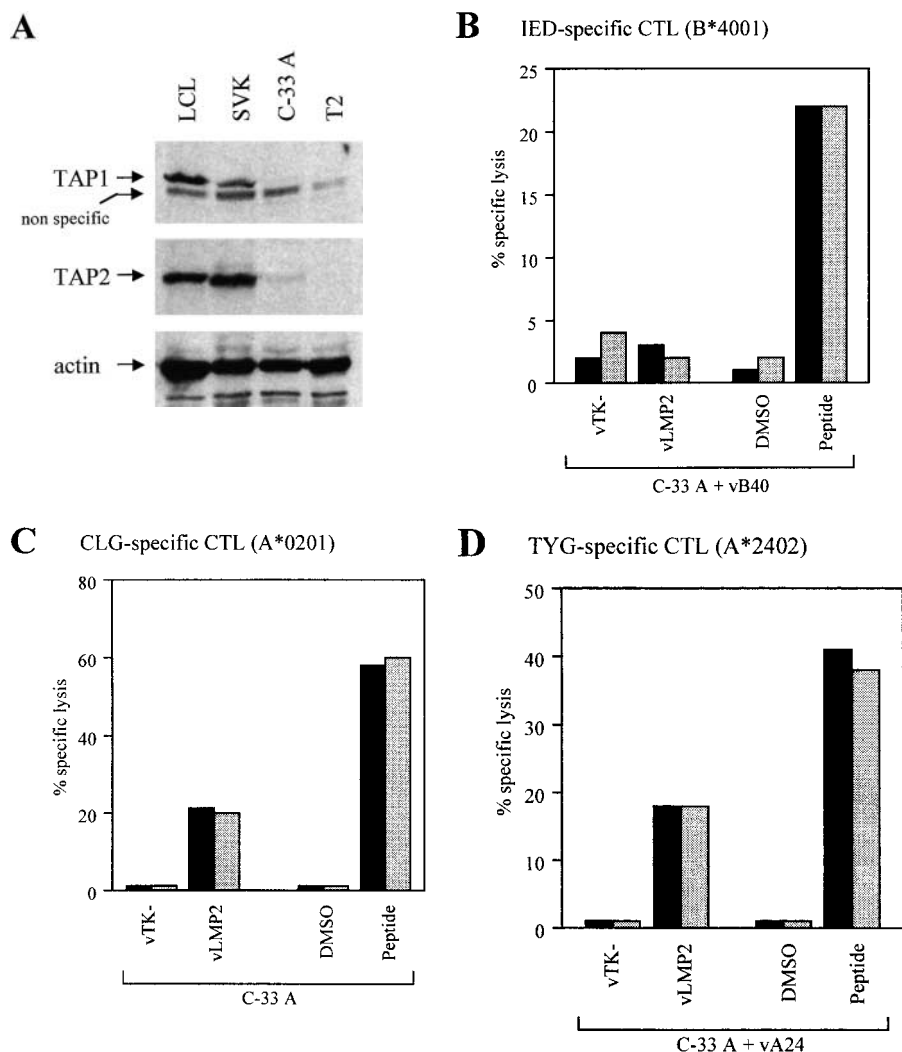
factors. As shown in Fig. 3 B, significant enhancement was observed with the original 14-mer peptide and also with the constituent 9-mer aa 330–338 and 8-mer aa 331–338 peptides, whereas other sequences were recognized poorly if at all. The predicted minimal epitope is therefore the 8-mer WTLVLLI, which accords well with the proposed HLA B\*63 binding-motif with a threonine at position 2 and isoleucine at the COOH terminus (41). This TAP-independent HLA B\*63-restricted epitope lies within a predicted transmembrane region of LMP2 and overlaps an-

other previously characterized TAP-independent epitope LLW/A2 (Fig. 1).

**TAP-independent Presentation of LMP2 Epitopes in an Epithelial Cell Background.** We were interested to see if the TAP-independent presentation of LMP2 epitopes observed in T2 cells could be reproduced in a different TAP-negative cell background. For this purpose we used C-33A, a human cervical carcinoma cell line, which in preliminary work was found to be deficient for presentation of standard TAP-dependent HLA class I epitopes. As shown by the immunoblots in Fig. 4 A, we analyzed the C-33A cell line for TAP expression and found that TAP1 was absent and TAP2 was barely detectable whereas both proteins were strongly expressed in the SVK cell line and in a standard LCL. Processing of LMP2 epitopes for CTL recognition was then investigated using C-33A cells as targets, again where necessary providing the appropriate HLA allele via a recombinant vaccinia virus. Representative results from such chromium release assays are shown in Fig. 4 B–D. Using CTLs specific for the IED/B40 TAP-dependent epitope, there was no significant lysis of C-33A cells coinfecting with vLMP2 and vB40 above that seen with vTK-/

vB40 coinfecting control targets, whereas vB40-infected targets pulsed with the IED epitope peptide were well recognized (Fig. 4 B). This same pattern of results was also observed in parallel assays using CTLs specific for the RRR/B27 TAP-dependent epitope (data not shown). However, when C-33A cells (naturally HLA A\*0201-positive) were infected with vLMP2 alone and used in chromium release assay with CTL effectors specific for the TAP-independent CLG/A2 epitope, there was significant lysis above that of the control targets (Fig. 4 C). Likewise when infecting C-33A cells with vLMP2 and coinfecting with vA24, there was clear recognition of the TAP-independent TYG/A24 epitope by epitope-specific CTLs (Fig. 4 D). By contrast, all four LMP2 epitopes described above were efficiently processed and presented in parallel assays conducted in the TAP-positive SVK cell line (data not shown). Thus, assays in epithelial cell backgrounds produced the same characterization of LMP2 epitopes as either TAP-dependent or TAP-independent as had the assays in T2 cells.

