

A Single T Cell Receptor Recognizes Structurally Distinct MHC/Peptide Complexes with High Specificity

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Summary

The 2C T cell is a CD8⁺, alloreactive T cell, which recognizes cells bearing L^d and K^{bm3} class I major histocompatibility complex molecules. Here, we characterize an allopeptide, designated dEV-8, that is a ligand in the K^{bm3} molecule for the 2C TCR but is not a ligand in the L^d molecule. By biochemical and immunological properties, dEV-8 is distinct from P2Ca, the L^d allopeptide that is also recognized by the 2C TCR. Using the deduced amino acid sequence of dEV-8, we isolate a candidate endogenous source of the peptide. The endogenous protein, MLRQ, contains a peptide sequence identical to dEV-8. This degenerate recognition of two distinct peptide/MHC complexes by a single TCR has important implications for understanding allorecognition.

Major histocompatibility complex (MHC) class I molecules bind 8–10 amino acid peptides from intracellular sources and display them at the cell surface for recognition by T cell receptors (TCR). If the peptide is from a viral or tumor specific protein, the peptide/MHC class I complex often elicits a CD8⁺, cytotoxic T lymphocyte (CTL) response. Peptides derived from normal self proteins are usually not antigenic and are thought to be involved in selective events during thymic development (1), but normal self peptides can play a role in recognition by TCRs when presented by non-self MHC antigen presenting molecules during an alloresponse (2, 3). Currently, the exact role of self peptides in an alloresponse is still being defined (4).

One distinguishing feature of allorecognition is the high precursor frequency of responding T cells (5). This phenomenon could be attributed to the innate ability of TCRs to associate with multiple MHC molecule/peptide complexes (6). Thymic development is the first situation where TCRs contact MHC/peptide complexes (7). T cells are presumably selected on self MHC/peptide structures, and if sufficient signals are generated, the T cells mature and exit the thymus. Once in the periphery, T cells are capable of being stimulated by MHC molecules presenting non-self peptides such as viral peptides. The fundamental, cross reactive nature of the TCR allows a single receptor to be selected on one set of peptide ligands in the thymus and stimulated by a second set of ligands in the periphery. This leads to the hypothesis that the ability of the TCR to engage MHC molecules presenting different peptides results in the high precursor frequency of alloreactive T cells.

Although MHC molecules are highly polymorphic, they all have the shared function of presenting peptides to

TCRs. While class I molecules differ from each other by up to 20% of their amino acids in the antigen binding domains (8), the amino acid residues believed to contact the TCR are highly conserved. It is the amino acids within the peptide binding groove where the major differences between MHC molecules exist. These differences can alter the type of peptide bound and the conformation of the MHC molecules. The inordinate number of alloreactive T cells may therefore be explained by the inherent ability of TCRs to combine with MHC molecules and the fact that different MHC molecules bind a variety of distinct peptides. Several labs have shown that these types of interactions do play a role in allorecognition. Alloreactive TCRs have the structural capability to recognize conformational differences (9); differences on the α helices (10, 11); and even multiple peptides (12). An allo-MHC molecule is in effect a complex set of many different antigenic entities, each of which can stimulate a small set of T cells. The sum of the T cell populations is responsible for the vigorous alloresponse.

While it is apparent that the elasticity of the T cell receptor is a central feature of this molecule, the structural basis for these interactions is not understood. To date there has been no defined set of distinct ligands that are recognized by a single alloreactive TCR. In this report, we identify the sequence of the allopeptide that is presented by a mutant class I molecule, K^{bm3} to the well-defined T cell receptor present on 2C T cells (13, 14). This is the second MHC/peptide complex that is an alloantigen for 2C T cells. The previously defined alloantigen is the p2Ca peptide in the context of the L^d class I molecule. This is the first description of a pair of distinct MHC/peptide complexes that are

recognized by a single TCR. These complexes will provide the basis for future studies to define the nature of TCR interactions with multiple MHC/peptide complexes.

Materials and Methods

Mice and Cell Lines. Mice used in these studies were C57BL/6K^h-H-2^{bm8} kindly provided by Dr. P. J. Wettstein, (Mayo Clinic, Rochester, MN) and maintained in the Mayo Clinic Animal Facility. BW5147 and T2 cells were transfected with K^{bm8} and K^{bm3} respectively using constructs described previously (15, 16). T2 and T2K^{bm8} cells were kindly provided by Dr. L. Sherman (Scripps Institute, La Jolla, CA). T2L^d cells were kindly provided by Dr. P. Cresswell (Yale University, New Haven, CT).

Peptides Isolation and Synthesis. Peptide extracts were generated by immunoprecipitation of the class I molecule from cell detergent lysates or dounce homogenized thymic lobes from K^{bm8} expressing cells from mice or BW5147 K^{bm8} transfected cells using two K^b specific antibodies Y-3 and K10.56 (17, 18). Protein A-Sepharose (Sigma Chemical Co., St. Louis, MO) was used to retain antibody-MHC complexes. The beads were washed and antibodies eluted using 0.2 N acetic acid. Peptides were eluted in 10% acetic acid. Eluted peptides were separated by a Centricon 3 column filtration system (Amicon, Beverly, MA) and lyophilized as previously described (19).

Peptides were synthesized using solid phase chemistry as described previously with N^α-9-fluorenylmethoxycarbonyl (Fmoc) protected L amino acids (20). Each synthetic peptide, excluding the two degenerate libraries, was purified to a single homogenous peak by reverse-phase HPLC.

