

Major Histocompatibility Complex Conformational Epitopes Are Peptide Specific

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Summary

Serologically distinct forms of H-2K^b are stabilized by loading cells expressing "empty" class I major histocompatibility complex (MHC) molecules with different H-2K^b binding peptides. The H-2K^b epitope recognized by monoclonal antibody (mAb) 28.8.6 was stabilized by ovalbumin (OVA) (257-264) and murine cytomegalovirus (MCMV) pp89 (168-176) peptides, but not by vesicular stomatic virus nucleoprotein (VSV NP) (52-59) and influenza NP (Y345-360) peptides. The H-2K^b epitope recognized by mAb 34.4.20 was stabilized by VSV NP (52-59) peptide but not by OVA (257-264), MCMV pp89 (168-176), or influenza NP (Y345-360) peptides. Immunoprecipitation of H-2K^b molecules from normal cells showed that 28.8.6 and 34.4.20 epitopes were only present on a subset of all conformationally reactive H-2K^b molecules. Using alanine-substituted derivatives of the VSV peptide, the 28.8.6 epitope was completely stabilized by substitution of the first residue and partially stabilized by substitution of the third or the fifth residues in the peptides. These results indicate that distinct conformational MHC epitopes are dependent on the specific peptide that occupies the antigenic peptide binding groove on individual MHC molecules. The changes in MHC epitopes observed may also be important in understanding the diversity of T cell receptors used in an immune response and the influence of peptides on development of the T cell repertoire.

Class I MHC molecules are polymorphic cell surface antigens with central roles in T cell-mediated immune responses (for review see reference 1). Stable class I MHC expression requires three components, a 45-kD polymorphic H chain, a nonpolymorphic L chain (β_2 -microglobulin), and a peptide bound to the antigenic peptide binding groove in the H chain. Currently, evidence indicates that nascent class I MHC molecules associate with β_2 -microglobulin (2-4) and antigenic peptide fragments (5-7) in the endoplasmic reticulum (ER)¹. This association is required for stable cell surface expression. By studying cells that have global defects in class I MHC expression, such as the murine RMA-S and the human B cell line .174 (8, 9), investigators have identified novel genes involved in antigen processing that regulate cell surface expression of class I MHC molecules (10-14). Under normal culture conditions these cells do not properly process antigen and fail to load their endogenous nascent class I MHC molecules with antigenic peptide fragments. Because of this

defect, these cells do not express normal levels of cell surface MHC class I molecules. However, "empty" MHC molecules are expressed on the surface of RMA-S cells when these cells are cultured between 20 and 28°C (15). Although these molecules are stable at lower temperatures, they rapidly disassociate or degrade at 37°C. Empty class I MHC molecules can be stabilized by the addition of antigenic peptides (15) or anti-class I MHC antibodies (16).

In the present investigation, we found that serologically distinct conformational epitopes on the class I MHC H-2K^b molecule were stabilized by loading these cells with different H-2K^b binding peptides. Although many determinants recognized by mAbs specific for H-2K^b molecules were reactive regardless of which antigenic peptide bound to H-2K^b, two $\alpha 2$ -specific epitopes recognized by mAbs 28.8.6 and 34.4.20 were dependent on the specific peptide bound to H-2K^b. An analysis of the $\alpha 2$ epitope recognized by mAb 28.8.6 indicates that specific amino acid residues at positions 1, 3, and 5 in the H-2K^b binding peptide control stabilization of that epitope. These results indicate that distinct H-2K^b binding antigenic peptides stabilize different conformational epitopes on H-2K^b. Changes in conformational

¹ Abbreviations used in this paper: ER, endoplasmic reticulum; MCMV, murine cytomegalovirus; NP, nucleoprotein; VSV, vesicular stomatic virus.

MHC epitopes, as detected serologically, may also be important in TCR recognition of a particular MHC molecule and may partially explain the diversity of TCRs used in responding to specific MHC antigens.

Materials and Methods

Cells and Culture Conditions. RMA, RMA-S, E3 K^b, and DAP K^b cells were maintained by 1:20 passage three times weekly in RPMI-1640 supplemented with 2 mM glutamine, nonessential amino acids, 50 µg/ml of gentamicin, 5 × 10⁻⁵ M 2-ME, and 10% FCS.