**Presentation of LMP2 CTL Epitopes Is Blocked by Proteasome Inhibitors.** The same epithelial lines were then used in experiments where, before infection with vLMP2 and

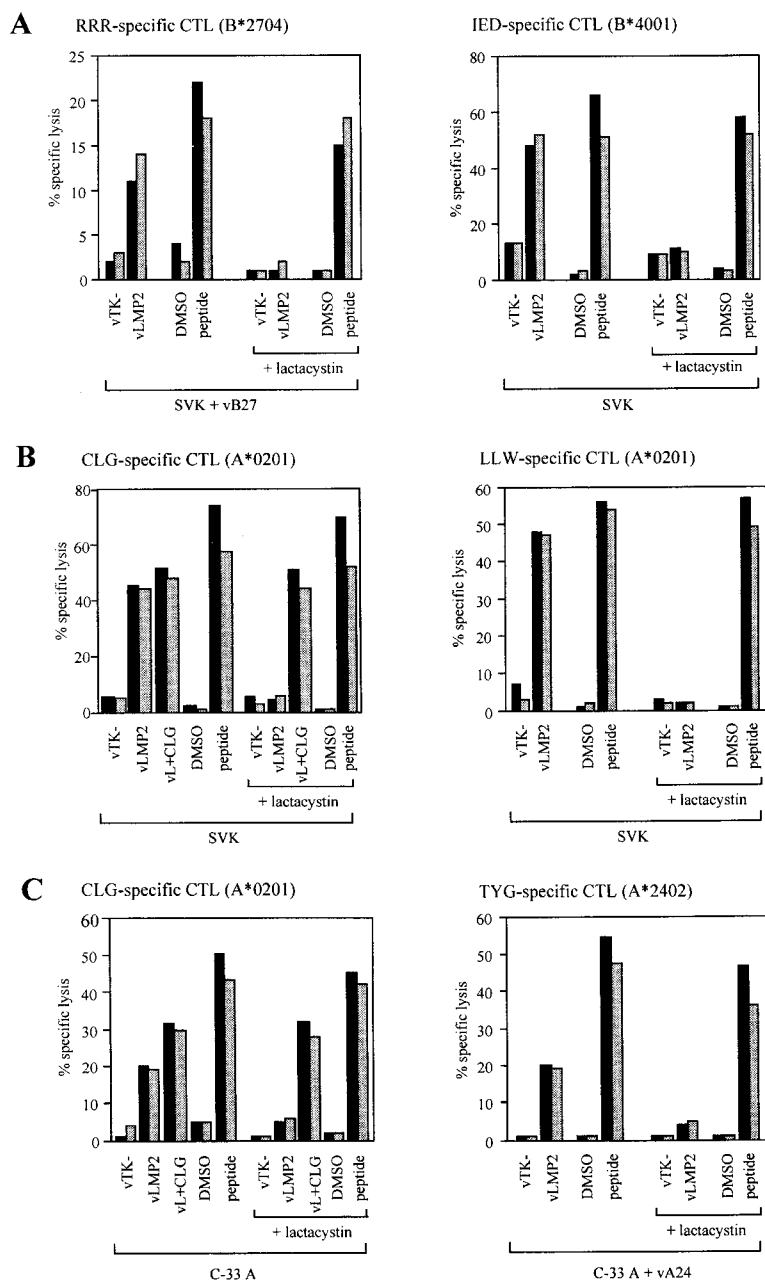


**Figure 4.** Results of assays conducted in the C-33A epithelial cell line. (A) Protein extracts from C-33A cells were compared with extracts from the TAP1/TAP2-negative T2 cell line, from a standard TAP-positive LCL, and from the TAP-positive SVK epithelial cell line in immunoblots probed with antisera to TAP1, TAP2, and actin. Note that the anti-TAP1 antisera also cross-reacts with a nonspecific protein running below TAP1 in the gel. (B) Results of a cytotoxicity assay using as effectors an IED/B40-specific CTL clone and as targets C-33A cells uniformly infected with vB40 and additionally either coinfecting with vTK- or vLMP2 or pre-exposed to IED epitope peptide or to DMSO solvent as a control. (C) Results of a similar cytotoxicity assay using a CLG/A2-specific CTL clone as effectors. (D) Results of a similar assay using a TYG/A24-specific CTL clone as effectors and vA24 to provide the HLA restriction element. Results are expressed as in Fig. 2 using effector:target ratios of 8:1 (black bar) and 4:1 (shaded bar).

where necessary with a vaccinia expressing the appropriate HLA restriction element, the cells were briefly pre-pulsed with the proteasome-specific inhibitor lactacystin (33). Fig. 5 A illustrates data from experiments in the TAP-positive SVK cell-line (naturally expressing A\*0201 and B\*4001) which show that two TAP-dependent epitopes, RRR/B27 and IED/B40, were presented efficiently from vaccinia-expressed LMP2 in the absence of lactacystin, but presentation of both epitopes was blocked when SVK cells were pretreated with the inhibitor. However, importantly, such lactacystin-treated cells clearly did show specific lysis if loaded with the epitope-peptide. In addition, lactacystin treatment itself did not affect vectored expression of LMP2 in these assays; equivalent levels of the protein were de-

tected in treated and untreated cells by immunofluorescence staining as well as by immunoblot analysis with LMP2-specific antibodies (data not shown).

Fig. 5 B presents similar experiments conducted in SVK target cells now using CTLs specific for the TAP-independent HLA A\*0201-restricted CLG and LLW epitopes from LMP2. These results clearly show that presentation of the TAP-independent epitopes was also blocked by pretreatment of SVK targets with lactacystin, whereas peptide loading of lactacystin-treated target cells allowed CTL recognition. These experiments were then extended to the TAP-negative C-33A cell line, this time analyzing presentation of the TAP-independent epitopes CLG/A2 and TYG/A24 (Fig. 5 C). Although both of these epitopes



**Figure 5.** Lactacystin inhibition of the presentation of LMP2 epitopes. (A) Assays conducted using effector CTLs specific for the TAP-dependent RRR/B27 and IED/B40 epitopes and the TAP-positive SVK epithelial cell line as a target. (B) Assays conducted using effector CTLs specific for the TAP-independent CLG/A2 and LLW/A2 epitopes and the TAP-positive SVK cell line as a target. (C) Assays conducted using effector CTLs specific for the TAP-independent CLG/A2 and TYG/A24 epitopes and the TAP-negative C-33A epithelial cell line as a target. All assays were conducted essentially as in Fig. 4, where necessary providing the HLA-restriction element by vB27 and vA24, but in the assays of CLG/A2 epitope presentation included targets infected with a leader plus epitope minigene construct (vL+CLG) and in all assays included a parallel set of targets prepared from cells exposed to lactacystin for 1 h immediately before the time of vaccinia infection. Results are expressed as in Fig. 2 from assays using effector:target ratios of 8:1 (black bar) and 4:1 (shaded bar).

were presented efficiently in TAP-negative C-33A cells infected with vLMP2 or coinfecting with vLMP2/vA24, there was again no significant lysis of target cells infected in the presence of lactacystin.

