Amino Acid Residues on the I-A^k α-Chain Required for the Binding and Stability of Two Antigenic Peptides

Christopher A. Nelson,* Nicholas Viner,* Steven Young,† Shirley Petzold,* Christophe Benoist,‡ Diane Mathis,‡ and Emil R. Unanue[1]*

The class II molecules of the MHC bind processed Ag fragments (peptides) for presentation to T cells, but the role of individual MHC residues in binding these peptides has not been entirely defined. A panel of 27 mutant I-A^k transfectants was analyzed for the capacity to bind 2 unrelated peptides. The main peptides examined were hen egg lysozyme residues 48–62 and heat shock protein (hsp70) to residues 28–41. Alanine substitutions of sites in the α-helical region of the I-A^k α-chain altered the ability of this class II protein to bind both peptides. Of the 27 substitutions tested, nine caused a decrease in peptide binding while only three caused an increase in peptide binding. The stabilities of these altered I-A^k-peptide complexes were also examined on SDS-PAGE. Complexes with lowered stabilities were observed after only four substitutions, and in all four cases this loss of stability was accompanied by a loss in hen egg lysozyme or hsp70 peptide-binding ability. Further, three of these residues lie in the short extended strand at the N terminus of the α-helix of the α1 domain, suggesting that this region of the I-A^k molecule may be critical for the formation of stable peptide-MHC complexes. *The Journal of Immunology,* **1996, 156: 176–182.**

X-ray crystallography has yielded a static picture of the structure of MHC class II proteins (1, 2) and suggests models of peptide-MHC interaction that can be tested experimentally. Unfortunately, limited information currently exists that measures the relative contributions toward peptide binding made by individual amino acids of the class II molecules that have been made to differ by single residues through site-directed mutagenesis (3–5). This is, in part, because T cell hybridoma assays do not always provide an accurate measurement of the amount of peptide bound by class II molecules.

In this study, we examined two parameters: peptide binding and the capacity of peptides to stabilize $\alpha\beta$ dimers of the class II molecule I-A^k. The peptides HEL[3] (48–62: DGSTDYGILQINSRW) and hsp70 (28–41: IIANDQGNRTTPSY) were selected for this analysis because their sequences correspond to naturally processed Ag fragments. The HEL peptide is thought to be the main fragment of lysozyme produced from this Ag by intracellular processing, and the hsp70 peptide results from the processing of an endogenously expressed murine hsp. Both peptides form stable complexes with I-A^k (6).

The first parameter examined was peptide binding by I-A^k. From work with mixed haplotype hybrids, it was known that polymorphic residues on the α-chain control the binding of the HEL peptide (7). To determine which residues on the I-A^k α-chain were used in binding the HEL and hsp70 peptides, we examined a panel of 27 class II transfectants, constructed in L cells, by replacing residues from positions 50 through 79 of the α-chain one at a time with alanine (8). These L cell lines were previously used by Mathis et al. (9) to define residues required for T cell hybridoma recognition of I-A^k-peptide complexes. This region of the α-chain is predicted to fold into an α-helix that forms one side of the Ag-binding groove.

Previous binding studies involved the purification of class II molecules from transfected cell lines. Here we used a procedure to measure peptide binding that does not require the large scale purification of the mutant molecules. The amount of iodinated peptide bound to surface-expressed class II molecules was measured directly by immunoprecipitation. This procedure closely parallels one developed by Racioppi et al. (3) in which the amount of peptide bound at the cell surface was measured by fluorimetry with the use of a biotinylated peptide.

The second parameter examined was the stability of peptide-MHC complexes on SDS-PAGE (10, 11). In previous work, using complexes recovered from APC, we found that peptides that stabilize complexes on SDS-PAGE also confer longer life spans to these complexes on APCs in tissue culture (12). Peptide-I-A^k complex stability is known to be dependent on the length and sequence of the bound peptide (13), but the molecular basis of this stability is not yet understood.

Finally, the class II residues found by this study to be required for peptide binding and complex stability were compared with those class II residues shown in previous studies to alter T cell recognition (8, 9).

Materials and Methods

Mutant APC lines

The panel of L cell lines bearing I-A^k molecules with alanine substitutions to the α-chain was described previously. In brief, expressible cDNA constructs encoding I-A$^k\beta$ and I-A$^k\alpha$ genes were cotransfected with the herpes simplex virus *tk* gene (*tk*$^-$) into L cells and selected with hypoxanthine-aminopterin-thymidine medium (8). The cells were carried in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% FCS. Before

*Department of Pathology and Center for Immunology, Washington University School of Medicine, St. Louis, MO 63110; †Department of Rheumatology, University of Birmingham, Birmingham, United Kingdom; and ‡INSERM-U.184, Molecular Biology and Genetics Unit, Strasbourg, France

Received for publication June 16, 1995. Accepted for publication October 24, 1995.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This study was supported by grants from the National Institutes of Health.

[2] Address correspondence and reprint requests to Dr. Emil Unanue, Department of Pathology and Center for Immunology, Washington University School of Medicine, St. Louis, MO 63110.

[3] Abbreviations used in this paper: HEL, hen egg white lysozyme; hsp, heat shock protein.

0022-1767/96/$02.00

the current experiments were begun, the cell lines were sorted for equivalent class II expression with the anti-I-$A^k\beta$ reagent 10.3.6.2 (14) and subcloned. The 10.3.6.2 Ab was labeled with FITC. Levels of class II expression on the resulting cell lines were quantitated by cytofluorimetry before each experiment. As a standard for comparison, we used the level of 10.3.6.2 staining on the wild-type I-A^k-expressing L cell line, RT7.3H3B (15).

Site-directed mutagenesis was also used to replace the arginine codon at position 56 of the I-A^k α-chain with alanine. This altered I-Aα^k cDNA (in pCEP4 (Invitrogen Co., San Diego, CA)) was co-transfected along with a wild-type I-Aβ^k cDNA (in pCEXV) (16) into the cell line M12.C3 (17). Stable transfectants were selected in hygromycin B and subcloned. One of these subclones, chosen at random, was given the name M12.C3.56A (or 56A). As a control in these experiments, we have used the wild-type I-A^k-expressing cell line M12.C3.F6 (18).