Cell Transfections. L cells expressing H-2K^b were previously described (17). EE2H3 cells were transfected by routine calcium phosphate DNA-mediated transfection. For high levels of expression, EE2H3 cells were transfected with a cDNA construct encoding H-2K^b driven by the human ubiquitin promoter (18). Transformants were selected for growth resistance in G418 (1 mg/ml, Gibco Laboratories, Grand Island, NY) from the G418-sensitive parental cell line and then screened by flow microfluorometric analysis for expression of H-2K^b.

Flow Microfluorometry Analysis. Approximately 10–20 × 10⁵ cells were incubated for 60 min at 4°C with 50 µl of indicated purified mAb diluted to 10 µg/ml final concentration or 100–200 µl of the specified mAb culture supernatants. Cells were washed twice in PBS and then incubated for an additional 40 min at 4°C in 50 µl of 1/40 dilution of fluorescent F(ab')₂ goat anti-mouse IgG (Cappel Laboratories, Cochranville, PA). Cells were then washed two additional times with PBS before analysis on an Epics Profile flow cytometer (Coulter Corp., Hialeah, FL).

mAbs. mAbs were either used as culture supernatants or further purified by protein A-Sepharose chromatography. See Table 1 for specificity of different mAbs used to recognize either α1 or α2 serological epitopes.

Peptide Loading of Cells. Cells (RMA, RMA-S, or E3 K^b) were incubated at either 25°C overnight or at 37°C for peptide-loading experiments. Cells that were maintained at 37°C were either incubated overnight in the presence of various antigenic peptides (100 µM final concentration) or in the absence of any known antigenic peptides. The following morning cells were harvested and processed for flow microfluorometry analysis. Cells that were maintained overnight at 25°C were incubated with antigenic peptides (100 µM) in the morning for 2 h and then incubated for an additional 2 h at 37°C. Cells were then harvested and processed for flow microfluorometry analysis as described.

All peptides except murine cytomegalovirus (MCMV) pp89 were made by the Johns Hopkins University biopolymer laboratory peptide synthesis facility. Peptides were made by F-moc chemical synthesis and then purified by preparative HPLC. Sequences were confirmed by amino acid analysis and protein sequencing. The MCMV pp89 peptide was the generous gift of Dr. David Margulies (National Institutes of Health).

Immunoprecipitation. For labeling of cells, 10⁸ cells were incubated at 37°C for 40 min in 2 ml methionine-free RPMI-1640 supplemented with 5% dialyzed FCS. [³⁵S]Methionine (0.5 mCi) was added for 15 min. Labeling was stopped by the addition of 2 mM cold methionine at 37°C. After incubation for an additional 2 h, the cells were harvested and 20 ml ice-cold PBS was added. The cells in PBS were centrifuged at 4°C and the pellet lysed in 1 ml lysis buffer (0.5% NP-40, 20 mM Tris, pH 8.0, 10 mM EDTA, pH 8.0, 0.1 M NaCl, and 1 mM PMSF). After 30 min on ice, the nuclei were sedimented at 14,000 rpm for 5 min at 4°C. Ly-

Table 1. mAb Specificity

Antibody	MHC specificity	Domain specificity	Reference no.
20.8.4	H-2K ^b	α1	(31)
B8.24.3	H-2K ^b	α1	(32, 33)
28.8.6	H-2K ^b	α2	(17, 31)
34.4.20	H-2K ^b	α2	(21, 32)
5F1	H-2K ^b	α2	(32, 33)
Y3	H-2K ^b	α1/α2	(32, 33)
28.14.8	H-2D ^b	α3	(34)

Summary of the reactivities of different monoclonal anti-MHC antibodies used in these experiments. The epitope recognized by Y-3 was not clearly mapped to either the α1 or the α2 domains of H-2K^b. Recently this antibody has been more definitively mapped to the α2 domain.

sates were precleared with 50 µl of protein A-Sepharose overnight. Protein A-Sepharose (50 µl) was incubated with various mAbs, 10 µg of purified 34.4.20, 28.8.6, or 20.8.4, at 4°C overnight, and then free antibody was washed away. Cell lysates were incubated with the protein A-Sepharose-antibody complex for 1 h at 4°C. Protein A-Sepharose-antibody beads were then washed at 4°C twice with 0.5% NP-40, 20 mM Tris (pH 8.0), 10 mM EDTA, 0.1 M NaCl, 0.1% SDS, 1% BSA, once with 10% lysis buffer in 0.5 M NaCl, and once in lysis buffer. They were then resuspended in sample buffer (50 µl) heated to 100°C for 3 min and centrifuged. Samples were analyzed by 12% SDS-PAGE.