HPLC Fractionation and Peptide Sequence. K^{bm8} class I eluted peptides were fractionated on two sequential RP-HPLC systems. The fractionation was carried out on an Applied Biosystems model 130A instrument, using an Aquapore OD-300 reverse phase column (2.1 × 220 mm) (Brownlee, Applied Biosystems Inc. [ABI], Foster City, CA) as previously described (19). The initial buffer system consisted of A and B, which were 1% trifluoroacetic acid (TFA)/5% acetonitrile/H₂O and 0.1% TFA/40% acetonitrile/H₂O, respectively. A linear gradient from 0–80% buffer B was used over 80 min. The flow rate was 0.2 ml/min and either 0.5 or 1 min fractions were collected. Biological activity of HPLC fractions was determined in chromium release assays as described below. The second HPLC buffer system was identical to the first except hydrochloric acid was used in place of TFA and a linear gradient starting at 10 min from 30 to 70% buffer B was used over 60 min. The final HPLC system that was employed used the same instrument, column, and flow conditions, but the buffers and gradient were as follows: buffer A and B compositions were 50 mM acetic acid/5% methanol, titrated to pH 3.9 with NH₄OH and 50 mM acetic acid/50% methanol, titrated pH 3.9 with NH₄OH, respectively. A linear gradient from 5 to 80% B was used over 75 min. Edman degradation was performed using an ABI Procysc 492 Protein Sequenator (Perkin Elmer, Norwalk, CT).

Cytotoxicity Assays. CTLs used in this study were either lines established from 2C transgenic mice (21) or the original 2C CTL clone (22) kindly provided by D. M. Kranz (University of Illinois, Urbana, IL). Briefly, splenocytes from the transgenic mice or the 2C clone (DK) were stimulated weekly with lethally irradiated BALB/c stimulators in the presence of rat concanavalin A supernatant and rIL-2. A standard 5-h ⁵¹Cr release assay with 3 × 10³ targets/well was employed. When peptide was added, targets were incubated with 2–7 μl of each HPLC purified peptide frac-

tion or synthetic peptides for 0.5–1 h before addition of effector cells. Percent specific lysis was calculated as follows: (experimental lysis – spontaneous lysis)/(maximum release – spontaneous lysis) × 100.

Mouse MLRQ-like Gene Sequence and Transfection. Total RNA was isolated from BW5147 and B6 spleen cells using either TRIzol reagent, (GIBCO BRL, Gaithersburg, MD) or guanidium-thiocyanate extraction followed by cesium chloride gradient purification (23), respectively. First strand cDNA was synthesized using oligo-dT primers with the cDNA Synthesis System Plus (Amersham International, Amersham, UK). cDNA was synthesized from 20 mg total RNA. The single stranded cDNA was used in a PCR reaction. The primers were designed based on the analogous sequences of human and bovine genes: 5'-CCTTCCAG-TAAGCTTCCTCTGCCGCAAACATGCTCCGCCAGATC-ATCGGG-3' and 5'-ATCGAGCTATGCCGGCCGCTGGT-TAAGTGGAAAATTGTGCGGAT-3'. The amplified product was cloned into the HindIII and NotI sites of pRc-CMV (Invitrogen, San Diego, CA). Sequence data was obtained using the fluorescent dideoxynucleotide method and run on an automated 377 DNA sequencer (ABI). The sequences were confirmed by two independent PCR amplifications and cloning of the MLRQ genes from both BW5147 cells (AKR) and C57BL/6 spleen cells.

K^{bm3} L cell generation was described previously (15). MLRQ cDNA was transfected into the K^{bm3} L cells, and 11 individual cell clones were picked after selection in 400 μg/ml G418. Cells were then screened for retention of the K^{bm3} molecule by flow cytometry to detect an engineered epitope using the monoclonal antibody, 28-14-8 (24).

Hybridoma Assay. The 2C CTL hybridoma was provided by P.D. Gottlieb (University of Texas at Austin, Austin, TX). This hybridoma, which was derived from the 2C T cell, has the same specificity as the 2C cytotoxic T cell clone and secretes IL-2 in response to cells bearing the alloantigens L^d and K^{bm3}. Hybridoma and target cells were combined in 96-well microtiter plates at an effector to target ratio of 1:2 (1 × 10⁵ effectors and 2 × 10⁵ targets). After incubation for 24 h at 37°, cells were pelleted and the supernatant removed for quantitation of IL-2 in a standard bioassay (25) using the IL-2-dependent T cell line, HT-2 (26). Briefly, 50 μl of the reaction supernatant was added to 1 × 10⁴ HT-2 cells in 100 μl, and incubated for 18 to 24 h at 37°. 2 μCi of [³H]thymidine (Amersham, Arlington Heights, IL) was added. HT-2 cells were allowed to incorporate radio label for 6 h before harvesting onto filters and quantitation in a Matrix96 Direct Beta Counter (Packard Instrument Co., Downers Grove, IL). Each data point represents the mean of triplicate samples.

RNA Hybridization Analysis. RNA from 11 cell lines transfected with the MLRQ cDNA was isolated using the TRIzol Reagent. Briefly, we loaded 15 μg of total RNA from each cell line onto a formaldehyde gel (1.5% agarose, 1.11% Formaldehyde, 1× MOPS buffer, pH 7.0; 10× MOPS is 0.2 M (3-(N-morpholino)-propanesulfonic acid), 0.05 M sodium acetate, 0.01 M EDTA). The size separated total RNA was transferred onto a GeneScreen Plus (NEN Research Products, Boston, MA) nylon filter, baked at 80°C for 2 h, and prehybridized in a water based solution (1.0% SDS, 1.0 M sodium chloride, 10% dextran sulfate) at 60°C for ~18 h. A ³²P-α-dCTP labeled probe was prepared using the Random Primers DNA Labeling System (GIBCO BRL). The template that we used to label the probe was a 262-bp SphI fragment isolated from the pRc/CMV vector. This fragment was located downstream of the MLRQ cloning site and included 95% of the polyadenylation signal. The labeled probe was specific for the 3' untranslated portion of the transfected MLRQ transcripts

