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Brain-Infiltrating Cytolytic T Lymphocytes Specific for Theiler's Virus Recognize H2D^b Molecules Complexed with a Viral VP2 Peptide Lacking a Consensus Anchor Residue

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Mice expressing the *H2^b* haplotype are resistant to infection with Theiler's murine encephalomyelitis virus (TMEV), which causes chronic demyelination in susceptible mice. The prominent cytolytic T-lymphocyte (CTL) response to the VP2 antigen encoded by TMEV led us to the identification of a class I-binding peptide derived from the VP2 antigen. *Escherichia coli* transformants overexpressing a series of 11 overlapping VP2 protein fragments were subjected to lysis and alkali digestion, and the resultant peptide pools were tested for their abilities to sensitize RMA-S targets for lysis by CTLs. The source of effector CD8$^+$ T cells for the assays was either freshly harvested central nervous system-infiltrating lymphocytes (CNS-IL) or CNS-IL-derived VP2-specific CTL clones and lines. A 10-residue peptide at VP2 positions 121 to 130 (VP2$_{121–130}$) (FHAGSLLVFM) was identified that sensitized targets for lysis and formed stable complexes with H2D^b class I molecules. The VP2$_{121–130}$ peptide sensitized target cells for lysis by freshly harvested CNS-IL CTLs at femtomolar concentrations. Despite its relative high level of biological activity, the VP2$_{121–130}$ peptide is distinguished from other D^b-binding peptides by its lack of an asparagine residue at position five, which had been previously proposed to be a requirement for D^b-peptide complexing.

Mice expressing the *H2$^{b, d,}$* or *^{k}* haplotypes effectively clear an intracerebral injection of the Daniels (DA) substrain of Theiler's murine encephalomyelitis virus (TMEV), whereas susceptible strains (*H2$^{s, q, r, v, f,}$* or *^{p}* haplotypes) develop persistent viral infection. The latter, TMEV-susceptible mouse strains, develop a chronic, progressive form of demyelination similar both clinically and pathologically to human multiple sclerosis (MS). TMEV-infected mice provide an excellent model for the study of MS (33), since epidemiologic studies consistently suggest an environmental pathogen(s) as an antecedent for MS (1, 5). Recent studies implicating a viral association of human herpesvirus 6 with MS plaques (6) also lend support to this hypothesis. Resistance to TMEV-induced demyelination is associated with specific alleles at the class I *H2D* region (32) and is further associated with differential regulation of class I products and the presence of virus-specific cytotoxic CD8$^+$ T cells (CTL) in central nervous system-infiltrating lymphocyte (CNS-IL) populations (10, 24, 28, 31). We previously determined that the VP2 capsid protein of TMEV is a prominent target of H2D^b-restricted CTLs (17). We also determined that cytolytic CD8$^+$ T cells are required for clearance of TMEV in resistant mouse strains (10, 24, 28, 31). The only TMEV-VP2 epitope identified to date is VP2 from positions 74 to 86 (VP2$_{74–86}$), which is a major histocompatibility complex (MHC) class II-restricted epitope encoded by another TMEV strain (BeAn) and recognized by T-helper cells in susceptible SJL/J mice (12).

The reproducibility of identifying VP2 region-specific, D^b-restricted CTL in CNS-IL populations from TMEV-infected mice suggested a prominent role for the CTL response to the VP2 antigen in virus clearance. Accordingly, the VP2 antigen should include at least one peptide that complexes with D^b molecules and is efficiently recognized by these CTL. An understanding of the fundamental difference between mice resistant and susceptible to TMEV infection, the former of which require CD8$^+$ CTLs for efficient virus clearance, depends on the identification of prominent peptides recognized by CTL restricted by class I genes associated with resistant *H2* haplotypes.

The goal of the present work was to identify a VP2-associated peptide that complexes with D^b molecules and is recognized by CNS-ILs from TMEV-infected resistant mice. A method for insertion of variable lengths of VP2 into a prokaryotic fusion protein gene followed by random base hydrolysis (11) led to the identification of a 10-amino-acid peptide that binds to D^b molecules and is recognized by freshly harvested CNS-ILs from TMEV-infected mice. The amino acid sequence of this peptide starkly contrasts with those of other identified D^b-binding peptides in its lack of an Asn at residue 5 which has been proposed to be part of an essential motif for D^b-binding peptides (8, 9, 29).