Results

Culturing RMA-S cells at 25°C overnight is known to enhance expression of empty class I MHC molecules (15). In our system, flow microfluorometric analysis of cells cultured at 25°C showed 2.5–3.5-fold increases in expression of conformationally intact H-2K^b molecules (Fig. 1). These empty H-2K^b molecules were reactive with the entire set of two α1-reactive anti-H-2K^b specific mAbs, 20.8.4 and B8.24.3, and four α2-reactive anti-H-2K^b specific mAbs, 5F1, 28.8.6, 34.4.20, and Y-3 (data not shown for Y-3).

To study epitopes expressed on peptide-stabilized class I MHC molecules, we analyzed the ability of four H-2K^b binding peptides to stabilize empty H-2K^b molecules expressed on RMA-S cells (Table 2). Three of the peptides used were previously reported to bind to H-2K^b, vesicular stomatitis virus nucleoprotein (VSV NP) (52-59), OVA (257-264), and influenza NP (Y345-360). The fourth peptide, MCMV pp89 (168-176) that was previously described as an H-2L^d binding peptide, also bound to empty cell surface H-2K^b molecules (Fig. 2). H-2K^b binding was analyzed by adding peptides to RMA-S cells that had been cultured overnight at 25°C. After allowing the peptide to bind and stabilize the cell surface MHC molecules at 25°C, cells were cultured for an additional 2 h at 37°C to degrade any remaining empty H-2K^b molecules. Compared to cells treated with either a control peptide, influenza NP (365-380), or no pep-

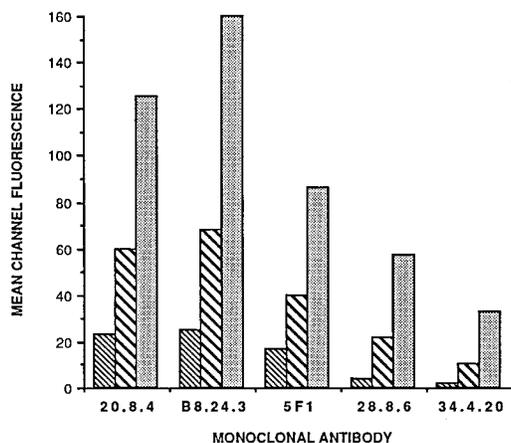


Figure 1. H-2K^b molecules expressed at 25°C are reactive with all H-2K^b-specific mAbs. RMA-S cells were incubated overnight at 25°C in a 5% CO₂ incubator. The next morning cells were either moved for two additional hours of incubation at 37°C (▨) or maintained at 25°C (▤) until they were all harvested and stained with different mAbs. RMA cells (▤) were simply maintained overnight at 37°C in a 5% CO₂ incubator and harvested immediately before staining with the different mAbs. Flow analysis was performed using an Epics fluorometer. Routinely, 5,000 cells were analyzed per histogram. Data are presented as mean channel fluorescence on a logarithmic fluorescence scale. Data presented are from a representative experiment that has been repeated at least three times.

tide at all, cells loaded with the H-2K^b binding peptides, VSV NP (52-59) OVA (257-264), influenza NP (Y345-360), and MCMV pp89 (168-176), all showed 2.5–4-fold increases in cell surface-expressed H-2K^b conformational epitopes as recognized by the α1-reactive antibodies (Fig. 2 A). The α1-reactive mAbs 20.8.4 and B8.24.3 were not differentially reactive with RMA-S cells treated with any of the four H-2K^b binding peptides. Stabilization of the H-2K^b molecule was peptide specific in that both a peptide that specifically bind to H-2D^b, influenza NP (365-380) (Fig. 2 A) and a randomly chosen 12 amino acid-long peptide (data not shown) did not stabilize cell surface expression of H-2K^b. Treatment of RMA-S cells with influenza NP (365-380) peptide increased H-2D^b reactivity twofold as seen by the H-2D^b-reactive mAb 28.14.8 (data not shown).