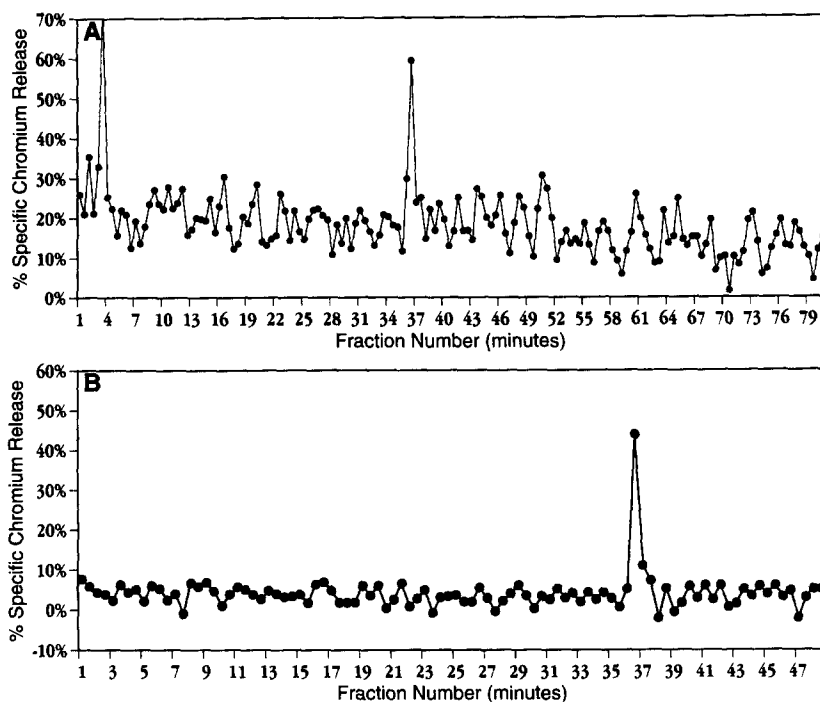


Figure 1. Preparative scale RP-HPLC fractionation and cytotoxicity assay of peptides eluted from K^{bm8} molecules reveals one 2C- K^{bm3} active peak. (A) Peptides were eluted from 1×10^{10} K^{bm8} transfected BW5147 cells and subsequently HPLC fractionated using the acetonitrile/TFA buffer system as described in the materials and methods. 5 μ l of each fraction was then tested in a cytotoxicity assay with 2C T cells from transgenic mice as effectors and T2K bm3 cells as targets. E:T was 6:1. The peak at 3.5 min is TFA and causes lysis of targets without addition of effector cells. (B) The active fractions from three independent TFA/acetonitrile separations of K^{bm8} eluted peptides were combined and fractionated using the HCl/acetonitrile buffer system described in Materials and Methods. A total of 2.2×10^{10} K^{bm8} cells and thymic extracts from 48 K^{bm8} mice were used in the peptide elution. Seven μ l of each fraction was then tested in a cytotoxicity assay as above.

and did not hybridize to the endogenous MLRQ transcript. We added the probe to the prehybridizing filters, and hybridized for 20 h, at 60°C. To wash the filters, we used two changes of 2 \times SSC (20 \times SSC is 3.0 M sodium chloride, 0.3 M sodium citrate) as an initial wash at 26°C, for 5 min each, then washed in 2 \times SSC, 1% SDS under stringent conditions at 60°C, for 30 min. The stringent washing was repeated until the level of background radioactivity was minimal. As a RNA hybridization control, we probed parallel blots with a random primer labeled probe specific for the ubiquitously expressed gene, GADPH.

Results

Isolation and Characterization of 2C- K^{bm3} Allopeptide. Previously, we have shown that K^b , K^{bm3} and K^{bm8} class I molecules all contain a single RP-HPLC fractionated peptide peak that sensitizes T2K bm3 cells to lysis by 2C T cells. We define this as the 2C- K^{bm3} allopeptide fraction. To identify and sequence the 2C- K^{bm3} allopeptide, a T cell cytotoxicity assay (chromium release) was used. As described previously (19), peptide eluates were added exogenously to TAP

Table 1. Edman Sequence Analysis

Peptide name	P1	P2	P3	P4	P5	P6	P7	P8
Endogenous peptide sequence analysis	A, E, G, Q, S ND	Q	Y	E, K	F	Y	Q, S	L, V
Synthetic L library	X	Q	Y	E, K	F	Y	Q, S	L
Synthetic V library	X	Q	Y	E, K	F	Y	Q, S	V
Synthetic peptide sequence analysis	A, D, E, S, T ND	Q	Y	K	F	Y	S	L
		10	10	7	6	4	0.6	0.1

Numbers after the single letter amino acid designation represent the yield (pmol) of each amino acid present in the sequence analysis. No concentration is shown for P1 due to the presence of background peaks commonly observed; A, S, and D. X indicates all 20 amino acids were represented in P1 of the peptide libraries.