Peptide labeling

Peptides were synthesized on a Synergy 430A peptide synthesizer (Applied Biosystems, Inc., Foster City, CA) by F-moc chemistry. The crude peptides were purified to homogeneity on HPLC, and the m.w. of the recovered material was confirmed by mass spectrometry. Both the HEL peptide and the hsp70 peptides were labeled with ^{125}I by the chloramine-T method. A typical reaction was as follows: 6 μl of a 1 mM stock of each peptide (6 nM, 10.3 μg of HEL or 9.3 μg of hsp70 peptide) were dissolved in 200 μl of 0.5 M sodium phosphate buffer, pH 7.5; then 40 μl (4 mCi) of ^{125}I (as NaI carrier free in NaOH) were added followed by the addition of 23 μl of chloramine-T at a concentration of 5 mg/ml in 0.5 M sodium phosphate buffer, pH 7.5. The reaction was incubated for 5 min on ice and then stopped by adding 23 μl of sodium metabisulfate, 5 mg/ml in phosphate buffer. The labeled peptides were purified by reverse-phase HPLC on a C_{18} column to remove unreacted iodine. Fractions containing the iodinated peptides were dried in a Speed Vac concentrator and then resuspended in sterile DMEM before use.

Quantitation of peptide binding

The I-A^k-expressing L cells were grown in 60-mm dishes until 90% confluent. All cell lines were checked for levels of I-A^k expression by flow cytometry with biotin-labeled 10.3.62 anti-I-A^k Ab. The medium was removed and the cells washed with DMEM. One milliliter of DMEM, 5% FCS was added to each plate followed by 5 ml of DMEM supplemented with 5% FCS and containing 5×10^8 cpm of ^{125}I-labeled peptide. Within each experiment, a single preparation of iodinated peptide was distributed among the different L cell lines to be tested. The cells were incubated overnight in medium containing the labeled peptide. (One aliquot of cells was used to recheck levels of I-A^k by incubating the dish with ^{125}I-labeled 40F Ab, in the absence or presence of 100-fold excess of unlabeled Ab. These data are not shown but they served as a control in each run.)

After the cells were washed twice with DMEM, 1 ml of lysis buffer was added to each plate. Each lysate was transferred to an Eppendorf centrifuge tube and rotated at 4°C for an additional 30 min to ensure complete lysis. The lysate was spun in an Eppendorf centrifuge at 10,000 $\times$ *g* for 30 min at 4°C, and the supernatant was transferred to a clean tube. As a preclear, 50 μl of a 50% slurry of protein A-Sepharose in PBS containing 1% Triton X-100 were added to each tube, and the mixture was rocked for 30 min at 4°C. The lysate was spun at 7,000 $\times$ *g* for 4 min, and the supernatant was transferred to a clean tube. The anti-I-$A^k\beta$ Ab 40F (19) was added to each tube to a final concentration of 25 μg/ml calculated to be about a 20-fold excess of Ab. After the mixture was rocked for 2 h at 4°C, we added 25 μl of a 50% protein A-Sepharose slurry in PBS containing 1% Triton X-100 and 1% BSA. The incubation was continued for 1 h longer. The immune complexes were precipitated by spinning for 4 min at 10K. Each immunoprecipitate was washed twice with PBS 1% Triton X-100, 1% BSA, and 0.5% deoxycholate. The samples were further washed three times with PBS, 1% Triton X-100, 1% BSA, 0.5% deoxycholate, and 0.5 M NaCl. SDS-sample buffer, 100 μl, was added to each tube and left to stand for 1 h at room temperature. The tubes were vortexed briefly and spun at 10K for 4 min, and the eluted I-A^k-peptide complexes were applied to nondenaturing SDS-polyacrylamide gel as described previously (6). We applied equal cell equivalents to the gel and not equal numbers of cpm.

After drying, the gels were used to expose storage phosphor "screens." The resulting images were captured digitally from the screens by scanning (PhosphorImager 425E, Molecular Dynamics, Sunnyvale, CA). Overnight exposures were generally sufficient. The amount of radiolabeled peptide in each lane was determined by integration of the recorded images using the software program ImageQuant as follows: for each set of complexes, a rectangular region containing the entire gel lane was drawn; the average pixel values across the small dimension of the rectangle were calculated and used to generate a line graph of average pixel height vs migration distance; the area under this curve was calculated at two positions (one corresponding to that of the $\alpha\beta$ dimer and one to that of the free peptide); the relative amount of labeled peptide bound by the different mutants was then determined by comparison of the areas obtained for the mutant I-A^k molecules to the area obtained for the wild-type I-A^k molecule on the same gel. (Most free peptides migrate to the front of the gel, although a few are slightly retarded. The areas of the gel containing free peptides are indicated by brackets in the figure.) The amount of peptide bound by each of the altered I-A^k α-chain expressing L cell lines was compared with the amount of the peptide bound by the wild-type I-A^k expressing L cell line. A relative value was then calculated as a percentage of wild-type binding. The ratio of stable to unstable peptide-I-A^k complex in each lane was also determined from these gels by integration.

One trivial explanation for the apparent loss of binding in our assay could be that the 40F Ab used in the immunoprecipitation no longer binds a particular combination of mutant class II and peptide. This is unlikely because 40F is specific for the β-chain of I-A^k (20), whereas these substitutions were made to the α-chain. Further, the mutants found to have reduced peptide binding were previously shown to react with four α-chain-specific and two β-chain-specific I-A^k Abs (9), suggesting that these mutations did not overly distort the class II structure.