The possibility that lactacystin might be blocking the presentation of LMP2-derived epitopes through some mechanism other than proteasomal inhibition led us to conduct two additional sets of experiment. In the first case, we constructed a recombinant vaccinia vL+CLG carrying a leader sequence-positive CLG minigene targeting the CLG epitope peptide directly to the ER. As shown both for SVK (Fig. 5 B) and C33-A (Fig. 5 C) targets, infection with the vL+CLG construct sensitized cells to CTL recognition whether or not they had been preexposed to lactacystin, thereby confirming that the drug did not block the presentation of a proteasome-independent epitope. Secondly, we repeated the assays with a different proteasome inhibitor, epoxomicin (34), and again observed that presentation of both the TAP-dependent and TAP-independent epitopes from LMP2 was blocked by pretreatment of target cells with the inhibitor (data not shown). These studies strongly suggest that both TAP-dependent and TAP-independent epitopes within LMP2 require the proteasome for their generation.

*LMP2 Epitope Hydrophobicity and TAP-dependence of Presentation from Minigene Constructs.* In view of the above results, we considered other possible explanations for the observed differences between LMP2 epitopes in their TAP dependence. In that context, Table I lists the epitopes according to their predicted degree of hydrophobicity (42), from which it is clear that the five most hydrophobic epitopes are also those which are TAP independent. This immediately suggested some form of selective accessing of the ER by hydrophobic sequences.

To investigate the ability of cytosolically expressed epitope peptides to access the HLA class I presentation pathway in TAP-positive and TAP-negative cell backgrounds, we generated minigene constructs in vaccinia vectors encoding two representative TAP-independent epitopes, CLG and TYG, and two representative TAP-

dependent epitopes, IED and RRR, in these cases without leader sequences. Fig. 6 presents the results of cytotoxicity assays in which a standard TAP-positive LCL and TAP-negative T2 cells were each infected across a range of moieties with epitope-encoding vaccinia recombinants, where necessary providing the relevant HLA restriction element to T2 cells via a second vaccinia recombinant. In the standard TAP-positive LCL background, all four minigene constructs sensitized target cells for epitope-specific lysis in a titratable fashion (Fig. 6 A). By contrast, in the TAP-negative T2 cell background, both the TYG- and the CLG-encoding constructs mediated clear CTL recognition whereas the IED- and the RRR-encoding constructs did not (Fig. 6 B). As a positive control T2 cells, where necessary infected with the relevant vHLA recombinant, were in all cases well recognized after exogenous loading of the epitope peptide. Therefore, the two epitopes which were known to be presented from endogenously expressed LMP2 in a TAP-independent manner also displayed a TAP-independent phenotype when expressed in the cytosol from minigenes; this strongly implicates the hydrophobic nature of these epitopes as one determinant of their ability to cross the ER membrane and bypass the TAP transporter.

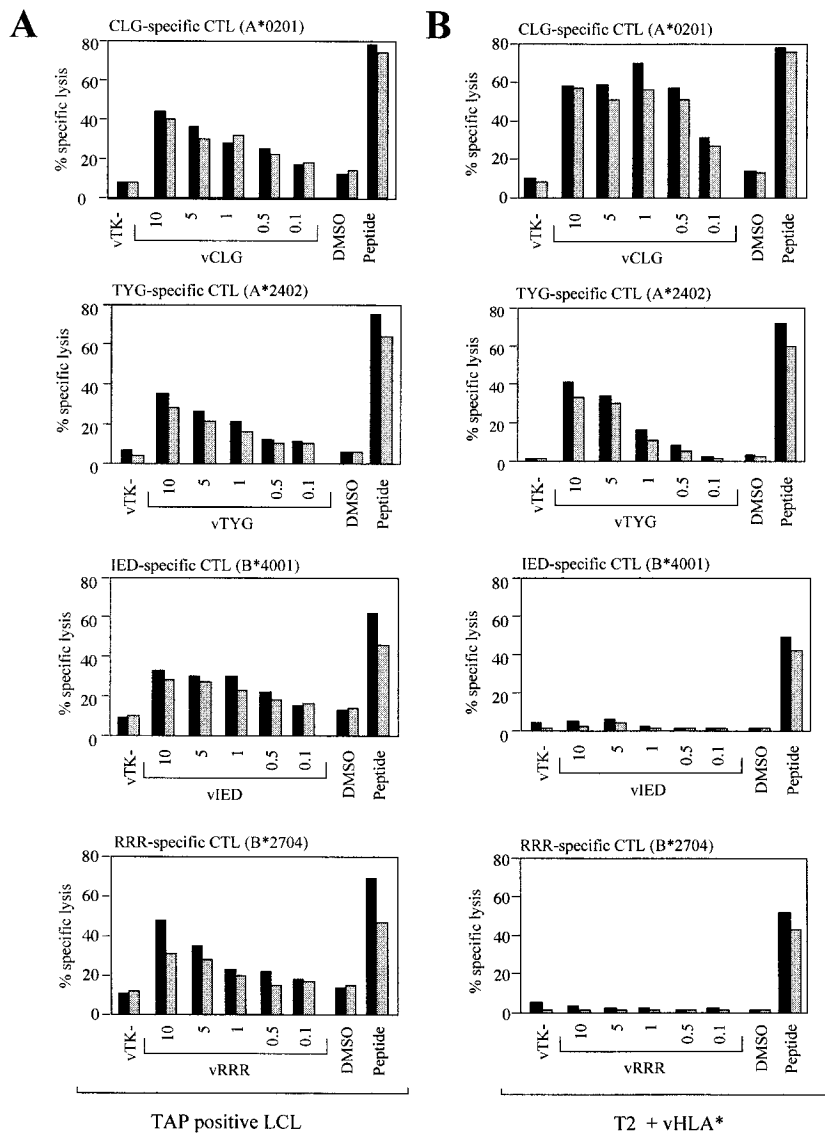
*Hydrophobic LMP2 Epitopes Remain TAP Independent when Inserted into a Nuclear Protein.* The next set of experiments sought to determine whether the TAP-independent presentation of epitopes from endogenously expressed LMP2 required not only intrinsic hydrophobicity of the epitope sequences but also their location within a native membrane protein. For this purpose we generated chimaeric constructs in which the TAP-independent CLG/A2 and TYG/A24 epitope sequences were introduced at residue 93 (CLG-N, TYG-N) or at residue 379 (CLG-E, TYG-E) of BMLF1, a 438 aa EBV nuclear protein of the early lytic cycle. After recombinant vaccinia construction, vectored expression of the chimaeric BMLF1 proteins was in each case confirmed by Western blot analysis and was localized to the nucleus by immunofluorescence staining; these patterns of expression were indistinguishable from that shown by a vaccinia recombinant vBMLF1 expressing the wild-type BMLF1 protein (data not shown). Target cells infected with the chimaeric antigen-encoding viruses, with vBMLF1 or with vLMP2 were then assayed for recognition by CLG- and TYG-specific effector CTL clones. The results of such assays are illustrated in Fig. 7, and follow the same consistent pattern for both epitopes. Assays conducted in a TAP-positive LCL expressing both the A\*0201 and the A\*2402 allele (Fig. 7 A) clearly confirmed that the BMLF1 chimaeric constructs could be processed to generate the relevant inserted epitopes. Thus CLG-specific effectors recognized TAP-positive LCL cells either expressing LMP2 as the source antigen or expressing the relevant chimaeric proteins, BMLF1/CLG-N and BMLF1-CLG-E. Likewise the TYG-specific effectors specifically recognized both BMLF1-TYG chimeras as well as LMP2. Surprisingly, we obtained exactly the same series of results when the assays were conducted in the T2 cell background (Fig.