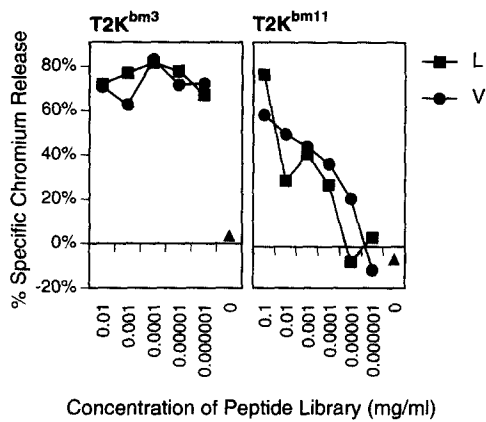


Figure 2. The leucine and valine degenerate libraries work equivalently in sensitizing T2K^{bm3} and T2K^{bm11} cells to lysis by 2C effectors. Targets were T2K^{bm3} (left) and T2K^{bm11} (right) cells, and effectors were 2C T cells from transgenic mice. The E:T was 6:1. Peptides were incubated with targets one h before addition of effector cells. A standard 5-h chromium release assay was used. V indicates valine library, and L indicates leucine library. See Table 1 for complete sequence information.

transporter-deficient target cells (T2) expressing the relevant MHC class I molecule and mixed with 2C effector cells. The ability of the peptides to sensitize the target cells to lysis was then determined. Only when the biologically active peptide was present did 2C T cells lyse the target cells. In repeated runs, we noted that K^{bm8} peptide eluates consistently yielded more 2C-K^{bm3} allopeptide than either K^b or K^{bm3} eluates; the number of cells required to generate detectable peptide was an order of magnitude lower than for K^b or K^{bm3}. Because of this observation, we chose cells

bearing the K^{bm8} molecule as the source for preparative scale isolation of peptides. Peptides from the thymuses of 48 K^{bm8} mice and 2×10^{10} K^{bm8}-bearing cells were eluted, subjected to HPLC separation (acetonitrile/TFA), and subsequently the sensitization assay with T2K^{bm3} cells as targets. Fig. 1 A is an example of one run of the sensitization analysis. The active fractions from each HPLC run were combined and fractionated on a second HPLC buffer system (acetonitrile/HCl). The sensitization assay was again used to identify the biologically active peptide fraction (Fig. 1 B). In both HPLC runs, only one active fraction was present, indicating that there was a single, dominant peptide.

Peptide Sequencing. The active HPLC fraction at 36.5 min was lyophilized and sequenced using Edman degradation. The results are represented in Table 1. Previous studies have indicated that fractions enriched by these procedures typically contain many peptides and often dominant peptides can be visualized by sequence analysis of enriched pools (27). The 2C-K^{bm3} allopeptide fraction had a distinct amino acid composition, and the amino acid assignments at P2, P3, P5, and P6 were unambiguously determined. At P4, P7, and P8, two amino acids were present in equimolar concentrations and definite residue assignments for those amino acid positions could not be determined. The amino acid present at P1 of the peptide was also unclear due to the common background peaks present in the first round of cleavage. To determine if the 2C-K^{bm3} allopeptide was among these peptide possibilities, we made two degenerate synthetic peptide libraries. Table I indicates the composition of the peptide libraries. We tested both libraries in a chromium release assay, and they sensitized T2K^{bm3} cells to

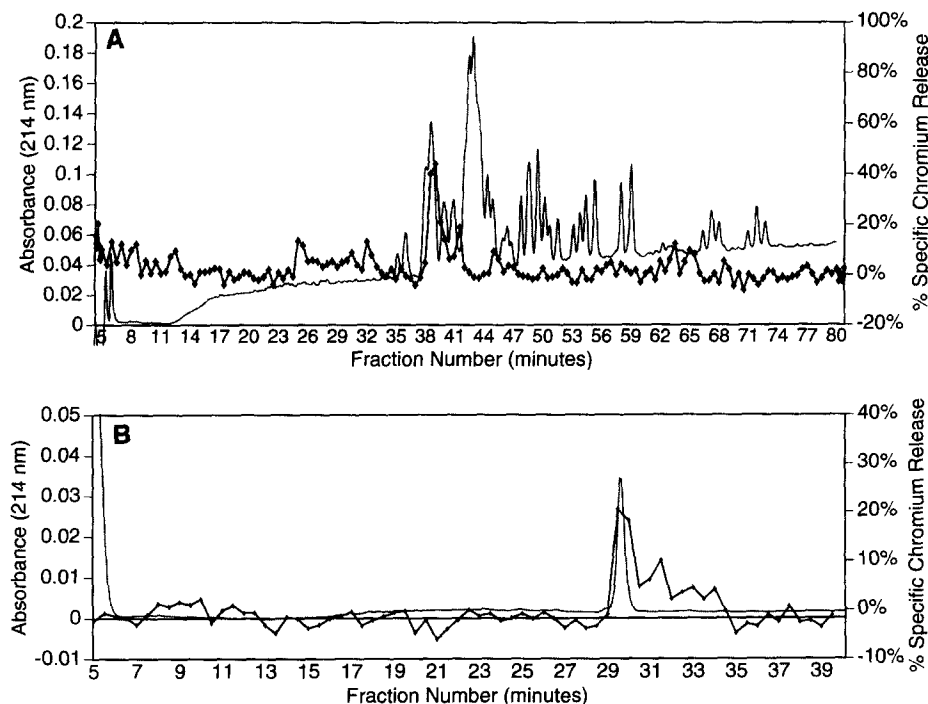


Figure 3. One major active peak is present in the degenerate synthetic peptide leucine gemisch after two rounds of sequential HPLC fractionation. (A) 20 μ g of the degenerate peptide library (leucine at P8) was fractionated using the TFA/acetonitrile buffer system, and 7 μ l of each HPLC fraction were then tested in a chromium release assay as described in Fig. 1 A. (B) The two major active HPLC fractions at 38.5 and 39 min were then run on the second acetonitrile/HCl HPLC buffer system and 7 μ l of each fraction tested in the chromium release assay. The fraction from 39 min is represented in the figure. Identical results were obtained for the fraction at 38.5 min. The continuous line indicates the absorbance at 214 nm, and the solid black line with discrete data points indicates the specific chromium release.