Metabolic labeling of I-A^k

Metabolic labeling experiments to measure the ratio of stable to unstable I-A^k were conducted in the two cell lines M12.C3.F6 and 56A. For each line, 1×10^7 cells/ml were suspended in methionine-free, cysteine-free DMEM medium containing 5% dialyzed FCS and incubated at 37°C for 1 h. Some cell samples then received HEL in PBS to a final concentration of 2 mg/ml, while others received only an equivalent amount of PBS. Trans^{35}S-Label was added (200 μCi/10^7 C3.F6 cells, or 400 μCi/10^7 and 56A cells), and the incubation was continued for another 30 min. After the addition of 100 μl/ml each of 10 mg/ml methionine and 10 mg/ml cysteine to stop the labeling, the incubation was continued for another 4 h. The cells were washed once in DMEM, and then 1 ml of lysis buffer was added to each sample. The lysates were processed as described above for the labeled peptides. The SDS-PAGE gels containing the labeled samples were first fixed for 1 h in 40% methanol and 10% acetic acid and then soaked in Enhance. The dried gels were used to expose phosphor storage screens, and the gel images were captured by scanning.

Molecular modeling

The three-dimensional coordinates for the human class II MHC-peptide complex were kindly provided by Dr. L. Stern (Harvard University, Cambridge, MA). Alignments between mouse I-A^k α- and β-chains and the corresponding sequences for human HLA-DR1 were made using QUANTA 4.1/Protein Workbench (Molecular Simulations, Inc., Burlington, MA). Alignments were made to take into account minor additions and deletions; an inserted glycine at position 10 in the α-chain of I-A^k and a two-amino acid gap (lysine and aspartic acid in DR1) after position 64 in the β-chain. Allowing for these gaps, there is a 74.2% similarity and 57.2% identity between the α-chains, and with the β-chains there is 76.6% similarity and 65.9% identity, using the GCG suite of programs. The I-A^k sequence was then superimposed on the x-ray coordinates of DR1, and the side chains and the regions around the insert and the gap were regularized. The whole structure was then minimized (5000 steps, steepest descent algorithm) using the CHARMM program (21). The structure was then subjected to a CHARMM molecular dynamics simulation for 50 ps at 300K with loosely constrained C-α. The final structure was again minimized with 100 steps using the Powell algorithm.

Results

Several amino acid side chains in the α1 domain of I-A^k contribute to peptide binding

L cells expressing either wild-type or altered I-A^k molecules were cultured in radiolabeled peptide. After 16 h, the cells were washed and the class II bound peptide was immunoprecipitated with an anti-I-A^k β-chain-specific Ab. The I-A^k-peptide complexes were eluted from the immunoprecipitates without boiling and resolved on SDS-PAGE. Gels from one set of experiments are shown in Figure 1*a* using the HEL peptide and in Figure 1*b* using the hsp70 peptide. The amount of radiolabeled peptide in each lane was determined by integration (see *Materials and Methods*). An average binding value for each cell line, calculated from at least two and usually three independent experiments, is shown in Figure 2 for

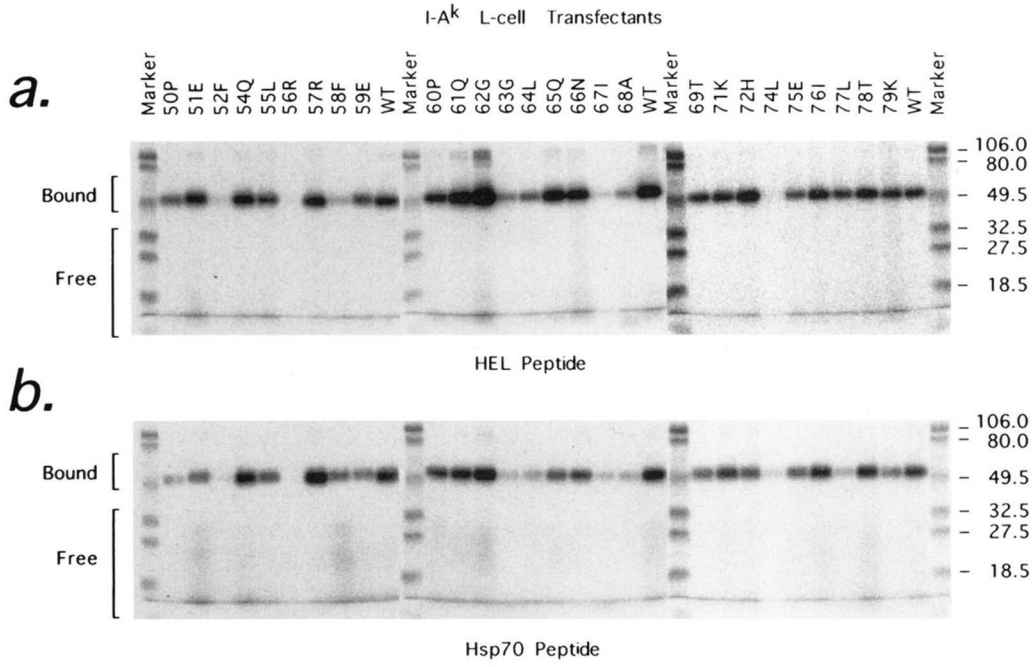
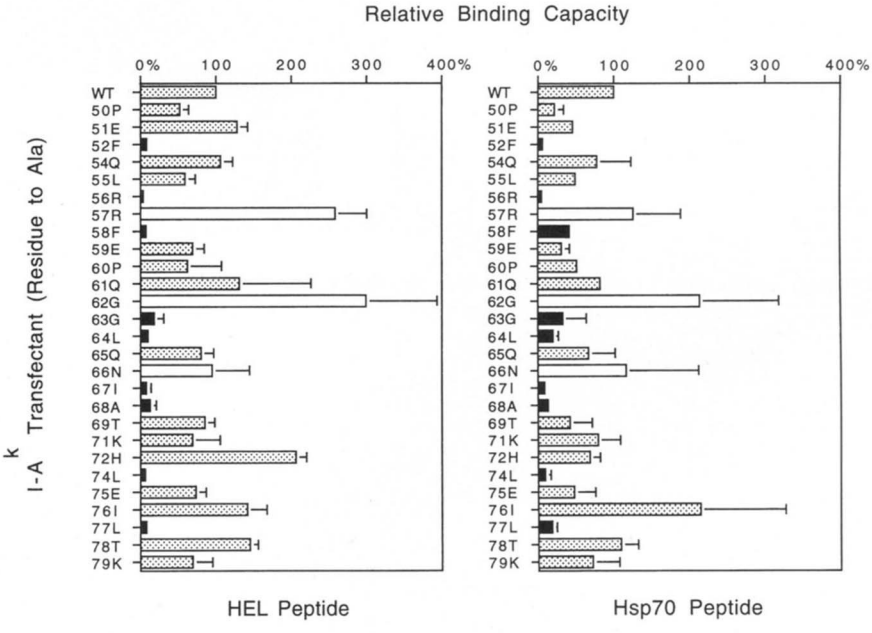