MATERIALS AND METHODS

PCR product generation. The template for variable-length VP2 PCR products was pDAFL3 plasmid (25), which includes the full-length sequence of the DA strain of TMEV (accession no. M20301). The left-hand primer VP2 Anchor (5-ACCGA GCTCGGATCCAGATCAAAACACAGAGGAGATGG-3) contains VP2 bases 1507 to 1528 at the 3′ end and a *Bam*HI site at the 5′ end. This primer was used with 11 right-hand primers (ranging from 18 to 21 bp), including *Xho*I sites at their 5′ ends, that allowed amplification of VP2 PCR products of increasing lengths (Fig. 1). The first 9 fragments increased sequentially by 60 bp, and the 10th and 11th fragments increased by 120 and 81 bp, respectively, as indicated in Fig. 1. The PCR was conducted with HotStart 50 reaction tubes according to the recommended protocol of the manufacturer (Molecular Bio-Products, Inc., San Diego, Calif.). All reaction mixtures included 20 pmol of each primer, 10 ng of template pDAFL3, and 0.25 μl of *Taq* DNA polymerase (Promega, Madison, Wis.). Cycling parameters were 25 rounds of 1 min of denaturation at 94°C, 1 min of annealing at 55°C, and extension for 1 min at 72°C. An initial round was denatured for 5 min, and a final round was extended for 7 min. All reactions were

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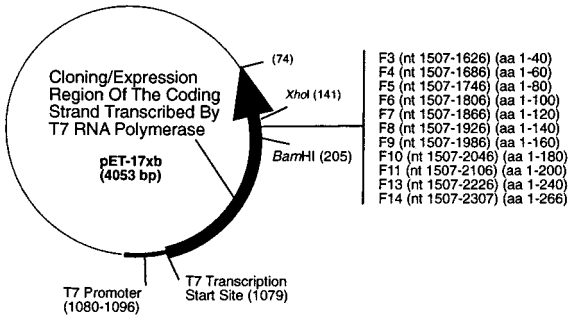

FIG. 1. Strategy for constructing TMEV-VP2 chimeric genes in pET17x-b. Eleven constructs were made, with each containing one of the indicated fragments of TMEV-VP2 inserted unidirectionally and in frame into the *Bam*HI/*Xho*I sites of pET17x-b. F3 to F14, fragments 3 to 14; nt, nucleotides; aa, amino acids.

performed with a DNA Thermo-Cycler (Perkin-Elmer Cetus, Foster City, Calif.).

Plasmid construction and fusion protein expression. Recombinant DNA manipulations were performed according to standard protocols (2). The 11 PCR products were unidirectionally cloned into the *Bam*HI and *Xho*I sites of pET-17xb (Novagen, Madison, Wis.) by placing each product in frame with the N-terminal and C-terminal sequences of the proprietary fusion protein (Fig. 1). Transformation hosts were NovaBlue cells selected for plasmid maintenance and BL21(DE3) cells (Novagen) for inducible T7 RNA polymerase transcription and subsequent fusion protein expression. Transformants in both hosts were selected for carbenicillin resistance according to the manufacturer's instructions. Inserts and cloning sites were sequenced on an ABI 373-A Automated Sequencer with an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer). Twenty-milliliter cultures in Luria-Bertani medium (LB) plus carbenicillin (100 μg/ml) were initiated with a fresh overnight colony from each cloning. Cells in mid-log growth were induced with isopropyl-β-D-thiogalactopyranoside (IPTG) (final concentration, 0.4 mM) and grown for 3.5 h postinduction. Fusion protein synthesis was confirmed with 500-μl samples taken every 30 min following IPTG induction; these aliquots were centrifuged, resuspended in 50 μl of sodium dodecyl sulfate (SDS) gel sample buffer, and frozen at −20°C. Following incubation of the samples at 70°C for 5 min, 6 μl of each sample was run on SDS-10% polyacrylamide gels (4). Protein band intensities subsequent to Coomassie blue staining confirmed that the TMEV fusion protein fragments were the dominant proteins expressed in their respective cultures and that the optimal times for harvesting cultures to acquire optimal yield were 1.5 to 2.0 h postinduction.

Cell lysis and hydrolysis. Three milliliters of induced cultures (prepared as described above for protein expression) was harvested by centrifugation (5,000 × *g* for 5 min at 4°C [Sorvall RC-5B; DuPont Instruments, Wilmington, Del.]), washed once in 50 mM Tris-HCl (pH 8.0)–2 mM EDTA, and centrifuged again. The pelleted cells were resuspended in 100 μl of fresh lysozyme solution (0.5 μg/ml in 10 mM Tris [pH 8.0]), and the suspension was incubated for 1 h at room temperature. After one freeze-thaw cycle in ethanol-dry ice, the lysozyme was inactivated by boiling. NaOH was added to a final concentration of 1 N, and lysates were boiled at 100°C for various time increments ranging from 30 s to 10 min. Hydrolyzed lysates were neutralized with HCl, placed on ice, and adjusted to pH 7.0 to 7.6. Samples were centrifuged briefly to remove insoluble material prior to use in CTL assays.