**Table I.** LMP2 Epitopes

Epitope	HLA restriction	Hydrophobicity	TAP status <sup>a</sup>
<u>LLWTLVVLL</u>	A*0201	8.14	Independent
<u>WTLVLLI</u>	B*63	6.95	Independent
<u>PYLFWLA AI</u>	A*23	6.49	Independent
<u>TYGPFMCL</u>	A*2402	5.47	Independent
<u>CLGGLLTMV</u>	A*0201	4.14	Independent
<u>IEDPPFNSL</u>	B*4001	-0.59	Dependent
<u>RRRWRRLTV</u>	B*2704	-0.60	Dependent
<u>SSCSSCLPSK</u>	A*1101	-2.35	Dependent

<sup>a</sup>TAP status as defined by the ability of the epitope to be generated from endogenously expressed LMP2 in TAP-negative cell backgrounds.





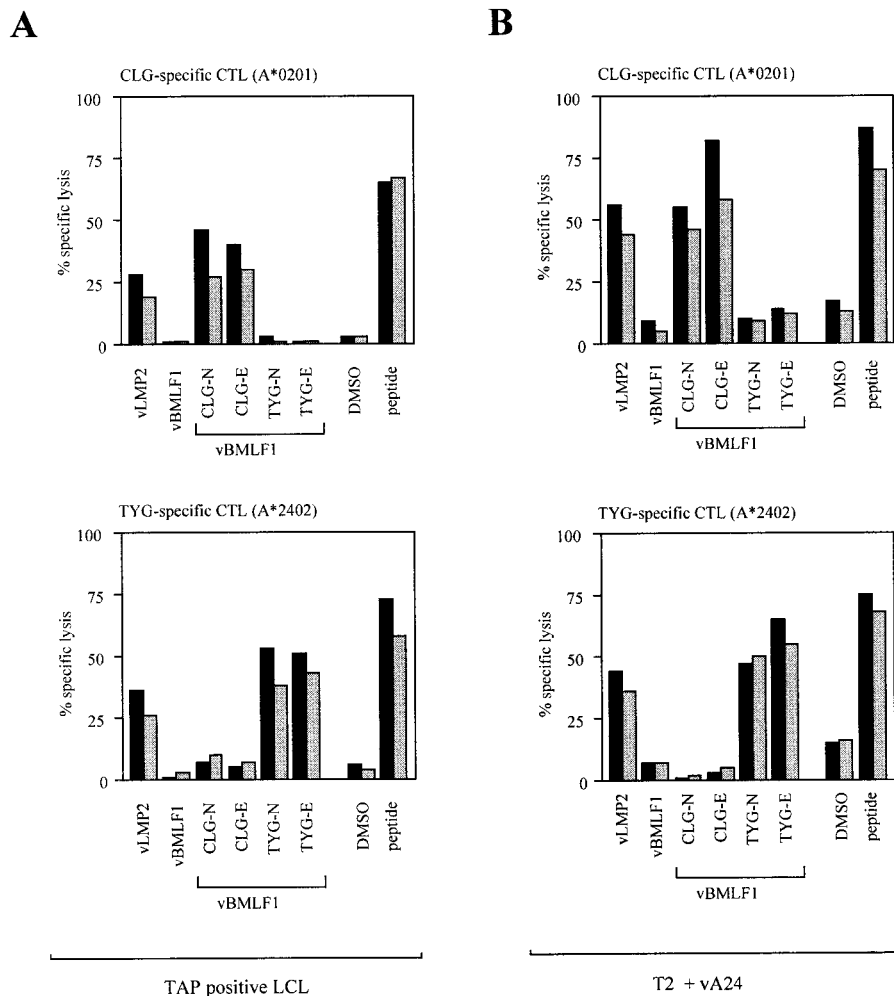
**Figure 6.** Results of cytotoxicity assays using vaccinia-minigene constructs in (A) a standard TAP-positive LCL background with the appropriate HLA-restricting element and (B) T2 cells. Targets were infected with vTK- as a control or with vaccinia-minigene recombinants expressing the TAP-independent CLG/A2 or TYG/A24 epitopes (vCLG, vTYG) or the TAP-dependent IED/B40 or RRR/B27 epitopes (vIED, vRRR); the minigene recombinants were used across a range of moieties from 10:1 to 0.1:1 as indicated. Target cells exposed to the epitope peptide or to an equivalent concentration of DMSO solvent served as positive and negative controls. \*Note that in the case of T2 targets, where necessary the relevant HLA restricting determinant was provided by coinfection with vA24, vB40, or vB27. Results are expressed as in Fig. 2 from assays using epitope-specific CTLs at effector:target ratios of 4:1 (black bar) and 2:1 (shaded bar).