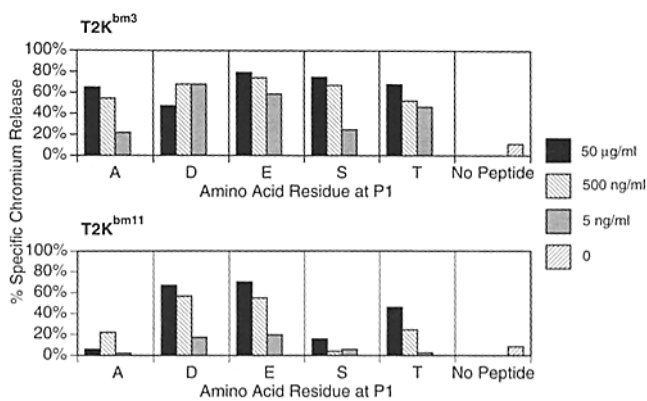


Figure 4. Acidic amino acid residues at P1 are to be more efficient at inducing lysis when presented in the $K^{\text{bm}11}$ molecule. Synthetic peptides containing the sequence QYKFYSL for amino acids at P2-P8, but varying at P1 were titrated in a chromium release assay with either T2K^{bm3} or T2K^{bm11} cells as targets. E:T was 6:1. Peptides were incubated one h with targets before addition of 2C effector cells from transgenic mice. Using the single letter amino acid code, the peptide is indicated by the residue present at P1.

lysis by 2C T cells efficiently and equivalently, indicating that the peptide of interest was a dominant peptide in the sequenced fraction. However, we could not discriminate between the two peptide libraries using 2C T cell recognition of T2K^{bm3} target cells (Fig. 2 A).

Previously, others have shown that the mutant class I molecule, $K^{\text{bm}11}$, is also a ligand for the 2C T cell receptor (21, 28). In these studies, 2C effectors were shown to have lower affinity for the $K^{\text{bm}11}$ molecule. The $K^{\text{bm}3}$ and $K^{\text{bm}11}$ molecules differ by two amino acids. While they have a common mutation at position 77, $K^{\text{bm}3}$ has an additional mutation at amino acid 89, and $K^{\text{bm}11}$ has a mutation at amino acid 80. It is this difference at position 80 which likely results in the difference of affinity in binding the 2C TCR. Assuming that $K^{\text{bm}11}$ presents the same allopeptide as $K^{\text{bm}3}$, we chose to test T2K^{bm11} as targets in the peptide sensitization assay because we anticipated that 2C TCR recognition of $K^{\text{bm}11}$ would be more discriminatory at the peptide level. Fig. 2 B illustrates that even on T2K^{bm11} targets, the two peptide libraries were equally efficient at inducing lysis.

We chose the leucine library to elucidate the definitive 2C- $K^{\text{bm}3}$ allopeptide sequence by biochemical means, because leucine is a more common amino acid residue at position 8 in the K^{b} binding motif (29). We ran the library of 80 degenerate peptides on the initial HPLC acetonitrile/TFA system (Fig. 3 A). One major active peak at 38.5–39.0 min was capable of sensitizing T2K^{bm3} to lysis by 2C effectors. This elution time was also commonly observed in the endogenously isolated allopeptide on the TFA/acetonitrile buffer system. We independently subjected the fractions at 38.5 and 39 min to a second round of HPLC separation (acetonitrile/HCl) and again tested the fractions in a chromium release assay (Fig. 3 B). Similar elution patterns in both absorbance and activity in the chromium release assay

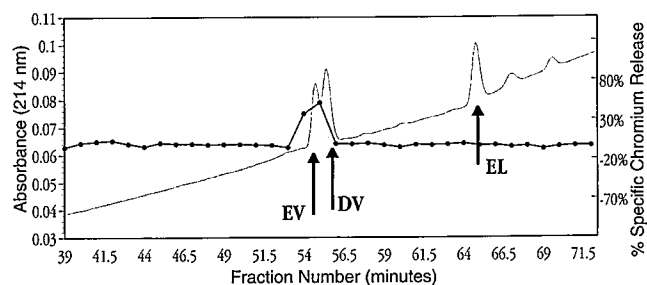


Figure 5. The biologically derived $K^{\text{bm}3}$ allopeptide elutes in the same fraction as the synthetic EV peptide. The third HPLC buffer system (methanol) with high resolving capacity was used to compare the elution of the biologically derived 2C- $K^{\text{bm}3}$ allopeptide to three synthetic peptides-EV, EQYKFYSV; EL, EQYKFYSL, and DV, DQYKFYSV. $K^{\text{bm}3}$ peptides from 1.3×10^9 $K^{\text{bm}3}$ Con A blast cells were eluted and subjected to the third HPLC system. Then, each fraction was tested on T2K^{bm3} cells in the standard chromium release assay (10 μl). 2C T cells from transgenic mice were used at an E:T ratio of 3:1. Solid line indicates the absorbance at 214 nm of the three synthetic peptides. Peptides were run independently and in pairs to identify their elution order before this separation.

were observed for the 38.5 and 39 minute fractions. Because these two fractions were indistinguishable, we chose to sequence the active peptide(s) from the fraction at 39 min by Edman degradation. The sequence analysis of the fraction is represented in Table 1. Mass spectrometry data and capillary electrophoresis independently revealed that there were between 1–4 peptides present in the sequenced HPLC fraction (data not shown). As before, the background in cycle one of the analysis prevented a definitive call for position one of the peptide. It should be noted, though, that the only amino acid residue present in both sequences at position one that was not a background peak was glutamic acid. In addition, the positions 4 and 6 were definitively lysine and serine, respectively. Only two positions remained to be identified, P1 and P8.