Extraction of CNS-ILs and establishment of lines and clones. Resistant C57BL/10 mice were infected with TMEV by an intracerebral injection of the DA strain of TMEV at a dose of 2×10^5 PFU in 10 μl. This DA viral stock was propagated in BHK-2 cells and plaqued on L2 cells. Fresh CNS-ILs were obtained as previously described (18, 19). Briefly, brains and spinal cords were harvested 7 days after infection, and cells were suspended by passage through fine-mesh stainless steel screens. Suspended cells were layered on a discontinuous Percoll (Pharmacia) gradient and centrifuged at 27,000 × *g* for 30 min. The lymphocyte fraction of the gradient was collected, washed twice with RPMI 1640, and counted. CNS-IL lines were established by the technique described by Bergmann et al. (3), which involved preincubation of CNS-ILs in plastic tissue culture vessels for 2 h at 37°C in order to remove adherent cells. Nonadherent cells were collected and mixed with 1.25×10^4 C57SV fibroblasts transfected with the TMEV *VP2* gene or with the left-hand portion (LP) of TMEV that includes the *VP1*, *VP2*, *VP3*, and *VP4* genes (17). These cells were grown in Iscove's medium plus 5% fetal calf serum and supernatant from rat splenocytes stimulated with concanavalin A as a source of interleukin-2. Limiting-dilution cloning was performed in 96-well plates (round bottom) with 20, 10, and 5 responders plus 10^4 irradiated VP2- or LP-transfected C57SV cells/well. CTL lines and clones were tested for specificity in a cell-mediated lympholysis (CML) assay as previously described (37). Target cells for testing the specificity of CTL were ^{51}Cr-labeled C57SV cells and C57SV cells transfected with VP2. Two thousand targets were mixed with CTL in microtiter wells to yield effector-to-target (E:T) ratios that varied according to the tested CTL, and the plates were incubated for 4 h at 37°C. One hundred microliters of supernatant was harvested for counting in a gamma counter, and the ^{51}Cr release from labeled targets was determined by the following equation:

$$\% \text{ specific release} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100$$

Maximum release and spontaneous release were the amounts of ^{51}Cr released during incubation with 2.5% Triton X-100 and medium alone, respectively; spontaneous release from RMA-S and fibroblast targets was routinely 10 to 20% of maximum release. Mean specific release (± standard errors) was determined for each E:T ratio, and standard errors were routinely <10% of the mean specific release. This standard CML assay was modified for RMA-S cells sensitized with peptides as described previously (23). ^{51}Cr-labelled RMA-S cells were sensitized with peptides for 40 to 60 min at room temperature in Iscove's medium plus 10% fetal calf serum. CTL were added to yield the required E:T ratio for a 4-h incubation at 37°C. Specific peptide sensitization was calculated as the difference between the percentages of ^{51}Cr release with and without CTL.

Peptide synthesis. Five peptides ($VP2_{112–121}$, $VP2_{112–131}$, $VP2_{121–140}$, $VP2_{121–130}$, and $VP2_{122–130}$) were synthesized on a Gilson AMS422 peptide synthesizer (Gilson Medical Electronics, Inc., Middleton, Wis.) by 9-fluorenylmethoxycarbonyl chemistry and purified by reverse-phase high-performance liquid chromatography. The purity and identity of the synthetic peptides were confirmed by mass spectrometry.

Peptide binding and class I stabilization assays. The specificity of synthetic peptide binding to K^b and D^b molecules of RMA-S cells was quantitated by a previously described stabilization assay (35). RMA-S cells were incubated overnight at 26°C, sensitized with peptides at a final concentration of 50 μM in the presence of Brefeldin A (BFA; 10 mg/ml; Sigma, St. Louis, Mo.) for 1 h at room temperature, washed, and incubated at 37°C for 2 h. Cells (3×10^5) were washed and stained for K^b or D^b class I expression by using Y-3 and B22.249 monoclonal antibodies (in appropriate dilutions of ascites), respectively (13, 14). Antibody-labelled cells were incubated for 30 min with a fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin antibody. All incubations were performed in 1% bovine serum albumin–phosphate-buffered saline at 4°C. The cells were washed, and the analysis of 10^4 gated events per sample was performed on a FACScan flow cytometer (Beckton Dickinson Co, Mountain View, Calif.). The 50% stabilization concentration corresponds to the peptide concentration producing half-maximal upregulation of class I molecules. The relative dissociation rate of bound peptide from cell surface class I D^b was estimated by incubating (3×10^5) RMA-S cells (26°C overnight) with or without BFA (10 mg/ml) for 1 h at room temperature, after which they were incubated with peptide for 1 h at room temperature. Free peptides were washed away, and fresh medium was added to the cells for incubation at 37°C. At different time points, samples were collected and stained for D^b complexes as described above. Samples not incubated at 37°C were taken as time zero.

RESULTS

Lytic activity of CTL with VP2-transfected C57SV cells. CTL lines and clones derived from CNS-IL populations were tested for specificity with C57SV cells transfected with the full-length *VP2* gene. C57SV-VP2 transfectants were lysed by all CTL lines and clones with killing ranging from 20 to 49% (Table 1). The clones and lines used in the experiments described here are indicated in Fig. 2. No significant lysis of nontransfected C57SV fibroblasts was observed. These results are consistent with previous observations pointing toward a prominent response of B6 mice to VP2 (17).