7 B). Again the CLG- and TYG-effectors were clearly capable of recognizing T2 cells expressing their cognate epitope within a chimeric nuclear protein at levels at least as high as those seen with targets expressing the native LMP2 protein. The TAP-independent phenotype of these epitopes is therefore apparent even when the source antigen is not a membrane-associated protein.

*Hydrophobicity and TAP Independence of Native Epitopes within Nuclear Proteins.* Therefore, we calculated the relative hydrophobicity of several already defined CTL epitopes lying within EBV-coded nuclear proteins. Table II lists seven such epitopes, their antigen of origin, their restriction element, and their hydrophobicity. From these we selected three epitopes for minigene experiments, GLC (A\*0201-restricted) whose hydrophobicity score of 4.41 was similar to the TAP-independent LMP2 epitope CLG, YPL (B\*3501-restricted) whose score of -0.66 was similar to the TAP-dependent LMP2 epitopes IED and RRR, and TLD (A\*0201-restricted) whose score of 1.72 was in-

termediate between the two extremes. Recombinant vaccinas carrying these epitope sequences as minigenes (vGLC, vYPL, vTLD) were used to infect both TAP-positive and TAP-negative target cells in CTL-detection assays. The results are illustrated in Fig. 8. All these minigene constructs were capable of sensitizing TAP-positive cells to recognition in a titratable manner across a range of moieties (Fig. 8 A). However, only the more hydrophobic epitopes GLC/A2 and TLD/A2 were presented in T2 cells (Fig. 8 B); in contrast the more hydrophilic YPL/B35 epitope was not presented either in T2 cells expressing HLA B35 from a vaccinia vector (Fig. 8 B) or in a stable HLA B\*3501-transfectant of T2 (data not shown).

Then, we sought to determine the TAP dependence of all seven EBV epitopes listed in Table II when expressed in their native viral antigen. In this case all four of the more hydrophilic epitopes (FRK/B27, HPV/B35, YPL/B35, and HRC/B27) proved to be TAP dependent. Representative data are shown for one such epitope, YPL/B35.



**Figure 7.** Processing of chimaeric BMLF1 proteins for recognition of inserted epitopes. Results of assays using vaccinia constructs encoding chimaeric proteins in which the TAP-independent CLG/A2 and TYG/A24 epitope sequences from LMP2 were introduced into the BMLF1 nuclear antigen at one of two locations (CLG-N, TYG-N inserted at residue 93; CLG-E, TYG-E inserted at residue 379). Assays were conducted in (A) a standard TAP-positive LCL naturally expressing the A2 and A24 restriction elements and (B) T2 cells naturally expressing A2 and infected with vA24. Target cells were infected with vLMP2, with vBMLF1, or with chimaeric antigen-encoding vaccinias (vBMLF1-CLG-N, vBMLF1-CLG-E, vBMLF1-TYG-N, vBMLF1-TYG-E). Target cells preexposed to the epitope peptide or to an equivalent concentration of DMSO solvent served as positive and negative controls. Results are expressed as in Fig. 2 from assays using CLG/A2- or TYG/A24-specific CTL at effector:target ratios of 4:1 (black bar) and 2:1 (shaded bar).

Thus a YPL epitope-specific CTL clone clearly recognized TAP-positive target cells expressing the relevant YPL-containing nuclear antigen EBNA3A (Fig. 9 A, bottom) whereas there was no significant lysis of vEBNA3A-infected T2 cells whether the HLA B35 restricting deter-

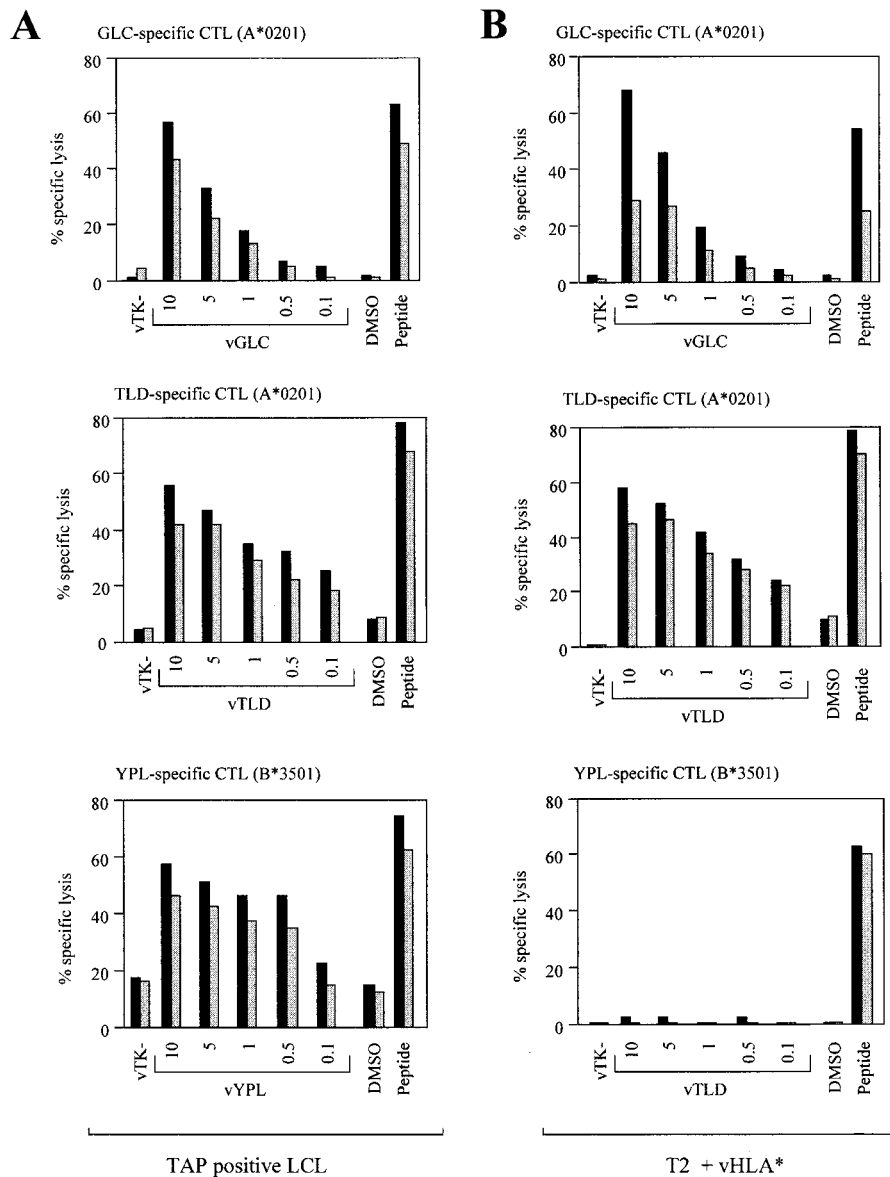
minant was supplied by stable transfection (data not shown) or by vB35 coinfection (Fig. 9 B, bottom); however, the B35 molecule was clearly expressed since exogenous loading of vB35-infected T2 targets with the YPL-epitope-peptide mediated strong lysis by CTLs. Parallel assays using