FIGURE 1. Example autoradiographs of gels containing complexes formed between the I-A^k L cell transfectants and the I^{125}-labeled HEL (*a*) or hsp70 peptides (*b*). *Top* of each lane, α-chain residue converted to Ala in the construction of that particular transfectant. Complexes in *lanes WT* were obtained from the wild-type I-A^k-expressing cell line, RT7.3H3B. All complexes were eluted from the immunoprecipitates at room temperature in SDS without boiling and ran on 10% SDS-PAGE (see *Materials and Methods*). *Left ordinate,* migration position expected for peptide running with the intact dimer (bound) or as released (free). *Marker lanes* contain m.w. standards. *Right ordinate,* corresponding m.w. values in kilodaltons. The values given in Figures 2 and 3 were generated by integration of these and similar gels. The figure contains three different experiments.

FIGURE 2. Many residues on the I-A^k α-chain are involved in peptide binding. The amount of HEL or hsp70 peptide bound by each of the mutant I-A^k expressing L-cell lines was determined. Briefly, each cell line was cultured in radiolabeled peptide to allow class II-peptide complexes to form. These complexes were immunoprecipitated and resolved on SDS-PAGE, and the amount of labeled peptide in each was determined by integration (see *Materials and Methods*). The amount of peptide bound by each of the mutant I-A^k-expressing cell lines was then compared with the amount of peptide bound by the wild-type I-A^k-expressing cell line, and this value is given as a percentage. WT indicates the amount of peptide bound by the wild-type expressing L cell; for comparison this amount was defined as 100%. *Dark bars,* transfectants that bound 50% less peptide than wild type. □, Transfectants that bound significantly more peptide than wild type. These values were determined from at least two and usually three independent experiments.



both peptides. Before beginning this series of experiments, the cell lines were sorted on a FACS for equivalent levels of class II expression and then subcloned. Most of the resulting cell lines expressed between 50 and 100% of the level of I-A^k found on the wild-type L cell line, RT7.3H3B (as measured by FACS; see *Materials and Methods*). Despite these efforts, three cell lines had consistently low levels of class II expression: 74L (i.e., residue 74 of the α-chain, a Leu that was changed to A; see Table I), 63G, and 67I with, respectively, 22 + 7, 36 + 16, and 39 + 5% of the wild-type level. Two cell lines had consistently higher levels of class II expression: 51E and 62G with, respectively, 149 ± 31% and 146 ± 18% of the wild-type level. The peptide-binding values given in Figure 2 were not adjusted to account for these differences.

On this panel of class II mutants, the two peptides behaved almost identically. The majority of substitutions to the I-A^k

Table I. *Summary of results*

Alanine Substitutions for Residues	Amount of I-A^k in L Cells	Binding	HEL Peptide Stability	T Cell Response to HEL Peptide	Predicted Amino Acid Side Chains
50P	—	—	—	No major effect	Away from groove
51E	High	—	—	No major effect	Away from groove
52F	—	Reduced	Reduced	Effects on some hybridomas	Under short extended strand
53A	nt[b]	nt	nt	No major effect	Away from groove
54Q	—	—	—	No major effect	Up
55L	—	—	—	Reduced with some hybridomas	Into groove
56R	—	Reduced	Reduced	Greatly reduced with all hybridomas tested	Into groove
57R	—	Increased	—	No major effects	Up
58F	—	Reduced	Reduced	Reduced with some hybridomas	Under short extended strand
59E	—	—	—	Reduced with some hybridomas	Up
60P	—	—	—	Reduced with some hybridomas	Away from groove
61Q	—	—	—	Effects on some hybridomas	Away from groove
62G	High	Increased	—	Greatly alters presentation	—
63G	Low	Reduced	—	Greatly alters presentation	—
64L	—	Reduced	—	Reduced to some hybridomas	Away from groove
65Q	—	—	—	Greatly reduced with all hybridomas tested	Up
66N	—	—	—	Greatly reduced with all hybridomas tested	Into groove
67I	Low	Reduced	—	Reduced to some hybridomas	Under helix
68A	—	Reduced	—	Greatly reduced	Up
69T	—	—	—	Greatly reduced	Into groove
70G	—	—	—	No major effect	—
71K	—	—	—	Reduced to some hybridomas	Away from groove
72H	—	[a]	—	Reduced to some hybridomas	Up
73N	nt	nt	nt	Reduced to some hybridomas	Into groove
74L	Low	Reduced	Reduced	Reduced to some hybridomas	Under helix
75E	—	—	—	Increased to some hybridomas	Away from groove
76I	—	Increased	—	No major effects	Into groove
77L	—	Reduced		No major effects	Into groove
78T	—	—	—	No major effects	Under helix
79K	—	—	—	No major effects	Away from groove
80R	nt	nt	nt	—	Into groove

[a] Binding increased with HEL (48–62) peptide but not with hsp70 peptide. Dashes mean that the parameter tested is within normal limits.
[b] nt, not tested.

α-chain (15 of 27) did not significantly alter the level of binding by either peptide. Substitutions at nine sites resulted in a notable reduction (i.e., <50%) in the amount of peptide bound: 52F, 56R, 58F, 63G, 64L, 67I, 68A, 74L, and 77L. The most impaired of these, 56R, bound <4% as much peptide as the wild type. The remaining bound between 10 and 50% as much peptide as the wild type (Fig. 2). It should be noted that amino acid 68A was changed to valine. Alanine substitutions at three other positions on the α-chain (57R, 62G, and 76I) caused an increase in the binding of both peptides (between 150 and 300% compared with wild type). The differences seen between the binding of these two peptides were found with residues 50P and 59E which had reduced binding of the hsp70 peptide; also, the 72H substitution favored HEL peptide binding but not hsp70 peptide binding. Table I contains a summary of these data with HEL peptide.