Determination of Amino Acid Position 1. Common background peaks present during the Edman sequencing process made the determination of the residue at amino acid P1 difficult. In an attempt to find the biologically relevant peptide, we synthesized all five possibilities that arose in the second sequence determination from the synthetic leucine library. These peptides were defined as AL, DL, EL, SL and TL. We tested the biological activity of all five peptides in our sensitization assay on T2K^{bm3}. Fig. 4 illustrates that all five peptides were effective in sensitizing T2K^{bm3} to lysis by 2C T cells, while the same peptides had no effect on T2L^d or T2K^{bm8} sensitization (data not shown). Fig. 4 also shows that unlike the T2K^{bm3} targets, T2K^{bm11} presented the five peptides to 2C T cells to varying degrees. The two most effective peptides were EL and DL, and the TL peptide also sensitized T2K^{bm11} to lysis but to a lesser extent.

Comparison of the Biologically Derived Peptide to Synthetic Peptide. To further define the 2C- $K^{\text{bm}3}$ allopeptide, we compared the candidate synthetic peptides to the biologically derived peptide. Because we had not conclusively determined the amino acids at position one or eight of the peptide, we ran peptides eluted from $K^{\text{bm}3}$ and $K^{\text{bm}8}$ on a

Table 2. Database Search Results

Peptide name	P1	P2	P3	P4	P5	P6	P7	P8
Synthetic peptide								
sequence analysis	A, D, E, S, T	Q	Y	K	F	Y	S	V, L
Rabbit lipopolysaccharide binding protein	A	Q	Y	K	F	Y	S	L
Murine lipopolysaccharide binding protein	G	Q	Y	E	F	H	S	L
Bovine MLRQ	D	Q	Y	K	F	Y	S	V
Murine MLRQ	E	Q	Y	K	F	Y	S	V

Sequence data for rabbit lbp and bovine mlrq were obtained from GenBank. The accession numbers are M35534 and X64897, respectively. Murine lbp sequence was kindly provided by P. Tobias, Scripps Institute, La Jolla, CA. The murine mlrq sequence was obtained from cDNA generated in our lab from C57BL/6 spleen cells.

HPLC instrument using methanol gradient solvent system and compared the elution time to that of several synthetic peptides. The methanol system was chosen because it provided sufficient resolution of three likely synthetic candidates; EL, DV, and EV. We then compared the retention time of the biologically derived, active peptide to the synthetic retention times. Fig. 5 illustrates that the biologically derived peptide from K^{bm3} eluted at the same retention time as the EV peptide with similar results for peptides derived from K^{bm8} and K^b (data not shown).

Data Base Homologies. We next searched databases for possible protein sources of the 2C-K^{bm3} allopeptide. Two homologous peptides were identified. One peptide was from bovine MLRQ. This protein contained a peptide that matched the 2C-K^{bm3} allopeptide sequence at six out of the eight definitive amino acids and corresponded with the possibilities at P1 and P8 (Table 2). MLRQ, is a nuclear encoded mitochondrial protein that is part of complex I of the NADH ubiquinone complex. Little is known about this protein's function or trafficking (30). The mouse homologue had not been characterized; we therefore cloned and sequenced the cDNA. Fig. 6 reveals a sequence from the mouse that is highly homologous to the bovine sequence and sequences that have been cloned as expressed sequence tags from human cDNA. The murine sequence is similar to both the human and bovine reported sequences, contains a peptide that is consistent with the partial amino acid sequence defined in our peptide eluates, and has the identical sequence as the synthetic peptide that migrates with the biologically active eluted peptides. The second peptide sequence match was from the rabbit lipopolysaccharide binding protein (LBP)¹ (Table 2). We obtained the sequence of the murine homologue of LBP, and unlike the rabbit peptide, it differed from our allopeptide sequence

¹ Abbreviation used in this paper: LBP, lipopolysaccharide binding protein.

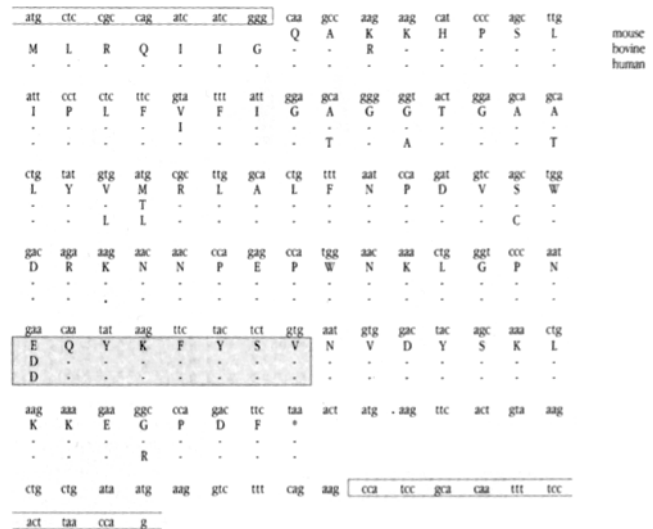


Figure 6. Partial sequence obtained for the mMLRQ protein with comparison to bovine and human homologues. The cDNA sequence of mouse is shown in the top line, flanked by the 5' and 3' primers from the bovine and human cDNA sequence (clear boxed region). The predicted amino acid sequence of mouse MLRQ is compared to the corresponding amino acid sequences from human and bovine. Dashes indicate the same amino acid is present as in the mouse. Gray shaded box region is the sequence that agrees with the 2C-K^{bm3} allopeptide sequence. * Represents a stop codon. • Indicates lack of corresponding amino acid. First seven amino acids were not determined for the mouse. These sequence data for human, bovine, and murine are available from GenBank under accession numbers z36856, x64897, and u59509, respectively.

data at three positions (Table 2) (P. Tobias, personal communication). To verify that this peptide was not a candidate, we synthesized it and tested its biological activity on T2K^{bm3} using 2C effectors (data not shown). Even at concentrations of 0.5 mg/ml the mLBP peptide, GQYEFHSL, did not sensitize T2K^{bm3} cells to lysis by 2C T cells.