VP2 fusion protein yields immunogenic peptides. Based on these results, the *VP2* gene was selected for construction of an inducible fusion protein as described above. Since H2 class I molecules preferentially bind 8- to 10-amino-acid peptides, it was necessary to cleave the induced fusion proteins prior to sensitization of RMA-S targets. RMA-S cells have defective TAP function, with the result that their class I molecules are unstable unless complexed with peptide or incubated at 26°C (21, 35). We chose to follow the previously described approach (5) to randomly hydrolyze the VP2 fusion protein with NaOH, neutralize the peptide mixture, and sensitize RMA-S cell targets with the peptide supernatant. Since that previous report cited the variable sensitivity of peptides to base hydrolysis (5), we subjected the full-length VP2 fusion protein to base hydro-

TABLE 1. CTL lines and clones derived from CNS-ILs from B6 mice infected with TMEV lyse fibroblast targets transfected with the *VP2* gene

CTL	Stimulator	Clone or line	Mean % ^{51}Cr Release at E:T ratio of 40:1 with the following targets:	
			C57SV-VP2	C57SV
B6	C57SV-LP	Clone	32	1
C5	C57SV-LP	Clone	21	1
D2	C57SV-LP	Clone	20	0
F7	C57SV-LP	Clone	49	0
9/95	C57SV-LP	Line	45	1
B8	C57SV-VP2	Clone	40	0
C4	C57SV-VP2	Clone	39	0
C7	C57SV-VP2	Clone	33	0
D6	C57SV-VP2	Clone	42	1
F5 and G10 pool	C57SV-VP2	Clone	44	8
212/95	C57SV-VP2	Line	27	0
269/95	C57SV-VP2	Line	45	0

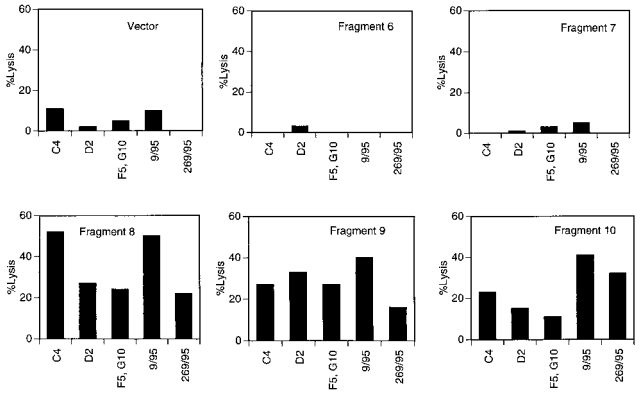

FIG. 3. CML assay of five VP2 fragments with multiple, VP2-specific CTL clones and lines. Five bacterial lysates (10 μl) containing hydrolyzed fragments of VP2 (F6 to F10) were used to sensitize ^{51}Cr-labeled RMA-S targets. CTL clones (C4, D2, and a pool of F5 and G10) and two lines (9/95 and 269/95) served as effectors at an E:T ratio of 40:1.

lysis for various lengths of time ranging from 15 s to 10 min. Only marginal cytolysis within a range of 2 to 11% was observed when the C4 VP2-specific CTL clone was mixed with RMA-S cells sensitized with 10 or 20 μl of bacterial supernatant hydrolyzed for 15 to 45 s, for 4 min, and for 10 min. However, 1 min of hydrolysis resulted in sensitization and 25% target lysis with 10 μl of hydrolyzed supernatant. Background lysis observed with the alkali-hydrolyzed pET-17xb vector transformant (without the VP2 insert) ranged from 3 to 11% over all time points.

Screening of the panel of VP2 fragments. Bacterial lysates containing VP2 fragments (F3 to F13 [Fig. 1]) from the respective transformants were hydrolyzed with NaOH for 1 min and were used to sensitize RMA-S cells for lysis by the C4 CTL clone (Fig. 2). No lysis was observed with fractions F3 to F7, but a significant level of lysis was observed with fragment F8 (52%). Further, lysis was maintained with all hydrolyzed fragments larger than F8. Fragment F8 differed from the F7 fragment by the addition of VP2 amino acids 121 to 140, localizing the region of an immunogenic VP2 epitope to amino acids 112 to 140 in that only part of the peptide may have been included in fragment F7. The recognition of fragments F8 to F10 was not restricted to the C4 CTL clone. VP2-specific CTL clone D2, a pool of CTL clones G10 and F5, and two VP2-specific CTL lines (9/95 and 269/95) recognized RMA-S targets sensitized with alkali-digested VP2 fusion protein fragments F8, F9, and F10 (Fig. 3). Alkali-digested *Escherichia coli* transformants containing vector without insert and VP2 fusion protein fragments F6 and F7 did not sensitize RMA-S targets for lysis by these clones and lines.