Alanine substitutions to the I-A^k α-chain that alter peptide-I-A^k complex stability

As a second test for peptide-MHC interaction, the stabilities of complexes made between the altered class II molecules and the radiolabeled peptides were examined on SDS-PAGE. Complexes were formed with the HEL and hsp70 peptides as described in Figure 1. The samples were eluted from the immune precipitates in SDS without boiling and run under reducing conditions. The amount of labeled peptide at two regions in each lane was considered: a region at the top of the gel where labeled peptide bound to the intact αβ dimer migrated; and a region at the bottom of the gel where free peptide migrated. For each complex, a percentage stability was calculated as the ratio of peptide found at the top position to the total peptide in each lane. An average stability value for complexes formed in each cell line was calculated (Fig. 3).

In general, complexes formed with the HEL peptide were more SDS-stable than complexes formed with the hsp70 peptide. For example, when the wild-type I-A^k molecule is used, 97% of the complexes formed with the HEL peptide were stable compared with 78% of the complexes formed with the hsp70 peptide. Alanine substitutions at four positions on the A^k α-chain (52F, 56R, 58F, and 74L) reduced the stability of the peptide-MHC complexes produced. For complexes formed with the HEL peptide, substitution at position 52F caused a drop in stability of about 40% (from 97 to 58%). Substitution at position 52F also caused a drop in stability with the hsp70 peptide of nearly 55% (from 78 to 23%). Similarly, substitution at 58F yielded complexes that were only 58% stable with the HEL peptide and 41% stable with the hsp70 peptide. The introduction of alanine at position 74L also lowered complex stability (73% stable with the HEL peptide and 44% stable with the hsp70 peptide). As described above, molecules with alanine substitutions at these positions do not bind peptide as well as the wild-type molecule (between 5 and 42% of wild-type binding with either peptide). We could not determine with certainty the stability of complexes formed with the class II mutant that contains an alanine substitution at position 56R because this mutant bound only a small amount of peptide. However, the small amount of labeled peptide that was recovered did suggest that the 56R mutant formed mostly unstable complexes.

A decrease in peptide-binding ability did not always lead to a decrease in complex stability. Many I-A^k, such as 63G, 64L, 67I, 68A, and 77L, bind reduced amounts of HEL peptide (18, 10, 8,

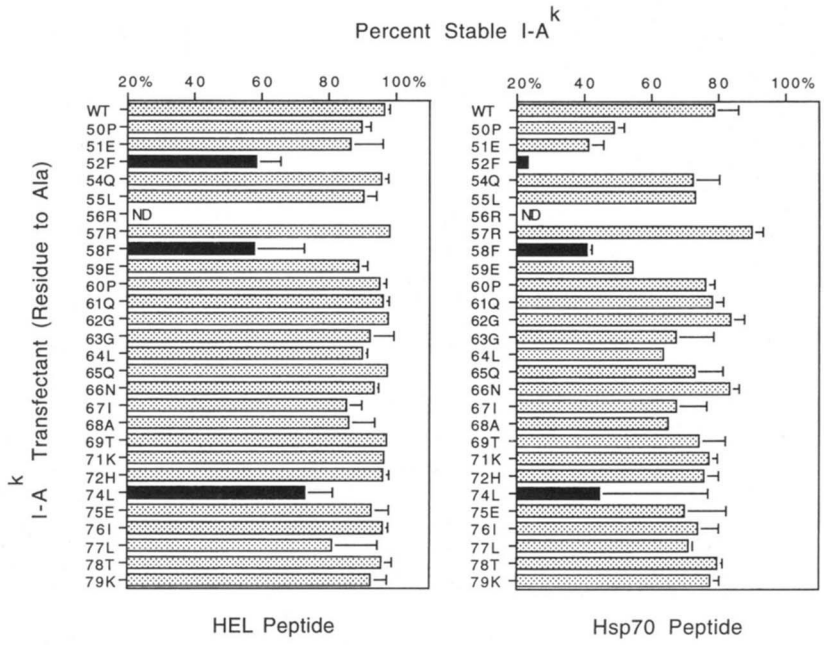


FIGURE 3. Alanine substitutions at least three positions on the I-A^k α-chain significantly decreased complex stability. The stabilities of complexes formed between the mutant I-A^k molecules from each transfectant and the labeled HEL or hsp70 peptides were measured on SDS-PAGE. The ratio of peptide at the *top* position to the total amount of peptide in each lane was calculated. This ratio is reported for each transfectant as the percentage stable I-A^k. *Dark bars,* the three positions where substitutions decreased complex stability: 52F, 58F, and 74L. ND indicates that the stability of complexes made with I-A^k molecules bearing an Ala substitution at position 56R could not be determined with certainty by this method, because this mutant did not bind enough HEL or hsp70 peptide (Fig. 2). Values were determined from at least two and usually three independent experiments.

13, and 8% as much as the wild-type A^k molecule, respectively). Yet these mutants formed complexes with the HEL peptide that were nearly as stable as the wild type (92, 90, 85, 86, and 81%). In parallel experiments, these same mutants bound only 33, 19, 8, 13, and 18% as much hsp70 peptide as the wild-type I-A^k molecule yet formed complexes that were 67, 64, 68, 65, and 71% stable with the hsp70 peptide (Table I).