**Table II.** Epitopes from EBV-encoded Nuclear Antigens

Antigen	Epitope	HLA restriction	Hydrophobicity	TAP status <sup>a</sup>
BRLF1	<u>Y</u> VLDHLIVV	A*0201	6.14	Independent
BMLF1	<u>GL</u> CTLVAML	A*0201	4.41	Independent
BMRF1	<u>TLD</u> YKPLSV	A*0201	1.72	Dependent <sup>b</sup>
EBNA3C	<u>FR</u> KAQIQGL	B*2705	0.24	Dependent
EBNA1	<u>HP</u> VGAEDYFEY	B*3501	0.22	Dependent
EBNA3A	<u>YPL</u> HEQHGM	B*3501	-0.66	Dependent
EBNA3B	<u>HRC</u> QAIRKK	B*2705	-2.35	Dependent

<sup>a</sup>TAP status as defined by ability of the epitope to be generated from endogenously expressed antigen in the TAP-negative T2 cell background.

<sup>b</sup>Note that the TLD epitope is TAP dependent by these criteria although this epitope is presented in TAP-negative T2 cells when expressed from a minigene construct (see Figs. 8 and 9).

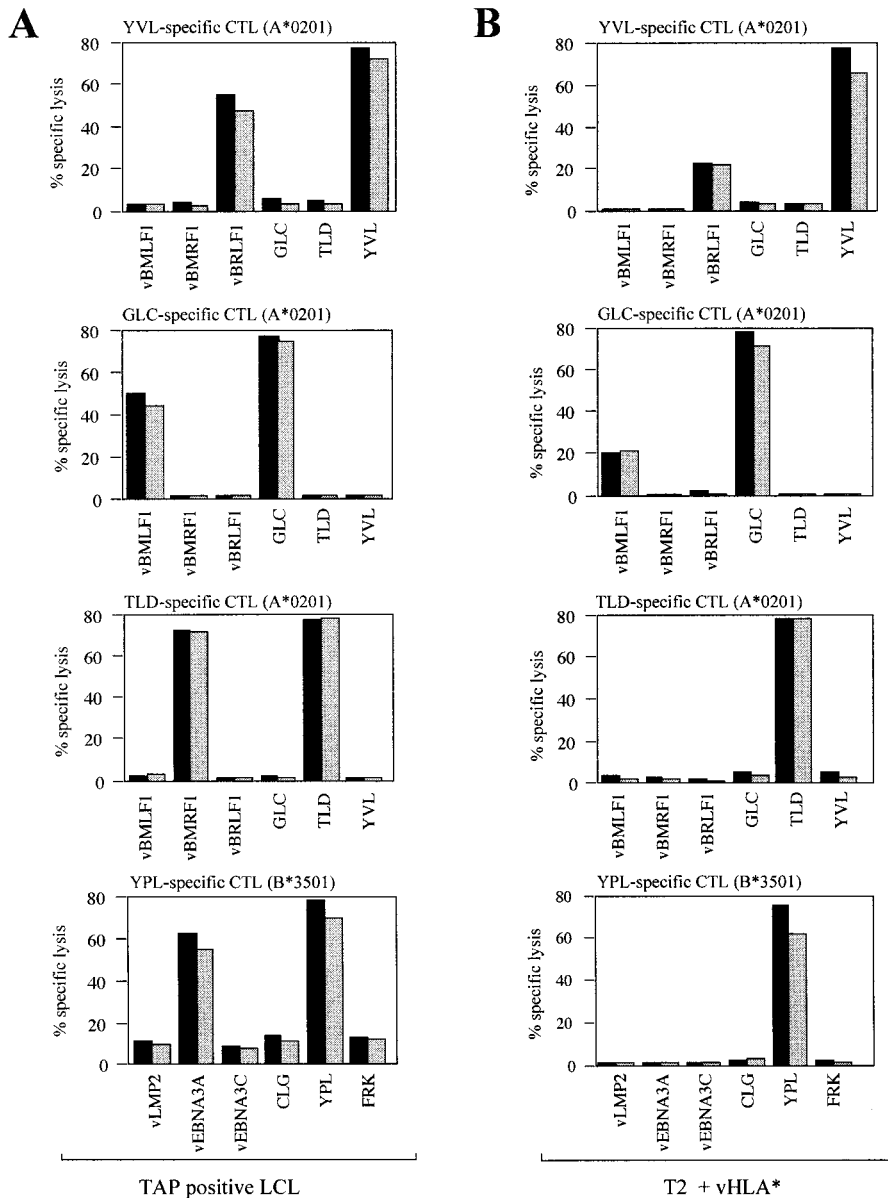


**Figure 8.** Results of cytotoxicity assays using vaccinia-minigene constructs in (A) a standard TAP-positive LCL background with the appropriate A2 and B35 restriction elements and (B) T2 cells. Targets were infected with vTK- as a control or with minigene recombinants expressing the GLC/A2, TLD/A2, or YPL/B35 epitope (vGLC, vTLD, vYPL) across a range of mois as in Fig. 6. Target cells preexposed to the epitope peptide or to an equivalent concentration of DMSO solvent served as positive and negative controls. \*Note that in the case of T2 targets tested with YPL/B35-specific effectors, the cells were coinfecting with vB35 to provide the relevant HLA restricting allele. Results are expressed as in Fig. 2 using epitope specific CTLs at effector:target ratios of 4:1 (black bar) and 2:1 (shaded bar).