Identifying the MLRQ protein as a potential precursor of the 2C-K^{bm3} allopeptide could not be taken as evidence that the biologically active peptide is processed from the protein. Previously, we had shown that the 2C T cell's recognition of K^{bm3} transfected L cells is often sub-optimal, therefore we predicted that by increasing the levels of the allopeptide we may be able to upregulate the recognition of K^{bm3} L cells as targets. To test this possibility we transfected K^{bm3} L cells with murine MLRQ cDNA under the control of the CMV promoter. We identified eleven individual clones that had levels of K^{bm3} expression that were comparable to the levels of expression on the parent K^{bm3} L cell (data not shown). We then tested the clones for enhanced recognition by 2C hybridomas and CTLs (Fig. 7, A and B, respectively). Clones 1, 2, 3, 4, and 8 had the ability to cause increased levels of cytokine production by the 3D9 hybridoma and were lysed more efficiently by the 2C CTL when compared to the parental L cell clone. We then isolated mRNA from the eleven MLRQ cell clones and the parental K^{bm3} L cell and determined the levels of transfected MLRQ mRNAs by Northern blot analysis (data not

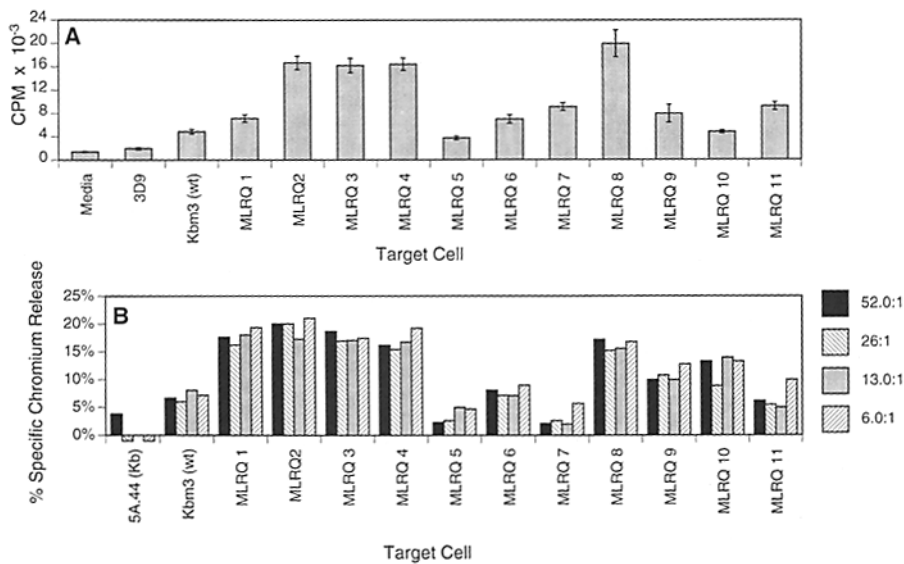


Figure 7. K^{bm3} L cells with over-expressed MLRQ are better targets than wild-type K^{bm3} L cells. K^{bm3} -expressing L cells transfected with MLRQ cDNA were used as targets in a hybridoma assay (A) and a chromium release assay (B) as described in Materials and Methods. Effector to target ratios were 2:1 for the 3D9 hybridoma assay and effector cells for chromium release assay were the original DK 2C clone.

shown). We found a complete correlation between the cells that had enhanced recognition as mentioned above and those that had detectable levels of pRc-CMV/MLRQ mRNA. These data indicate that not only does mMLRQ contain a peptide with identical sequence to the 2C- K^{bm3} allopeptide, but that this peptide can be processed from MLRQ and presented by the K^{bm3} class I molecule to 2C T cells. The sum of these results supports the hypothesis that the peptide, EQYKFYSV, is the 2C- K^{bm3} allopeptide, henceforth known as dEV-8.

Discussion

In recent years, the techniques of peptide isolation and the understanding of T cell recognition have allowed a detailed view of allorecognition at the molecular level. Clearly, there are a variety of possible TCR and MHC/peptide complex interactions in allorecognition. This study describes an additional aspect; the ability of a single TCR to recognize two very different MHC/peptide structures with high specificity. The 2C T cell is a highly studied cytotoxic T cell, whose allopeptide in the L^d molecule was identified previously (31). We have determined a second allopeptide, designated dEV-8, for the 2C TCR that is presented in the K^{bm3} molecule. The dEV-8 sequence is distinct from P2Ca, and the recognition of dEV-8 by the 2C TCR is specific to the K^{bm3} molecule. How a single TCR can interact effectively with two very different MHC/peptide complexes is an interesting question. One possible explanation for this cross recognition is that there is a one to one correspondence between conserved residues of K^{bm3} and L^d , and these amino acid residues are contact points for the 2C T cell receptor. However, another possibility is that the 2C TCR contacts different residues on K^{bm3} and L^d , and cross recognition is simply a result of the T cell receptor's inherent ability to associate with an MHC molecule (32). T cells are presumably selected to have moderate affinity/

avidity for the MHC molecule structure (33, 34), and therefore it may not be surprising that more than one peptide may provide the added contacts to increase the affinity of the TCR for any given MHC molecule to the point of antigenic recognition. Information about the orientation of the TCR on these class I molecules will come from structural studies using site directed mutagenesis and x-ray crystallography. No matter which of these scenarios causes the cross recognition, understanding this phenomenon may explain the high frequency of alloreactive T cells.