It is interesting that not all the α2 epitopes on H-2K^b were stabilized by each of the H-2K^b binding peptides. Whereas the epitope recognized by mAb 28.8.6 was stabi-

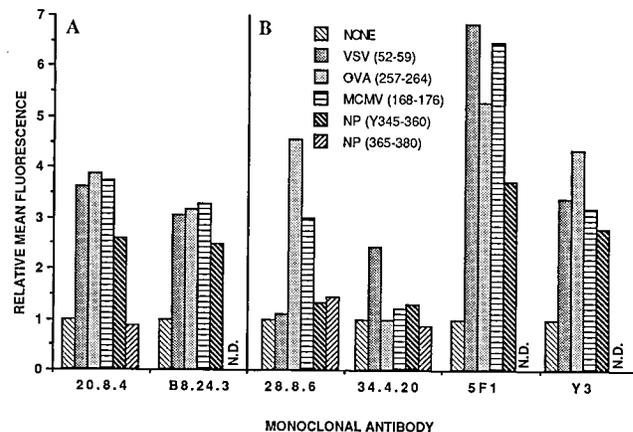


Figure 2. Cell surface expression of H-2K^b epitopes on RMA-S cells treated with several H-2K^b binding peptides. RMA-S cells were incubated overnight in a 5% CO₂ incubator at 25°C. The next morning cells were pulsed either with peptides (100 μM final concentration) derived from: VSV (52-59), (▀); OVA (257-264), (▥); MCMV pp89 (168-176), (▧); or influenza NP (Y345-360), (▨); or influenza NP (365-380), (▩); for 1 h at 25°C, or without any added peptides (▤). Cells were then incubated for an additional 2 h at 37°C. At the end of the incubation period, cells were harvested and stained with various α1-reactive mAbs (A), or α2-reactive mAbs (B). Data are presented as relative mean channel fluorescence, where the reactivity with each of the mAbs has been normalized to untreated cells. The mean channel fluorescence of the various antibodies for untreated RMA-S cells in the experiment shown are: 20.8.4, 36.2; B8.24.3, 31.3; 28.8.6, 9; 34.4.20, 7; Y3, 24.9; and 5F1, 11. Data presented are representative of three experiments.

lized by OVA (257-264) and MCMV pp89(168-176) peptides, H-2K^b binding peptides derived from VSV (52-59) and influenza NP (Y345-360) did not stabilize the 28.8.6 epitope (Fig. 2 B). The effects of the different peptides on the epitope recognized by mAb 28.8.6 was also seen when RMA-S cells were incubated with the H-2K^b binding peptides overnight at 37°C (data not shown). H-2K^b binding peptides derived from OVA (257-264) and MCMV pp89(168-176) increased reactivity with mAb 28.8.6 3.5–4.5-fold. Mean channel fluorescence increased from 9 to 30 for MCMV pp89(168-176) treated and to 41 for OVA (257-264) treated cells.

The H-2K^b epitope recognized by the α2-reactive mAb 34.4.20 was also peptide specific. In this case, while the VSV (52-59) peptide stabilized the epitope seen by 34.4.20, peptides derived from OVA (257-264), MCMV (168-176), and

Table 2. MHC Binding Peptides

Peptide source	Known restriction element	Sequence	Reference no.
1. VSV NP (52-59)	H-2K ^b	RGVYVYQGL	(35)
2. OVA (257-264)	H-2K ^b	SIINFEKL	(25, 36)
3. Influenza NP (Y-345-360) ('68)	H-2K ^b	YSFIRGTKVSPRGKLSL	(37)
4. MCMVpp89 (168-176)	H-2L ^d /H-2K ^b	YPHFMPNTNL	(38, and this report)
5. Influenza NP (365-380)	H-2D ^b	IASNENMETMESSTLE	(39)

influenza NP (Y345-360) did not stabilize the epitope recognized by mAb 34.4.20 (Fig. 2 B). The VSV (52-59) peptide increased 34.4.20 reactivity 2.5-fold.

Other $\alpha 2$ -specific epitopes on H-2K^b were not dependent on the specific H-2K^b binding peptide resident in the H-2K^b molecule (Fig. 2). The $\alpha 2$ -specific epitopes recognized by antibodies 5F1 and Y3 were stabilized by all four H-2K^b binding peptides tested. H-2K^b binding peptides increased reactivity with mAb 5F1 4–7-fold and increased reactivity with mAb Y3 3–5-fold. To summarize, the VSV (52-59) peptide stabilized all H-2K^b epitopes except the epitope recognized by mAb 28.8.6. The OVA (257-264) and MCMV (168-176) peptides stabilized the epitopes recognized by all mAbs except the epitope recognized by 34.4.20. The influenza NP (Y345-360) peptide stabilized all the epitopes except the 28.8.6 and 34.4.20 epitopes.