CTLs specific for the relatively hydrophilic FRK/B27, HRC/B27, and HPV/B35 epitopes from EBNA3C, EBNA3B, and EBNA1, respectively, gave similar results (data not shown).

Fig. 9 (top three panels) presents the corresponding data from experiments with CTL effectors specific for three A\*0201-restricted epitopes of different hydrophobicities derived from EBV nuclear antigens of the lytic cycle. All three epitopes were presented from their source antigen in TAP-positive cells (Fig. 9 A). On testing in TAP-negative cells we found that the least hydrophobic epitope TLD/A2, although capable of TAP-independent presentation when expressed from a minigene, was nevertheless TAP-dependent in assays requiring presentation from its native antigen BMRF1 (Fig. 9 B). By contrast, we observed significant

presentation of the more hydrophobic YVL/A2 and GLC/A2 epitopes when their native nuclear antigens, BRLF1 and BMLF1, respectively, were expressed from vaccinia constructs in T2 cells (Fig. 9 B). Although levels of lysis of vBRLF1- and vBMLF1-infected targets were always lower in the T2 cell background than a standard TAP-positive LCL, they were nevertheless reproducibly observed on five successive assays of this kind at levels >20%, compared with <3% for T2 targets infected with the control vaccinia constructs. TAP-independent presentation of the native GLC epitope was also observed from all four BMLF1/epitope chimaeric constructs described previously (data not shown). This indicates that, if sufficiently hydrophobic in sequence, epitopes native to nonmembrane-associated proteins can be presented in a TAP-independent manner.



**Figure 9.** Processing of EBV nuclear antigens BRLF1, BMLF1, BMRF1, and EBNA3A for recognition of native epitopes. Results of assays using native nuclear antigen-encoding vaccinia constructs in (A) a standard TAP-positive LCL with the appropriate A2 and B35 restriction elements and (B) in T2 cells. Targets were either infected with recombinant vaccinia encoding the native antigens vBMLF1, vBMRF1, vBRLF1, vEBNA3A (with vLMP2 and vEBNA3C as controls), or pre-exposed to their epitope peptides GLC, TLD, YVL, YPL (with CLG and FRK as controls). \*Note that in the case of T2 targets tested with YPL/B35-specific effectors, the cells were coinfecting with vB35 to provide the relevant HLA restricting allele. Results are expressed as in Fig. 2 using epitope specific CTLs at effector:target ratios of 4:1 (black bar) and 2:1 (shaded bar).

## Discussion

Apart from early work in the TAP2 mutant mouse line RMA-S where findings were complicated by a suggestion of residual TAP function (43), published examples of TAP-independent presentation from endogenously expressed antigens all involve epitopes derived from secreted proteins or from integral membrane proteins with ectodomains which naturally access the ER. All such TAP-independent epitopes appear to be generated either from signal sequences liberated by the signal peptidase enzyme (10, 15, 16) or from breakdown from within the secretory pathway by other ER-resident or Golgi-resident proteases (17–20). By contrast the LMP2 protein represents a source antigen which contains at least some TAP-independent epitopes yet which has no conventional signal sequence and no significant projection into the ER. Indeed to our knowledge LMP2 is the only such multiple membrane spanning struc-

ture to be analyzed as a CTL target. Our studies, conducted in both lymphoid and epithelial target cell backgrounds and involving an extended range of LMP2 epitopes, clearly identify a subset of epitopes which are consistently TAP-independent (Figs. 2–5). Furthermore this TAP-independent processing is observable when LMP2 is expressed at physiological levels and not just at the enhanced levels seen from vaccinia viral vectors. Thus, clones specific for the TAP-independent CLG/A2 epitope show significant baseline killing of unmanipulated T2 cells (Fig. 2 D), consistent with the fact that the T2 line is A\*0201 positive and expresses low levels of LMP2 from its endogenous EBV genome. Furthermore, an individual with an inactivating mutation in the TAP2 gene was capable of mounting LMP2-specific CTL responses after natural EBV infection in vivo (23), and CTL clones derived from this individual allowed us to

map a new TAP-independent LMP2 epitope, WTL/B63; such clones again showed significant baseline killing of TAP-negative LCL cells expressing physiological levels of LMP2 and HLA B\*63 (Fig. 3).

Our first objective was to determine whether there was any relationship between epitope location in the native LMP2 protein and TAP dependence. One possible scenario, originally influenced by models of membrane protein breakdown (44, 45), was that LMP2 might be cleaved on its cytosolic face, possibly by a nonproteasomal mechanism, and particular transmembrane/luminal loop fragments released directly into the ER, thereby allowing some epitopes to access HLA class I molecules via a TAP-independent route (21). Contrary to such a model, however, we found that one epitope naturally lying on a luminal loop (IED/B40) was TAP dependent whereas one of the epitopes predominantly situated on a cytosolic loop (TYG/A24) was TAP independent. Furthermore, the processing of TAP-independent epitopes from LMP2 showed the same sensitivity to two proteasomal inhibitors, lactacystin (33, 46), and epoxomicin (34), as seen for conventional TAP-dependent epitopes. These compounds are among the most specific of the known proteasome inhibitors, functioning via covalent linkage to particular catalytic subunits of the proteasome (33, 46). Although widely used in antigen processing experiments to demonstrate involvement of the proteasome (12, 13, 47), lactacystin also has some inhibitory effects on cathepsin A (48), but these are reversible and would therefore not be effective under the conditions of lactacystin pulsing used in the present experiments. Likewise lactacystin has been reported to show partial inhibition of tripeptidyl-peptidase-II (TPPII) (49), a cytosolic protease which can substitute for proteasome-mediated general protein turnover in certain circumstances and may substitute, albeit inefficiently, for the proteasome's antigen processing function (49, 50). However, the concentration of lactacystin used in the present work had little effect on protein turnover in cells where TPPII activity is dominant whereas turnover was strongly inhibited in cells with regular proteasome function (51).

