Structural Basis for Helper T-cell and Antibody Epitope Immunodominance in Bacteriophage T4 Hsp10

ROLE OF DISORDERED LOOPS*

Guixiang Dai, Stephanie Carmicle, N. Kalaya Steede, and Samuel J. Landry‡

From the Department of Biochemistry, Tulane University Health Sciences Center, New Orleans, Louisiana 70112-2699

Antigen three-dimensional structure potentially limits the access of endoproteolytic processing enzymes to cleavage sites and of class II major histocompatibility antigen-presenting proteins to helper T-cell epitopes. Helper T-cell epitopes in bacteriophage T4 Hsp10 have been mapped by restimulation of splenocytes from CBA/J and C57BL/6J mice immunized in conjunction with mutant (R192G) heat-labile enterotoxin from Escherichia coli. Promiscuously immunogenic sequences were associated with unstable loops in the three-dimensional structure of T4 Hsp10. The immunodominant sequence lies on the N-terminal flank of the 22-residue mobile loop, which is sensitive to proteolysis in divergent Hsp10s. Several mobile loop deletions that inhibited proteolysis in vitro caused global changes in the helper T-cell epitope map. A mobile loop deletion that strongly stabilized the protein dramatically reduced the immunogenicity of the flanking immunodominant helper T-cell epitope, although the protein retained good overall immunogenicity. Antisera against the mobile loop deletion variants exhibited increased cross-reactivity, most especially the antisera against the strongly stabilized variant. The results support the hypothesis that unstable loops promote the presentation of flanking epitopes and suggest that loop deletion could be a general strategy to increase the breadth and strength of an immune response.

The structure of protein antigens controls the immunogenicity of helper T-cell epitopes at the level of antigen processing as well as binding of peptides to class II MHC proteins. Analyses of published structural data and epitope maps for several model antigens suggested that local structural instability increases immunogenicity in the adjacent sequences (1, 2). In this work, we test this hypothesis by analyzing the immune response to bacteriophage T4 Hsp10 (T4Hsp10).

As in all Hsp10s examined thus far, T4Hsp10 has a flexibly disordered Hsp60-binding mobile loop (3) that we suspected would favor presentation of flanking sequences to helper T-cells. T4Hsp10 is homologous to bacterial and mammalian Hsp10s by comparison of the respective three-dimensional structures, although it exhibits less than 20% sequence identity with any of these Hsp10s (4). Earlier studies have mapped helper T-cell epitopes to sequences flanking the mobile loop of Mycobacterium leprae Hsp10 infected humans and immunized mice (5–7), but the possibility that the mobile loop should be responsible for the immunodominance of these epitopes has only been hypothesized (1).

Preferred proteolytic cleavage in Hsp10 mobile loops could enhance presentation of sequences flanking the mobile loop. Evidence indicating the proteolytic sensitivity of Hsp10 mobile loops is presented in a companion paper (8). The proteolytic sensitivity of the mobile loop in divergent Hsp10s was verified by mapping peptides generated by limited proteolysis with several proteases. Moreover, proteolytic sensitivity of T4Hsp10 is reduced when the mobile loop is made smaller in recombinant variants of T4Hsp10. Low resolution structural analysis of the mobile loop deletion variants reveals little difference in three-dimensional structure from wild-type T4Hsp10, suggesting that the changes were confined to the mobile loop.

We examined the propensity for three-dimensional structure to influence processing and presentation of T4 Hsp10 by mapping T-cell epitopes in mice. In two strains of mice, immunogenic sequences were found adjacent to each of three disordered loops of T4Hsp10, with the single immunodominant sequence located on the N-terminal flank of the mobile loop. The immunogenicity of the dominant sequence was almost eliminated when a deletion in the mobile loop substantially reduced proteolytic sensitivity. Surprisingly, the immunogenicity of distal epitopes was increased by mobile loop deletions that only modestly affected proteolytic sensitivity. The deletions also affected the antibody response. Antibody titers induced by all three deletion variants were greater than those induced by T4Hsp10. Furthermore, antibodies raised against the deletion variants exhibited increased cross-reactivity, indicating that antibody epitope immunodominance shifted from epitopes within the loop to epitopes outside of the mobile loop.