To determine if differential stabilization of H-2K^b epitopes is a general feature of class I-deficient cells or unique to RMA-S cells, the effects of peptide binding on class I MHC expression in EE2H3 embryonic cells (19) which also express empty cell surface class I MHC molecules (20) were determined. For these experiments, EE2H3 cells, which are from an H-2K^k mouse, were transfected with an expression vector encoding H-2K^b (E3 K^b cells). As seen with the RMA-S cells, the H-2K^b epitope recognized by mAb 28.8.6 was stabilized by both OVA (257-264) and MCMV pp89 (168-176) peptides, but not by VSV (52-59) and influenza NP (Y345-360) peptides (Fig. 3). Mean channel fluorescence increased 2.5–4.5-fold for cells treated with MCMV pp89 (168-176) and OVA (257-264) peptides, respectively. The epitope recognized by 34.4.20 was also only stabilized by VSV (52-59) peptide and

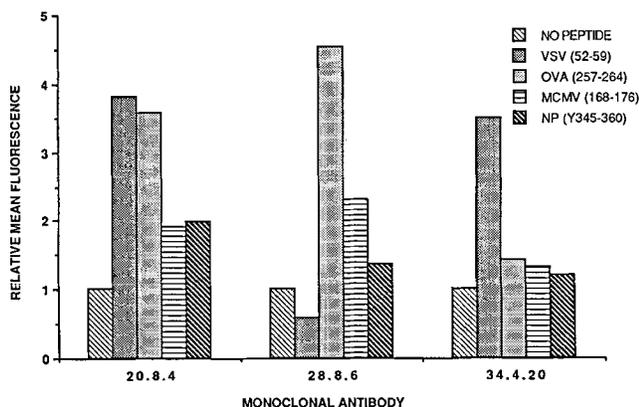


Figure 3. Stabilization of cell surface expression of H-2K^b epitopes on E-3K^b cells by H-2K^b binding peptides. E-3K^b cells were incubated overnight in a 5% CO₂ incubator at 25°C. The next morning cells were pulsed either with peptides (100 μ M final concentration) derived from: VSV (52-59), (▨); OVA (257-264), (▤); MCMV (168-176), (▥); or influenza NP (Y345-360), (▧) for 1 h at 25°C, or without any added peptides (▩). Cells were then incubated for an additional 2 h at 37°C. At the end of the incubation period, cells were then harvested and prepared for staining with various mAbs. Data are presented as relative mean channel fluorescence. Mean channel fluorescence of the various antibodies for untreated E-3K^b cells in the experiment shown are: 28.8.6, 16.4; 34.4.20, 10.5; and 20.8.4, 22.6.

not by OVA (257-264), MCMV pp89 (168-176), or influenza NP (Y345-360) peptides (Fig. 3). Since mAb 28.8.6 cross-reacts with H-2D^b which is present on RMA-S cells, analysis of the E3 K^b cells also confirmed that the increased reactivity of mAb 28.8.6 for peptide-stabilized MHC on RMA-S cells was due to binding to H-2K^b and not to H-2D^b.

Although antigenic peptide loading of H-2K^b in both RMA-S and E-3K^b cells established that serologically distinct forms of H-2K^b are peptide specific when empty cell surface H-2K^b molecules are loaded with peptides, we wanted to determine if serologically distinct forms of H-2K^b exist in normal cells that do not have defective class I MHC expression. To assess this possibility, sequential immunoprecipitations were used to determine the relative amounts of H-2K^b immunoprecipitated by various anti-H-2K^b-specific mAbs. Because of the crossreactivity of the H-2K^b mAbs 28.8.6 for H-2D^b and 20.8.4 for Qa2, H-2K^b was precipitated from L cells transfected with H-2K^b. These mAbs only recognize H-2K^b on the transfected L cells (17), and all have comparable avidities for H-2K^b (B. Catipovic, unpublished data). mAb 28.8.6 precipitated large amounts of H-2K^b from labeled H-2K^b transfected L cells (Fig. 4). Two rounds of immunoprecipitation depleted the 28.8.6-reactive material from the cell lysates (Fig. 4 A, lanes A–C). Even after three rounds of immunoprecipitation of the 28.8.6-reactive material, significant amounts of 20.8.4-reactive H-2K^b remained in the lysates (Fig. 4 A, lane D). Whereas significantly less material was immunoprecipitated by mAb 34.4.20 (Fig. 4 B, lanes A–C), immunoprecipitation with 34.4.20 produced essentially similar findings. Although two rounds of immunoprecipitation depleted all the 34.4.20-reactive material, significant amounts of 20.8.4-reactive material remained in cell lysates (Fig. 4 B, lane D). Thus both anti-H-2K^b-specific mAb 28.8.6 and 34.4.20 precipitated subsets of the total cellular conformationally intact H-2K^b molecules.