Localization of an active 10-amino-acid peptide. Two 20 residue overlapping synthetic peptides ($VP2_{112–131}$ and $VP2_{121–140}$) were synthesized to provide an 11-residue overlap. $VP2_{112–131}$ included the last 10 amino acids of F7. Both peptides sensitized RMA-S target cells for lysis by VP2-specific CTL clone C4 (Fig. 4), suggesting that the active peptide was included in the 11-amino-acid overlap. Based on the preponderance of Asn residues at position five of D^b-binding peptides (30), a 10-amino-acid peptide comprised of VP2 residues 112 to 121 was synthesized and tested for sensitization (Fig. 4). No lysis was observed with $VP2_{112–121}$ in the parallel analysis with the two active overlapping 20-amino-acid peptides. The lack of a second Asn residue in the overlap region suggested that the

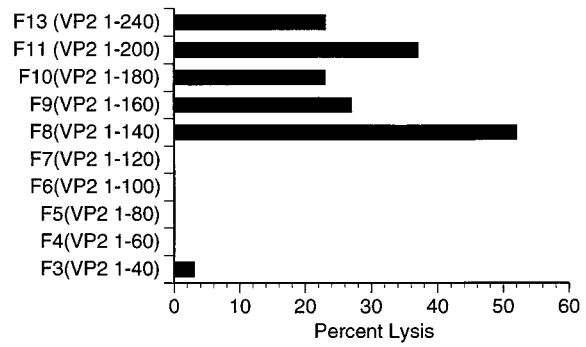

FIG. 2. CML assay of 10 NaOH-hydrolyzed VP2 fragments with CTL clone C4 derived from CNS-IL populations 7 days after TMEV infection of B10 mice. Bacterial lysates (10 μl) containing hydrolyzed fragments of VP2 of increasing size (amino acid locations indicated in parentheses) were mixed with ^{51}Cr-labelled RMA-S targets and clone C4 CTL (E:T ratio, 40:1). Spontaneous release of ^{51}Cr from RMA-S targets was 9% of maximum release.

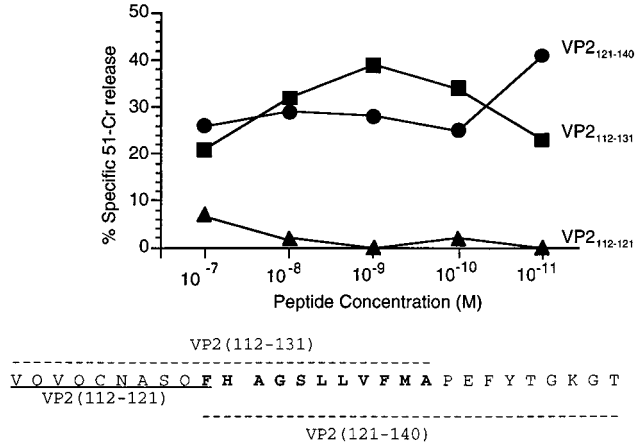

FIG. 4. CML assay of two partially overlapping 20-residue synthetic peptides that span potentially informative regions of fragments F7 and F8 as well as a 10-residue synthetic peptide from fragments F7 and F8 with Asn at position 5. Titrated concentrations of the three VP-2 peptides were incubated with ^{51}Cr-labelled RMA-S cells in a CML assay with freshly harvested CNS-IL CTLs from TMEV-infected B10 mice (E:T ratio, 100:1).

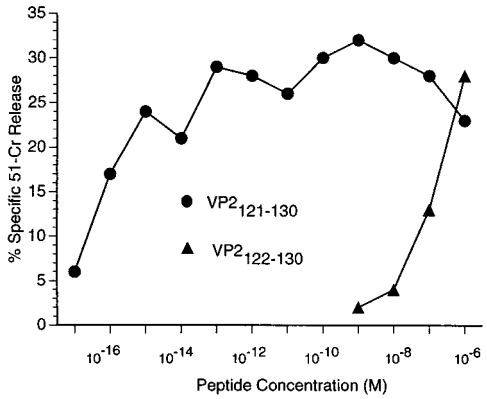

FIG. 5. CML analysis of the $VP2_{121–130}$ and $VP2_{122–130}$ peptides that lie in the region of overlap between the $VP2_{112–131}$ and $VP2_{121–140}$ 20-residue peptides. ^{51}Cr-labeled RMA-S cells were incubated with titrated concentrations of peptides prior to the addition of CNS-IL CTL (E:T ratio, 50:1) freshly harvested from TMEV-infected B10 mice.