Even though 2C TCR recognition is degenerate regarding its two alloantigens, it appears to be very specific for the peptides within a given MHC molecule. Only a single HPLC peptide fraction from K^{bm3} or K^{bm8} class I molecules was observed in the highly sensitive cytotoxicity assay, even when peptides from 1×10^{10} cells were analyzed. A similar circumstance was seen for the L^d allopeptide, where only three active HPLC fractions were present, and one of the peaks was simply a longer precursor of P2Ca (35). The identity of the third peak is unknown. In both systems, a discrete number of peptides from the entire repertoire of isolated peptides were identified for activity in their respective class I molecules. Further support for the 2C TCR's fidelity was observed when an analogue of P2Ca known as P2Ca-Y4 was identified. P2Ca-Y4 differs from P2Ca at P4, where a tyrosine replaces a phenylalanine, and yet the 2C TCR can distinguish between these two similar peptides in the context of K^b (36). Similarly, the mLBP peptide with homology to dEV-8 was an ineffective allopeptide when bound to K^{bm3} , providing more evidence that although 2C TCR has degenerate recognition of two class I molecules, the peptide recognition is extremely specific.

We are confident that dEV-8 is at least one 2C- K^{bm3} allopeptide for several reasons. The sequence of dEV-8 agrees with the predicted K^b motifs; Y or F at P5, Y at P3 and a hydrophobic residue at P8. Even though we derived the sequence from the K^{bm8} bound peptides, we have pre-

viously shown that a single HPLC fraction from K^b , K^{bm3} and K^{bm8} eluates contained the 2C- K^{bm3} allopeptide (19) demonstrating that the same peptide species can be found in K^{bm3} . This peak eluted in identical positions on two very different HPLC systems, and the peptide we have identified co-migrates with the biologically isolated species. Finally, identifying a potential protein precursor, MLRQ, which when expressed in cells bearing the K^{bm3} class I molecule can up-regulate recognition by 2C cells also adds support that this sequence is the endogenous peptide. While all this data favors the hypothesis that MLRQ is the dEV-8 peptide source, it is still possible that more than one protein contains the same peptide or a similar peptide capable of stimulating 2C T cells. This possibility is accentuated by the fact that MLRQ is a member of a multi-gene family in the mouse (data not shown).

It is interesting that P2Ca and dEV-8 are derived from mitochondrial proteins. While this may be a coincidence, it may be significant that a third allopeptide, ND-1, for a non-classical class I molecule is also mitochondrially derived (37). Because three of the identified allopeptides have a mitochondrial origin, there is a possibility that organelles such as mitochondria play a predominant role in providing polymorphic peptides to the class I presentation pathway, though this is not evident from peptide elution studies (38, 39). Another possibility is that these peptides have been identified because they are abundant, ubiquitous, and/or have high turnover rates. Only further investigation will determine if there is a unique aspect to mitochondrial allopeptides.

The finding that a self peptide that is present in the thymus is also an allopeptide when presented in a mutant molecule suggests that there may be a relationship between mutant recognition and thymic selection. K^{bm3} and K^b differ by only two amino acid positions, 77 and 89. According to crystal structure data and mutational analysis, amino acid position 89 plays no role in peptide binding or T cell recognition (15, 21, 40). If dEV-8 is bound to both K^b and K^{bm3} and seen by the 2C TCR, the amino acid at position

77 is the residue responsible for the difference in recognition. Assuming that K^b and K^{bm3} bind dEV-8 with similar affinities and that the affinity/avidity model of thymic selection pertains to 2C thymocytes, one would predict that the 2C TCR would have a higher affinity for K^{bm3} than K^b when dEV-8 is bound. Thymocytes of 2C transgenic mice are negatively selected in the presence of K^{bm3} , but positively selected in the presence of K^b . If the affinity of the thymocytes for K^b is increased by addition of a CD8 transgene, then 2C thymocytes become negatively selected (41, 42). This data suggests that K^b may bind the same peptide as K^{bm3} but that the affinity of the 2C TCR for K^b under normal conditions only elicits positive selection. Currently, 2C TCR affinity information is available for P2Ca bound in both L^d and K^b (43, 44), but there is no data for 2C TCR affinity for K^b with dEV-8 bound. This affinity information, in addition to thymic organ culture studies, may reveal if dEV-8 plays a role in selecting 2C thymocytes when bound to K^b and if it is solely responsible for the selection of 2C thymocytes. While only one allopeptide peak was present in the HPLC separation of the eluted peptides, selection of 2C thymocytes may rely on a group of peptides being presented for positive selection. A sum of low affinity interactions totaling a moderate avidity would also fit the current models of positive selection. Therefore, dEV-8 may be one of many peptides bound in K^b capable of selecting 2C thymocytes. This possibility can only be addressed by creating a mouse deficient in MLRQ.

We have illustrated a unique system where one T cell receptor sees two distinct MHC/peptide complexes as antigens. This system demonstrates the versatility of a single TCR to recognize two different MHC molecules, but maintain its peptide specificity. If it can be assumed that all TCRs possess a similar ability to cross react with multiple MHC/peptide complexes, this may be one explanation for the high frequency of alloreactive T cells. In addition, because the allopeptide is also a ubiquitous self peptide, this system may prove to be a useful tool to address questions regarding positive selection, tolerance, and autoimmunity.

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