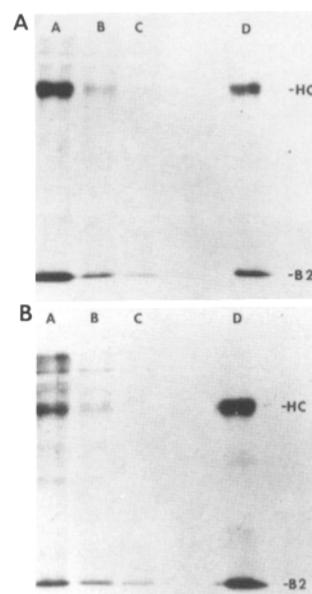


Figure 4. H-2K^b epitopes recognized by mAbs 28.8.6 and 34.4.20 are present on a subset of all H-2K^b molecules. H-2K^b expressing L cells were processed as described and immunoprecipitated with Sepharose coupled to mAb 28.8.6 (A, lanes A–C) or mAb 34.4.20 (B, lanes A–C). Lysates were initially precleared with one round of protein A-Sepharose (50 μ l). After preclearing, lysates were immunoprecipitated with three rounds of the mAb bound to Sepharose (lanes A–C). Note that almost all the 28.8.6- (A, lane c) or 34.4.20- (B, lane c) reactive material was depleted by the third round of immunoprecipitation. Lysates were then precipitated with mAb 20.8.4 bound to Sepharose (A and B, lane d). The positions of H chain and $\beta 2$ microglobulin are indicated on the side.

The influence of individual residues of the H-2K^b binding peptides on peptide-specific stabilization of H-2K^b epitopes was analyzed using alanine substituted VSV (52-59) peptides to stabilize empty cell surface H-2K^b molecules. All of the alanine-substituted VSV (52-59) peptides stabilized the epitope recognized by mAb 20.8.4 (Fig. 5). Only alanine substituted at position 8 (VSV [A8]) significantly decreased reactivity with 20.8.4. The inability of the wild type VSV (52-59) peptide to stabilize the H-2K^b epitope recognized by 28.8.6 was completely reversed by an alanine substitution at the first position in the VSV peptide (VSV [A1]) (Fig. 5). Relative mean fluorescence increased more than fourfold. Reactivity of cells treated with VSV (A1) was comparable to the reactivity seen with OVA (257-264) treated cells. Alanine substitution at positions three and five of the peptide also partially stabilized the epitope recognized by mAb 28.8.6. In three independent experiments, reactivity with 28.8.6 increased 1.5–1.75-fold when H-2K^b was stabilized with VSV (A3), and increased 1.9–2.5-fold when H-2K^b was stabilized with VSV (A5). None of the other alanine substitutions significantly increased reactivity with mAb 28.8.6.

The epitope recognized by mAb 34.4.20, which ordinarily is stabilized by treatment with wild type VSV (52-59) peptide, was not very sensitive to the alanine substitutions of the VSV peptide. Only partial loss of the 34.4.20 epitope was seen in cells treated with VSV (A5).

Discussion

This and a companion paper (20a) are the first demonstrations that distinct conformational MHC epitopes are dependent on the antigenic peptide that resides in the peptide binding groove of the MHC molecule. The experiments presented in this manuscript show that when empty, H-2K^b molecules are loaded with peptides, H-2K^b epitopes associated with the

α 2 helix identified by mAbs 28.8.6 and 34.4.20 are stabilized by certain peptides, but not by all H-2K^b binding peptides. Specifically, the epitope recognized by 28.8.6 was not stabilized by peptides derived from VSV (52-59) or influenza NP (Y345-360), and the epitope recognized by 34.4.20 was not stabilized by OVA (257-264), MCMV pp89 (168-176), or influenza NP (Y345-360) peptides. The influence of peptides on conformational MHC epitopes is not only seen with empty class I molecules loaded exogenously with peptides, but may also occur in MHC molecules expressed on normal cells. Evidence for this comes from our finding that the epitopes recognized by mAbs 28.8.6 and 34.4.20 are only present on subsets of all conformationally intact H-2K^b molecules in cells that express normal levels of class I MHC molecules.