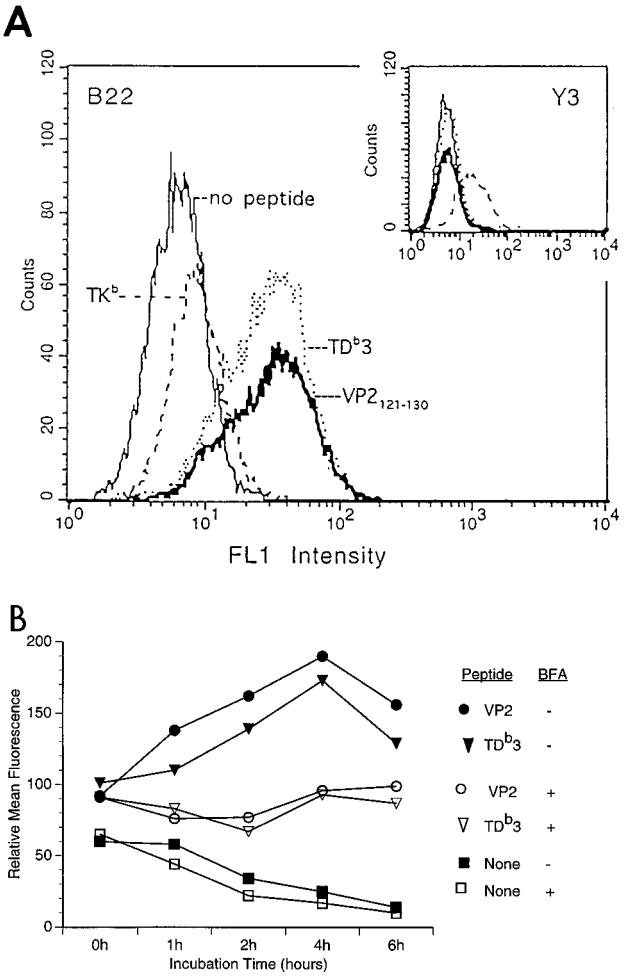

FIG. 6. The $VP2_{121–130}$ peptide specifically stabilizes cell surface expression of D^b molecules on RMA-S cells. (A) RMA-S cells were cultured overnight at 26°C, aliquoted in medium, and mixed with the following peptides at a final peptide concentration of 50 mM: $VP2_{121–130}$ (——), TD^b3 (· · ·), TK^b (– – –), and no peptide (——). Pulsed RMA-S cells were incubated at room temperature for 1 h after which they were stained with B22.249 (anti-D^b) or Y-3 (anti-K^b), followed by fluorescein, isothiocyanate conjugated anti-mouse immunoglobulin. (B) RMA-S cells, incubated overnight at 26°C, were incubated for 1 h at room temperature with $VP2_{121–130}$ (1 mM), TD^b3 (1 mM), and no peptide, after which the cells were pelleted and the medium was replaced with fresh medium. The cells were transferred to 37°C, and aliquots were removed at individual time points ranging from 0 to 6 h for staining with B22.249 as described above. Peptide pulsing and subsequent incubation at 37°C were performed in the absence or presence of BFA (10 μg/ml).

active peptide must not include a canonical D^b-binding anchor residue. Based on the previously cited similarity between the D^b and L^d molecules in regions of alpha helices and beta strands that impact the carboxy termini of bound peptides (38), viral peptides known to bind L^d were surveyed for similarities with the VP2 overlap region. An L^d-binding peptide from LCMV includes a VYM sequence at the carboxy terminus that is similar to the VFM sequence in the overlap (34). Therefore, we synthesized two peptides that had the VFM sequence at carboxy termini and included residues 121 to 130 and 122 to 130. The two peptides were titrated for sensitization of RMA-S cells for lysis by freshly harvested CNS-ILs from TMEV-infected B10 mice (Fig. 5). CNS-IL effectors were chosen to evaluate the recognition of these peptides by CTL; since they had not been subjected to long-term in vitro manipulation. Although both peptides sensitized RMA-S cells for lysis at 1 μM, only FHAGSLLVFM maintained activity throughout the titration, with >50% maximal lysis still observed at a concentration of 1 fM. The activity of the $VP2_{122–130}$ peptide rapidly declined at 100 and 10 nM. These results indicate that the FHAGSLLVFM peptide is effectively recognized by CNS-IL CTL despite its lack of a canonical anchor residue in the fifth amino acid position.

$VP2_{121–130}$ efficiently binds D^b molecules. Although CNS-IL CTL directly harvested from TMEV-infected B10 mice preferentially recognize TMEV peptides in the context of D^b (17), it was essential to confirm formation of D^b-$VP2_{121–130}$ complexes. The ability of $VP2_{121–130}$ to bind to D^b molecules was examined by quantitating the stabilizing effect of the peptide on these molecules at the surfaces of RMA-S cells. These cells have defective TAP function, and their class I molecules are stable at 26°C and labile at 37°C without the addition of exogenous, binding peptides (21, 35). The binding of $VP2_{121–130}$ was compared with those of a simian virus 40 T-antigen peptide presented by D^b (TD^b3 [GQINNLDNL]) and a simian virus 40 peptide that binds to K^b (TK^b [VVYVDFLKC]) (7, 36). Peptide-D^b and peptide-K^b complexes on the cell surface were detected with monoclonal antibodies B22.249 and Y-3, respectively. After incubation of the cells with the various peptides and elimination of the free MHC molecules at the cell surface by incubation at 37°C for 2 h, the cells were stained to detect the peptide-MHC complexes. Both the $VP2_{121–130}$ and the TD^b3 peptides prevented the decay of D^b, but not K^b, molecules, at 37°C (Fig. 6A). The opposite pattern was observed with the TK^b peptide that stabilized K^b, but not D^b, surface expression. These results indicate that $VP2_{121–130}$ does bind to D^b molecules and is presumably presented to D^b-restricted CTL as predicted by our previous studies (17).