The arginine side chain at position 56R is required for stable complex formation with endogenous peptides

Because alanine substitution at position 56R caused a dramatic loss in the ability of this I-A^k molecule to bind the radiolabeled peptides, the ability of this mutant to form stable complexes with constitutively presented endogenous peptides was examined. I-A^k molecules carrying an alanine substitution at position 56 were introduced into M12.C3 cells by DNA-mediated transfection. The resulting cell line M12.C3.56A (56A) expressed mutant I-A^k at the cell surface by flow cytometry (data not shown). This altered molecule appears to be correctly folded, as shown by the ability of the 56A cell line to present the HEL peptide to the I-A^k-restricted T cell hybridoma 3A9, although at a low efficiency (data not shown). Next the 56A cell line was metabolically labeled with [^{35}S]methionine/cysteine, and the mutant I-A^k molecules were isolated by immunoprecipitation for analysis on SDS-PAGE. Although >45% of the wild-type I-A^k molecules purified from the control cell line M12.C3.F6 ran as stable under these conditions (Fig. 4, *lane 1*), only 12% of the altered I-A^k molecules purified from the 56A cell line ran as stable (Fig. 4, *lane 2*). The addition of 2 mg/ml of lysozyme during the label and chase period increased the ratio of stable molecules (from 45 to 55%) on the wild-type I-A^k-expressing cell line, but this source of peptides could not increase the ratio of stable molecules on the 56A mutant cell line.

Other peptides

Three other peptides were tested. Peptide 51–61 (T-52–61) of HEL binds to wild-type I-A^k but is not effective in inducing a stable dimer; only 40 to 70% are bound stable (13). Our previous studies showed that changing Thr, the natural residue, at 51 to Glu

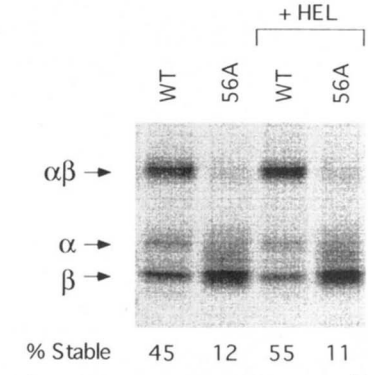


FIGURE 4. The arginine at position 56 is required for stable complex formation with endogenous peptides. Complexes in *lanes WT* were recovered from the wild-type I-A^k expressing cell line M12.C3.F6. Complexes in *lanes 56A* were recovered from the mutant I-A^k expressing cell line M12.C3.56A. The 56A complexes differ from the wild-type complexes by a single alanine substitution to the I-A^k α-chain at position 56R. Both cell lines were pulse-labeled with [^{35}S]-methionine/cysteine either with (+) or without (−) HEL for 1 h and then chased for an additional 4 h in the continued presence or absence of 2 mg/ml HEL. The labeled I-Aα^k and I-Aβ^k proteins were immunoprecipitated from the lysates of these cells with an anti-I-Aβ^k Ab 40F. After elution for 1 h at room temperature, the complexes were analyzed on SDS-PAGE (see *Materials and Methods*). The wild-type complexes were 45% stable on cells cultured without HEL and 55% stable on cells cultured with HEL. The 56A-mutant complexes were 12% stable on cells cultured without HEL and 11% stable on cells cultured with HEL.

(E-52–61) resulted in a peptide with a higher propensity to form a stable dimer, while changing it to Lys resulted in a more unstable complex (13). The results with the E-52–61 and T-52–61 peptides were similar to those depicted in Figures 2 and 3 with the HEL and hsp70 peptides. Substitution at the 51E position of the A^k molecule reduced the percentage stable to 49% of wild type (n = 3) while not affecting the percentage bound (i.e., 26% increase). The K-52–61 peptide, on the other hand, caused a consistent reduction

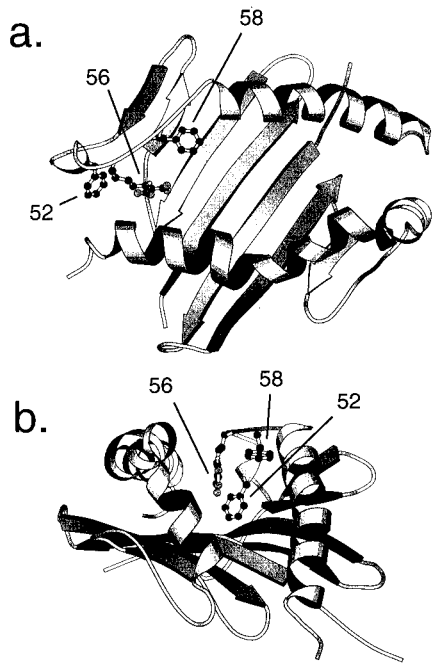


FIGURE 5. Three residues involved in the formation of stable complexes all cluster in a single region of the I-A^k α-chain. Predicted locations of the side chains from residues 52F, 56R, and 58F, on a hypothetical model of the I-A^k peptide binding site. These residues are shown from above in *A*, with the α-chain across the *top* and the β-chain across the *bottom*, and from the side in *B*. This short extended strand at the N terminus of the α-helix of the α1 domain may be critical for the formation of stable peptide-MHC complexes.

with the class II mutant 51E and 52F but resulted in an increase on the class II mutant 57R.

Discussion

This study, together with earlier studies from the laboratories of Mathis, Benoist, and Allen, identify amino acid residues in the I-Aα^k chain involved in peptide binding, SDS-complex stability, and T cell recognition (Table I). One purpose of this study was to determine to what extent these three functions are related to each other. To help in interpreting our results, we have constructed a model for the structure of the I-A^k molecule based on the published HLA-DR1 crystal structure coordinates (Fig. 5).

Alanine substitution of at least nine residues affected the level of peptide binding (Fig. 2 and Table I). It is possible that the low level of peptide binding with substitutions at positions 63G, 67I and 74L could be due to the low level of I-A^k expression by these lines. Five of the other residues that showed decreased peptide binding—52F, 58F, 64L, 68A, and 77L—are conserved among class II I-A α-chain sequences (I-A^k, I-A^b, I-A^f, I-A^d, I-A^u, I-A^s, I-A^r, I-A^q, I-A^a, and I-A^e) (20). These five residues may form contacts directly required for peptide binding, or they may form part of the I-A^k molecule framework and disrupt peptide binding by disturbing the orientations of other residues.