It has previously been shown that MHC determinants expressed on erythrocytes and blastocysts are serologically distinct from the same MHC determinants expressed on lymphocytes (21). In retrospect, this may reflect a different spectrum of peptide-specific MHC determinants expressed by blastocysts and by erythrocytes than those found on lymphocytes that were used to make the mAbs. It is interesting that it was also the α 2 epitopes that were missing from the MHC molecules expressed by the erythrocytes.

A likely explanation of our data is that class I MHC molecule conformations are peptide dependent. Empty MHC molecules, which are reactive with all mAbs tested, are conformationally unstable. Presumably, these empty molecules exist in a temperature-dependent floppy configuration. In this case, MHC molecules can explore conformational states and can be trapped in a specific conformational state by mAbs (16). The recent three-dimensional x-ray crystallography structure of H-2K^b cocrystallized with two different peptides shows that small but significant differences in the H-2K^b class I MHC molecule were seen when crystallized with the two different peptides (22). Our results indicate that when antigenic peptides bind to either empty cell surface MHC molecules or newly formed MHC molecules in the ER, the peptide may also fix the MHC molecule in one of several serologically distinct configurations. Our strongest support for this hypothesis is from the effects of alanine substitution at position 5 in the VSV peptide on stabilization of the epitope recognized by 28.8.6. Both positions 3 and 5 are anchor residues that interact directly with amino acid residues in the MHC molecule. Position 5 in the VSV peptide is a buried residue whose side chain faces down and is not exposed to solvent (22). It is unlikely that position 5 in the VSV peptide can directly interact with the surface of the MHC molecule. Thus the influence of position 5 and in the VSV peptide on 28.8.6 reactivity is probably not secondary to steric interactions between that position in the peptide and the mAb. These changes may be indicative of conformational changes in the MHC molecule.

Although we favor the hypothesis that the differences seen in the serological reactivity with different H-2K^b binding peptides indicates a conformational change in the MHC molecule, there are other interpretations of the data. An alternative hypothesis is that individual antigenic peptides, when bound to MHC molecules, can obscure specific mAb reac-

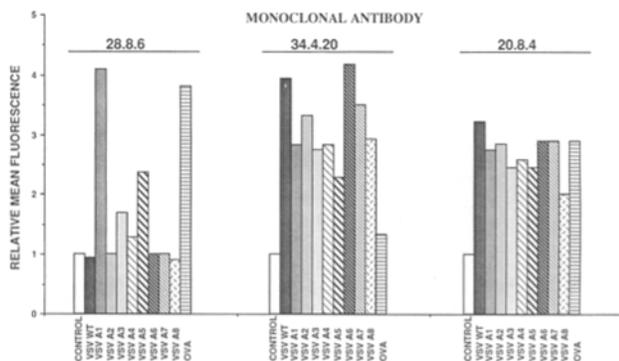


Figure 5. Effects of alanine substituted VSV peptides on stabilization of H-2K^b epitopes. E-3K^b cells were incubated overnight in a 5% CO₂ incubator at 25°C. The next morning cells were pulsed either with wild type VSV, VSV-substituted peptides, OVA (257-64) peptide (all at 100 μ M final concentration), or without any added peptides. Cells were then incubated for an additional 2 h at 37°C. At the end of the incubation period, cells were then harvested and prepared for staining with various mAbs. Data are presented as relative mean channel fluorescence. Mean channel fluorescence of the various antibodies for untreated RMA-S cells in the experiment shown are: 28.8.6, 17.8; 34.4.20, 12; and 20.8.4, 31.