Dissociation rates of D^b-$VP2_{121–130}$ complexes. Given that the $VP2_{121–130}$ peptide binds to D^b molecules, we investigated the rates of dissociation of $VP2_{121–130}$-D^b complexes detected by B22.249. RMA-S cells were incubated overnight at 26°C and pulsed with either $VP2_{121–130}$ or TD^b3 peptide at 1 μM concentrations for 1 h at 26°C in the presence or absence of BFA. Unbound peptide was washed away, and the pulsed RMA-S cells were incubated at 37°C for 0 to 6 h (with or without BFA) prior to labelling with B22.249 for flow cytometric analysis (Fig. 6B). Both the $VP2_{121–130}$ and the TD^b3 peptides stabilized D^b expression in the presence of BFA over the entire

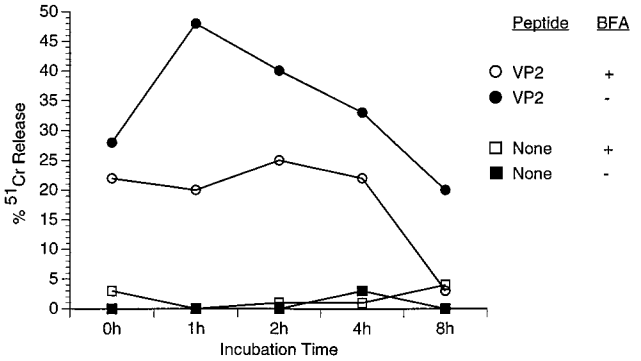

FIG. 7. Stability of $VP2_{121–130}$-D^b complexes assayed by lysis of sensitized RMA-S cells by CNS-IL CTL. ^{51}Cr-labelled RMA-S cells (2×10^3/well) were cultured at room temperature in the presence (open symbols) or absence (closed symbols) of BFA (10 mg/ml) for 1 h before addition of the $VP2_{121–130}$ peptide (1 nM). After 1 h of incubation at room temperature, the medium was discarded, fresh medium with or without BFA was added, and the cultures were transferred to 37°C. Samples were harvested at time points ranging from 1 to 8 h and, after the medium was changed, a 4-h CML assay was performed with CNS-IL CTL (E:T ratio, 50:1).

tested 6-h interval in comparison with unpulsed RMA-S cells. The absence of BFA resulted in comparable, initial D^b expression with both peptides, but expression increased significantly over the remainder of the incubation interval relative to expression in the presence of BFA. Comparable results were observed in three replicate assays, suggesting that newly synthesized and translocated D^b molecules were stably expressed, presumably due to their binding of both peptides.

We subsequently evaluated the dissociation rates of $VP2_{121–130}$-D^b complexes at 37°C as recognized by CNS-IL CTL with a peptide concentration (1 nM) of less than the limits of detection with anti-D^b antibody. The D^b-$VP2_{121–130}$ complexes remained stable for 4 h in the presence of BFA as indicated by pulsed target lysis; sensitizing activity disappeared by 8 h of incubation (Fig. 7). Consistent with the results of flow cytometry experiments described above, we observed an increase in $VP2_{121–130}$ activity after 1 h at 37°C in the absence of BFA, which was not observed in the presence of BFA. Furthermore, detectable, sensitizing activity was still observed after 8 h of incubation at 37°C.

DISCUSSION

Alkali-hydrolyzed products of a panel of TMEV-VP2 recombinant fusion proteins were successfully screened in CTL assays to identify a unique $H2D^b$-restricted CTL epitope encoded by the VP2 region of the DA strain of TMEV. The identified peptide, $VP2_{121–130}$ (FHAGSLLVFM), binds $H2D^b$ molecules and is recognized by freshly harvested CNS-IL CTL from TMEV-infected mice. The truncation of this peptide to HAGSLLVFM lowers both the ability to sensitize targets for lysis and the affinity for D^b molecules. It was important to confirm the identification of the synthetic peptides by using CTL that were harvested directly from TMEV-infected mouse brains. Potential problems with drift and selection during in vitro stimulation and expansion of CTL, which could conceivably give either false-negative or -positive results, were thus avoided.

The alkali hydrolysis method employed here was demonstrated previously to be useful for epitope analysis with the identification of an L^d-restricted nonamer from *E. coli* β-galactosidase that is known to be endogenously processed and presented (11). This method provides a rapid and inexpensive means to potentially produce all immunogenic epitopes for any gene (prokaryotic or eukaryotic) for which the sequence is known. The ability to restrict the origin of the peptide pool is clearly advantageous over the use of peptide libraries as an epitope source. For our purposes, fusion protein constructs were selected because the small VP2 protein fragments could potentially be unstable in an expression system. Further, neither the non-VP2 portion of the fusion protein nor the bacterial proteins interfered with our assays as sources of immunogenic peptides or toxicity.