One striking finding of this study was the loss of peptide binding caused by the alanine substitution at position 56R. As shown in Figure 5, our molecular modeling suggests that the 56R side chain points to the bottom of the binding cleft. Because the aspartic acid residue in the fifth position of the HEL peptide is known to be required for I-A^k binding (23), it has been proposed that the aspartic acid side chain at position 52D of the HEL peptide forms a salt bridge with the arginine side chain at residue 56 of I-A^k (24). The loss of HEL peptide binding upon removal of the 56R side chain supports the proposal of a salt bridge between the peptide and the class II molecule. Perhaps the 56R side chain contributes to the formation of a deep pocket at this end of the groove, as seen in HLA-DR1 (2). Further, the hsp70 peptide, which also contains an aspartic acid as its fifth residue, does not bind the 56R mutant. In studies to be reported, we have concluded that the aspartic acid residues, at position 52 of the HEL (48–62) peptide and at position 32 of the hsp70 (28–41) peptide, are essential for high affinity binding to I-A^k and for the induction of stable I-A^k $\alpha\beta$ dimers.

Using as a guide the structure of an influenza peptide (HA 306–318) bound to the human class II molecule HLA-DR1 determined by x-ray crystallography (2), we would predict that five residues make hydrogen bonds with main chain atoms of the bound peptide, residues 55L, 57R, 66N, 73N, and 80R. Residues 55L and 57R lie in the short extended strand at the N terminus of the α1-helical region. These residues are expected to hydrogen bond through their main chain atoms to main chain atoms of the bound peptide (using the same pattern of hydrogen bonding observed in parallel β-strands). Substitution of these residues with alanine, which changes only the side chain atoms, should not have altered peptide binding. On the other hand, removal of the bulky arginine side chain from position 57R might allow better access by the peptide backbone to the main chain atoms of 57R which would help peptide binding, a possible explanation for the increase in peptide binding observed after this alanine substitution. Unfortunately, L cell lines with alanine substitutions at positions 73N and 80R were not available for this study, but the mutation at position 66N was tested. Position 66N is important because this conserved asparagine is believed to make bidentate hydrogen bond through its carbonyl oxygen and amide hydrogen atoms to similar atoms in the peptide backbone. Removal of this side chain by alanine substitution did not cause a decrease in peptide binding. This is surprising because removal of this side chain has been shown to abolish presentation of the HEL peptide to T cell hybridomas (9). To bind the 13-amino acid-long influenza virus peptide, HLA-DR1 forms 15 hydrogen bonds with the peptide main chain. It is possible that the loss of only two hydrogen bonds (caused by removal of the 66N side chain) was undetectable in this binding assay.

Also striking was the behavior of the mutant class II MHC molecules on SDS-PAGE, where stable and unstable conformations could be distinguished. Stable complex formation depends on both the length and the amino acid composition of the bound peptide. In this study, four class II mutations lowered the stability of peptide-MHC complexes formed with our labeled peptides, 52F, 56R, 58F, and 74L. In each case, the decrease in stability was accompanied by a decrease in peptide binding, although this was not necessarily the case with all residues. As mentioned, this loss of peptide binding ability was particularly noticeable with 56R. In addition, removal of the 56R side chain from the class II molecule reduced the ability of the I-A^k molecule to form stable complexes with endogenous peptides. It is still unclear whether removal of the 56R side chain altered the intrinsic stability of the class II molecule or resulted in the binding of a distinct set of endogenous peptides that do not favor stable complex formation. This I-A^k mutant could fold in a conformation that is less favored to peptide binding. However, because it is still recognized by mAbs, we do not favor this interpretation. Three of the four I-A^k α chain residues required for complex stability in this study (52F, 56R, and 58F) cluster in a single region on the I-A^k molecule (see Fig. 5). This suggests that the short extended strand at the N terminus of the α-helix of the α1 domain may be critical for the formation of stable peptide-MHC complexes.

In previous studies (8, 9), six residues on the I-Aα^k chain (56R, 62G, 65Q, 66N, 68A, and 69T) were shown to consistently reduce T cell responses to both HEL (46–61) and RNase (41–61) peptides. It was apparent from competition experiments that some of these positions did not influence peptide binding. Indeed, several points can now be made by comparing the present binding studies with the reported T cell responses. First, of the six substitutions shown to lower T cell recognition, only two reduced peptide binding (56R and 68A, of which only 56R affected stability). However, a decrease in peptide binding did not always lead to a decrease in recognition. For example, 63G, although it bound only 18% as much HEL peptide as wild-type I-A^k, was seen 2.5-fold better by the T cell hybridoma kLy4.10. Because the remaining four residues (62G, 65Q, 66N, and 69T) had no negative effect on the amount of peptide bound or on the formation of a stable complex, we must conclude that the loss of any of these side chains alters the determinant recognized by the HEL-specific T cell receptors used in their study (i.e., from hybridomas 3A9, 2A11, kLy11.10, and kLy4.10). 62G in fact led to an increase in peptide binding, although this cell line expressed 50% more A^k molecules. In general, an increase in peptide binding rarely led to an increase in recognition. Consider the three L cell lines that bound more HEL peptide than wild type; two (57R and 76I) showed a slight decrease in the ability to stimulate all four T cell hybridomas tested (down less than 10-fold), while the third, 62G, was not recognized by three of the hybridomas (although it was seen 2.5-fold better by a fourth). These data again underscore that the relative amount of peptide bound, in complexes formed with selectively altered class II molecules, is difficult to determine by comparing the relative size of the T cell responses to those complexes.

The biologic significance of stable and unstable peptide-MHC complexes is still to be determined. The four substitutions that reduced the formation of a stable complex had diverse effects on T cell presentation. We have argued that APCs discriminate between such conformations and retain the stable complexes for longer periods of time (12). Although important in vivo, such decreases in the persistence of peptide-I-A^k complexes may not be revealed by T cell assays performed with peptide continuously present in the culture (as reported by Peccoud et al. (9)).

In summary, for the first time we have been able to quantitate peptide binding and stability with the T cell response using a panel of I-Aα^k mutants. Clearly, there are profound effects of some residues on global T cell responses that cannot be explained either by an impairment of binding or by impairment of MHC-peptide complex stability. Finally, we have been struck by the similarities in the behavior of the two peptides used in this study. This similarity raises the interesting question of whether or not peptides that stabilize I-A^k bind in a particular way and therefore share a common binding motif, a point that we are currently exploring.