tivities. The strongest argument for this idea is from the finding that all the serological epitopes are expressed on conformationally unstable empty H-2K^b molecules. Thus one might argue that when the antigenic peptide derived from VSV NP (52-59) bound to the H-2K^b molecule, it obscured the epitope recognized by 28.8.6 but not the epitope seen by 34.4.20. The three-dimensional x-ray crystallographic data indicate that the nonamer peptide is embedded in the antigenic peptide binding groove and that the peptide backbone is thought to lie deep in the antigenic peptide binding groove (23, 24). Thus it seems unlikely that nonamer peptides could entirely mask serological epitopes that have been mapped to polymorphisms on the surface of the MHC helical regions (23). However, peptide side chains could still interact with the surface of the MHC molecule (24) and interfere with access to mAb binding sites. The effect of an alanine for arginine substitution at position 1 of the VSV (52-59) peptide seen in analysis of the H-2K^b epitope recognized by mAb 28.8.6 could be due to steric inhibition. This H-2K^b epitope is known to map to the terminal portion of the α helix of the $\alpha 2$ domain (17). Alteration of amino acid positions 167 or 173 of H-2K^b completely abolishes the 28.8.6 epitope on H-2K^b (J. P. Schneck, unpublished data). Based on the crystal structure of antigenic peptides bound to MHC molecules, we know that the NH₂-terminal portion of antigenic peptides lie in close proximity to the beginning of the α helical region of $\alpha 1$ and the terminal portion of the α helical region of $\alpha 2$. Thus arginine in position 1 could directly influence the relevant area of the MHC molecule and may sterically interfere with binding of mAb 28.8.6. Ultimately, a structural analysis of MHC molecules loaded with either one of two different peptides that have been shown to stabilize serologically distinct forms of that MHC molecule will help determine if the MHC is fixed in two different conformational states, or if peptides obscure different serological epitopes.

Another hypothesis we entertained was that these mAb binding sites were combinational binding sites that were part MHC and part peptide. This seems unlikely since all the mAbs react with the empty H-2K^b molecule. Even if the empty MHC molecules are only functionally empty, it is still unlikely that the antibody binding sites are combinational. Were this the case, it is surprising that reactivity with mAb 28.8.6 could be stabilized by incubation with the two different peptides derived from either OVA (257-264) or from MCMV pp89. These peptides have no apparent homology to each other. Even the H-2K^b peptide binding motif (25) is not easily identifiable in the MCMV peptide. Since peptides derived from VSV, MCMV, and OVA do not occur naturally

in the mouse, it also seems unlikely that we would have stumbled on three peptides that were crossreactive with actual endogenously occurring peptides that could potentially form part of the binding cleft for the mAbs. If these antibodies actually have combinational binding sites, they are qualitatively different than other mAbs that have combinational binding sites (26-28). These previously described mAbs with combinational binding sites are specific for a single peptide in the context of an MHC molecule. The unique aspect of the antibodies reported in this study is that they are not specific for one peptide, but recognize a class of peptides associated with H-2K^b.

Independent of the mechanism by which peptides alter serological MHC epitopes, presumably, these changes are also important for TCR recognition. Whereas antigenic peptide binding to MHC molecules clearly confers a specific conformation that antigen-specific TCRs recognize, our data shows that a prominent effect of antigenic peptide binding is loss of certain serological conformational MHC epitopes. Recent work analyzing TCR interaction with the antigenic peptide-MHC complex has focused on the direct interaction between the third hypervariable region of the α and β chains of the TCRs and the antigenic peptide bound to the MHC molecule (29, 30). However, this does not explain why T cells often use many different α and β chains when interacting with a specific MHC restriction element. Different TCR α and β chains not only have changes in the third hypervariable domain, but also differ in the first two hypervariable regions. The loss of conformational epitopes seen with peptide binding may also influence contact regions between the first two hypervariable regions on the α and β chains of the TCR and the MHC portion of the antigen-MHC complex. Thus a TCR may use different α and β chains to accommodate specific antigenic peptide-MHC complexes that have either stabilized the MHC in a specific conformation or limited access to certain area of the MHC molecule. Peptide-dependent alterations in the MHC molecule may also influence the way endogenous peptides in the thymus shape the development of the T cell repertoire during thymic maturation.

The experimental system described here uses cells that express empty MHC molecules to probe the influence of specific peptides on MHC structure. This system has allowed us to analyze the effects of uniform loading of MHC molecules with specific peptides on the conformational states of MHC molecules. These findings indicate that peptide binding to MHC molecules may affect the T cell repertoire by topographically altering the areas available for interaction between MHC molecules and TCRs.

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