TMEV-infected mice provide a model for virus-induced demyelination with resistance and susceptibility to demyelination mapping to polymorphic genes linked to the H2 complex. Mice expressing $H2^b$, which were investigated here, effectively clear TMEV and do not demyelinate. An understanding of the specific mechanism by which mice possessing D^b class I molecules can clear the virus is critical to the issue of why susceptible strains cannot do so. Also, knowledge of a TMEV epitope that lends an advantage to mice expressing the $H2^b$ haplotype can be used for the design of therapeutic strategies to establish increased resistance by preimmunization. A $VP2_{121–130}$-vaccinia virus-based vaccine is now under development for this purpose; $VP2_{121–130}$-vaccinia virus-infected cells will be used to stimulate syngeneic CTL for adoptive transfer into TMEV-infected mice to evaluate the role of $VP2_{121–130}$-specific CTL in resistance to TMEV.

While we have now shown that a resistant $H2^b$ mouse expands CTL that recognize one peptide bound to D^b, it remains to be determined if other class I-restricted TMEV epitopes are recognized by $CD8^+$ T cells in resistant mice. Sequentially larger VP2 fragments originating at the amino terminus were constructed here, and the identification of the epitope at amino acids 121 to 130 does not rule out the possibility that one or more active peptides exist between amino acid 130 and the carboxy terminus of the VP2 capsid protein. Furthermore, other TMEV genes may encode peptides presented by D^b and K^b molecules. The VP1 capsid protein of TMEV is a target of H2D-restricted CTL in the CNSs of resistant B10 mice (17). A search for immunogenic peptides in the remainder of the VP2 protein as well as the remaining TMEV genes is in progress.

Although the $VP2_{121–130}$ peptide binds to D^b molecules and is recognized by anti-TMEV CTL, it is not certain that this is the exact peptide that is processed by either TMEV-infected cells in the CNS or fibroblasts transfected with the *VP2* gene. The increase in both $VP2_{121–130}$-D^b complexing and sensitization activity in the absence of BFA suggests a potential role for endocytosis and processing of $VP2_{121–130}$ for augmented presentation in the absence of BFA. There is precedent for such endocytosis and processing of antigens for presentation by class I molecules (16). The reduction in sensitizing activity to the micromolar range associated with removal of the Phe at residue 1 suggests that the naturally processed peptide is not HAGSLLVFM; this assumption is based on the observation that peptides derived from D^b molecules of 10^8 fibroblasts transfected with VP2 and separated by high-performance liquid chromatography sensitize targets for lysis by TMEV-specific CTL (data not shown). However, we have not investigated the activity of the FHAGSLLVF peptide differing from $VP2_{121–130}$ by removal of the Met at residue 10. These experiments and efforts to identify the natural peptide processed by CNS cells and bound to D^b molecules by mass spectrometry are under way.

Recognition of the $VP2_{121–130}$ peptide by TMEV-specific CTL raises the possibility that this peptide and/or closely related homologs are involved in stimulating the CTL response to other

murine picornaviruses. An alignment of the $VP2_{121–130}$ sequence with database sequences identified two TMEV strains that differ in this immunogenic region by only single amino acids. One of the variants, the Vilyuisk virus, is a poorly characterized, highly divergent Theiler's virus (27) and encodes the FHAGSLLVFL peptide that differs from $VP2_{121–130}$ by an M>L interchange at position 10. The highly virulent GDVII strain of Theiler's virus encodes the FHAGSLLVLM peptide (F>L interchange at position 9) (26). All tested mouse strains, including those of the b haplotype, rapidly develop fatal encephalitis when infected with low dosages of GDVII (20, 22). Further, mengovirus and the B and D variants of encephalomyocarditis virus (EMC-B and EMC-D) contain regions of 100% identity to $VP2_{121–130}$. Interestingly, EMC-D causes virus-induced diabetes in SJL/J mice (susceptible to TMEV-induced demyelination), whereas C57BL/6J mice are resistant to both EMC-D and TMEV (15). The potential involvement of the $VP2_{121–130}$ peptide in differential susceptibility to murine picornaviruses is of particular interest.

The $VP2_{121–130}$ peptide is distinguished from other known D^b-binding peptides by its lack of a consensus motif residue, Asn at position 5 (30); in fact, this peptide would have most certainly been overlooked in a search for conventional D^b-binding motifs in the VP2 amino acid sequence. Examination of the $VP2_{121–130}$ sequence for other amino acids predicted to be important for D^b-peptide complexing indicates that the Met at the carboxy terminus of the peptide (position 10) is potentially a strong candidate for a hydrophobic end residue. However, if Met is assumed to occupy this hydrophobic position, a Ser occupies anchor position 5 where an Asn is motif dominant. Of the remaining amino acids in the candidate 10-residue peptide, the Phe at position 1 and the Leu at position 6 could be described as weakly and strongly binding residues, respectively, in D^b-binding peptides (8, 9, 29). Preliminary observations indicate that deletion of the Phe residue at the first position eliminates the ability of this peptide to stabilize D^b expression on RMA-S cells (data not shown). It is clear from our results with $VP2_{121–130}$ that not all D^b-binding peptides have an Asn at position 5, and the combination of alternative amino acids at positions important for binding and T-cell receptor recognition can endow a peptide with the ability both to bind to class I molecules and to be efficiently recognized by CTL. This conclusion supports the utility of class I motif information only as a valuable guide, and the principal reliance on motif data may result in a failure to identify immunogenic epitopes.

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