From the above we infer that, unlike all other published TAP-independent epitopes within endogenously expressed proteins (10, 16), those within LMP2 are dependent upon the proteasome for their generation. The processing of LMP2 might occur either in its membrane location (since membrane associated proteasomes have been detected in several systems; references 52–54) or possibly as a recently synthesized but misfolded polypeptide marked for degradation in the cytosol (55, 56). LMP2 has been reported to bind Nedd4-like ubiquitin-ligases through PPPY motifs in the cytosolic NH<sub>2</sub>-terminal domain, thereby producing a ubiquitination signal which could target LMP2-associated src kinases or even LMP2 itself to the proteasome (57, 58). However, this interaction is not required in the present context since a naturally expressed version of LMP2 lacking the NH<sub>2</sub>-terminal domain (so called LMP2B) is processed to TAP-dependent and TAP-independent epitopes as effectively as the full-length protein (reference 21 and

unpublished data).

Insights into the mechanism of TAP-independent epitope presentation came from experiments expressing LMP2 epitopes as cytosolic peptides from vaccinia minigene constructs. This produced the same clear discrimination between TAP-dependent and TAP-independent epitopes as observed with LMP2 processing itself (Fig. 6), implying that TAP independence was a feature of the epitope sequence per se. An apparently strong correlation between TAP-independence and LMP2 epitope hydrophobicity (Table 1) led us to construct minigenes encoding epitopes native to nonmembrane associated proteins but with hydrophobicity indices (GLC/A2, 4.41; TLD/A2, 1.72; YPL/B35, -0.6) which spanned the same range as the LMP2 epitopes. Again the two most hydrophobic epitopes GLC/A2 and TLD/A2 were clearly TAP-independent when expressed as cytosolic peptides whereas the hydrophilic YPL/B35 epitope was not (Fig. 8). Other studies expressing epitope minigenes either from plasmid or vaccinia viral vectors have reported discrimination between different epitopes in their ability to be presented in TAP-negative cells (59, 60). Close inspection of the epitopes in one such study (60) indicates that the three TAP-independent sequences had hydrophobicities of 1.44 to 2.63, compared with a TAP-dependent epitope hydrophobicity of -3.68. In another earlier study where the data was interpreted as showing no such correlation (59), recalculation of hydrophobicities in fact confirm the relationship between TAP-independence, and hydrophobicity scores for 9 of the 10 peptide sequences examined, the exception being a 12-mer peptide which did not represent the minimal epitope sequence. We infer that hydrophobicity is indeed an important determinant of the TAP-independent status of epitope sequences, at least when expressed from minigenes.

Finally, our experiments showed that the TAP-independent presentation of hydrophobic epitopes from endogenously expressed antigen does not require the source antigen to be a membrane-associated protein. Thus transfer of the CLG/A2 and TYG/A24 epitope sequences into an EBV nuclear antigen BMLF1 still allowed their efficient presentation in T2 cells (Fig. 7). Furthermore two equally hydrophobic epitope sequences (GLC/A2 and YVL/A2), native to BMLF1 and to another EBV nuclear antigen BRLF1 respectively, were also presented from their vaccinia-expressed native antigen via a TAP-independent route (Fig. 9). Given an apparent discrepancy between the above BMLF1 result and that seen in earlier work (21), we rechecked BMLF1 processing in several different HLA class I-transfected subclones of T2 as well as in different passages of T2 itself. All showed clear presentation of the GLC/A2 epitope with the single exception of the T2: B\*2705 transfectant, the target line used in our original study (data not shown); why T2:B27.05 cells give atypical results in BMLF1 processing assays is unclear but appears to reflect a peculiarity of this transfectant. Throughout these experiments on GLC/A2 and YVL/A2 processing, epitope-specific recognition in T2 cell backgrounds was ~50% that seen for the same constructs in TAP-positive

LCL cells. However, this appears to reflect a less efficient generation of these epitopes by proteolysis in the immunoproteasome-negative T2 environment (rather than less efficient epitope transport) since the same comparatively low levels of lysis vis-à-vis that seen in LCL cells were also observed in the TAP-transfected T3 reference line (data not shown). However, we did note a clear difference between the results of minigene assays and of native antigen processing assays with the TLD/A2 epitope from another EBV nuclear antigen, BMRF1. This epitope, with an intermediate hydrophobicity of 1.72, was TAP-independent as a minigene-encoded peptide but TAP-dependent when expressed within its native protein (Figs. 8 and 9), results which mirror the data obtained by Norbury et al. (60) using epitopes with hydrophobicities in the 1.44–2.63 range. This reemphasizes the fact that vaccinia minigene constructs produce much larger numbers of cytosolic epitope peptides than are generated from processing of a vaccinia-encoded antigen (61), and such quantitative differences can become particularly important determinants of epitope representation at the T2 cell surface when epitope hydrophobicity is itself suboptimal.

Therefore, this study highlights the existence of a proteasome-dependent, TAP-independent pathway of epitope processing/presentation which appears to be restricted to highly hydrophobic epitopes that can be drawn either from membrane-associated or from nonmembrane-associated proteins. The pathway of TAP-independent access into the ER remains to be determined. One possible route could involve the ER translocon Sec61p, since this is involved not only in the recognition of hydrophobic signal sequences for protein translocation into the ER but also in the retrograde transport of peptides from the ER back into the cytosol (62). Another possibility would be the postulated transporter whose existence was inferred from studies of TAP-independent presentation of epitopes from chimaeric Jaw-1 constructs (18); however, there is no evidence that such a transporter would have selectivity for hydrophobic sequences. Alternatively it may be that hydrophobicity allows peptides to penetrate membranes by an energy-independent mechanism rather than via a specific transporter pathway. Indeed this is supported by earlier findings of an ATP-independent transport into microsomes which was selective for 2 of 3 epitope peptides (63) and where inspection of sequences clearly shows a correlation between transport and hydrophobicity indices.

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