Acknowledgments

We thank Anthony Combs for his work in preparing the synthetic peptides used in this study.

References

1. Brown, J. H., T. S. Jardetzky, J. C. Gorga, L. J. Stern, R. G. Urban, J. L. Strominger, and D. C. Wiley. 1993. Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1. *Nature 364:33.*
2. Stern, L. J., J. H. Brown, T. S. Jardetzky, J. C. Gorga, R. G. Urban, J. L. Strominger, and D. C. Wiley. 1994. Crystal structure of the human class II MHC protein HLA-DR1 complexed with an influenza virus peptide. *Nature 368:215.*
3. Racioppi, L., F. Ronchese, R. H. Schwartz, and R. N. Germain. 1991. The molecular basis of class II MHC allelic control of T cell responses. *J. Immunol. 147:3718.*
4. Krieger, J. I., R. W. Karr, H. M. Grey, W. Y. Yu, D. O'Sullivan, L. Batovsky, Z. L. Zheng, S. M. Colon, F. C. Gaeta, J. Sidney, et al. 1991. Single amino acid changes in DR and antigen define residues critical for peptide-MHC binding and T cell recognition. *J. Immunol. 146:2331.*
5. Rosloniec, E. F., Beard, K. S., and Freed, J. H. 1993. Functional analysis of the antigen binding region of an MHC class II molecule. *Mol. Immunol. 30:491.*
6. Nelson, C. A., R. W. Roof, D. W. McCourt, and E. R. Unanue. 1992. Identification of the naturally processed form of hen egg white lysozyme bound to the murine major histocompatibility complex class II molecule I-Ak. *Proc. Natl. Acad. Sci. USA 89:7380.*
7. Lee, J. M., D. J. McKean, and T. H. Watts. 1991. Functional mapping of MHC class II polymorphic residues: the alpha-chain controls the specificity for binding an A d- versus an A k-restricted peptide and the β-chain region 65–67 controls T cell recognition but not peptide binding. *J. Immunol. 146:2952.*
8. Dellabona, P., J. Peccoud, J. Kappler, P. Marrack, C. Benoist, and D. Mathis. 1990. Superantigens interact with MHC class II molecules outside of the antigen groove. *Cell 62:1115.*
9. Peccoud, J., P. Dellabona, P. Allen, C. Benoist, and D. Mathis. 1990. Delineation of antigen contact residues on an MHC class II molecule. *EMBO J. 9:4215.*
10. Dornmair, K., B. Rothenhausler, and H. M. McConnell. 1989. Structural intermediates in the reactions of antigenic peptides with MHC molecules. *Cold Spring Harbor Symp. Quant. Biol. 54(Pt. 1):409.*
11. Germain, R. N., and L. R. Hendrix. 1991. MHC class II structure, occupancy and surface expression determined by post-endoplasmic reticulum antigen binding. *Nature 353:134.*
12. Nelson, C. A., S. J. Petzold, and E. R. Unanue. 1994. Peptides determine the lifespan of MHC class II molecules in the antigen-presenting cell. *Nature 371: 250.*
13. Nelson, C. A., S. J. Petzold, and E. R. Unanue. 1993. Identification of two distinct properties of class II major histocompatibility complex-associated peptides. *Proc. Natl. Acad. Sci. USA 90:1227.*
14. Oi, V. T., P. P. Jones, J. W. Goding, L. A. Herzenberg, and L. A. Herzenberg. 1978. Properties of monoclonal antibodies to mouse Ig allotypes, H-2, and Ia antigens. *Curr. Top. Microbiol. Immunol. 81:115.*
15. Lechler, R. I., M. A. Norcross, and R. N. Germain. 1985. Qualitative and quantitative studies of antigen-presenting cell function by using I-A-expressing L cells. *J. Immunol. 135:2914.*
16. Lechler, R. I., F. Ronchese, N. S. Braunstein, and R. N. Germain. 1986. I-A-restricted T cell antigen recognition. *J. Exp. Med. 163:678.*
17. Glimcher, L. H., D. J. McKean, E. Choi, and J. C. Seidman. 1985. Complex regulation of class II gene expression: analysis with class II mutant cell lines. *J. Immunol. 135:3542.*
18. Wade, W. F., Z. C. Zheng, R. Maki, S. McKercher, E. Palmer, J. C. Cambier, and J. H. Freed. 1989. Altered I-A protein-mediated transmembrane signaling in B cells that express truncated I-A^k protein. *Proc. Natl. Acad. Sci. USA 86:6297.*
19. Pierres, M., C. Devaux, M. Dosseto, and S. Marchetto. 1981. Clonal analysis of B- and T-cell responses to Ia antigens. I. Topology of epitope regions on I-Ak and I-Ek molecules analyzed with 35 monoclonal alloantibodies. *Immunogenetics 14:481.*
20. Landias, D., B. N. Beck, J. M. Buerstedde, S. Degraw, D. Klein, N. Koch, D. Murphy, M. Pierres, T. Tada, K. Yamamoto, et al. 1986. The assignment of chain specificities for anti-Ia monoclonal antibodies using L cell transfectants. *J. Immunol. 137:3002.*
21. Brooks, B. R., R. E. Bruccoleri, B. D. Olafson, D. J. States, S. Swaminathan, and M. Karplus. 1983. CHARMM: a program for macromolecular energy minimization and dynamics calculations. *J. Comp. Chem. 4:187.*
22. Kabat, E. A., T. T. Wu, H. M. Perry, K. S. Gottesman, and C. Foeller. 1991. *Sequences of Proteins of Immunological Interest,* 5th Ed. NIH Publication 1:838. U. S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Bethesda, MD.
23. Allen, P. M., G. R. Matsueda, R. J. Evans, J. B. Dunbar, Jr., G. R. Marshall, and E. R. Unanue. 1987. Identification of the T-cell and Ia contact residues of a T-cell antigenic epitope. *Nature 327:713.*
24. Brown, J. H., T. Jardetzky, M. A. Saper, B. Samraoui, P. J. Bjorkman, and D. C. Wiley. 1988. A hypothetical model of the foreign antigen binding site of class II histocompatibility molecules. *Nature 332:845.*