MATERIALS AND METHODS

Proteins and Peptides—Preparation of T4Hsp10 was as previously described (9), except that the gel-filtration step was substituted with hydroxyapatite chromatography employing a 20–300 mM gradient of sodium phosphate, pH 6.8. Construction, expression, and characterization of mobile loop deletion variants have been described in a companion paper (8). Overlapping peptides spanning the sequence of T4Hsp10 were synthesized by Core Laboratory, Louisiana State University Health Sciences Center (New Orleans, LA). All cysteine residues were incorporated as the acetamide derivative.

Immunization and Necropsy Procedure—Pathogen-free CBA/J (H-2k) or C57BL/6J (H-2b) mice, female, 8–12 weeks of age (Jackson), were...
intranasally administered T4Hsp10, T4Hsp10dLIG, T4Hsp10d8, or T4Hsp10d8C in combination with mutant R192G heat-labile toxin (mLT, kindly provided by John Clements, Tulane University Health Sciences Center) as adjuvant (10). Each mouse received 10 µl of protein (2 mg/ml) and 10 µl of mLT (0.5 mg/ml) in PBS. Control mice received the same amount of mLT only. Mice were boosted twice using the same protocol at intervals of 7 days. One week after the second boosting, mice were euthanized with drops of Metofane into their noses. The abdominal cavity of each mouse was opened aseptically, and the spleen was removed for lymphocyte isolation by gently teasing against a stainless steel mesh. The cells were treated with red blood cell lysis buffer (Sigma) for 3 min at room temperature and washed three times in RPMI 1640 medium. Viability was determined by trypan blue exclusion, and the number of cells was adjusted to the desired concentration in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine (working medium).

Lymphocyte Blastogenesis—Splenocytes were distributed into 96-well flat-bottom tissue culture plates at 4 × 10⁶ cells/well in RPMI 1640 working medium. Triplicate (CBA/J) or duplicate (C57BL/6J) cultures were stimulated with peptide or protein at a final concentration of 15 µg/ml. In an initial experiment, we found that cultures stimulated with peptide at 50 µg/ml gave only slightly higher levels of proliferation. Thus, the level of stimulation probably reflects the population of epitope-specific helper T-cells, which was primed during the immunization. In no case was the proliferation reduced at the higher peptide concentration, indicating that differences in stimulation are not caused by an inhibitory activity in any of the peptides. The cultures were incubated for 3 days at 37 °C in a 5% CO₂ atmosphere, labeled with 1.0 µCi of tritiated thymidine/well for another 18 h, and harvested onto glass wool fiber filters using a cell harvester. Tritiated thymidine incorporation into cellular DNA was measured in a liquid scintillation counter. Outliers in the triplicate assays were eliminated from the analysis if the standard deviation exceeded the average cpm. Using this criterion, 60 of 1500 assays were eliminated. The result for each peptide was expressed as the stimulation index (SI), which is the quotient of the average cpm for stimulated wells divided by the average cpm for medium-only control wells.

Biotinylation of gp31 Peptides—Peptides bind poorly to enzyme-linked immunosassay/radioimmunoassay plates and therefore were tethered to avidin-coated plates by covalently attached biotin. Immediately prior to use, sulfo-NHS-LC-biotin (Pierce) was dissolved in distilled water and mixed with peptides in PBS, pH 7.4, at a molar ratio of 1:5.1. The mixture was placed at room temperature for 30 min. Tris-HCl, pH 8.0, was added to a final biotin:Tris ratio of 1:20 to quench unreacted biotin. After 30 min at room temperature, sodium azide was added to achieve a final concentration of 0.1%. The resulting solutions were treated with red blood cell lysis buffer (Sigma) for 3 min at room temperature and washed three times in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine (working medium).

Serum IgG Measurement—Sera from immunized mice were assessed for IgG antibodies against T4Hsp10, T4Hsp10dLIG, T4Hsp10d8, T4Hsp10d8C, and T4Hsp10d8C peptides by ELISA as described previously (11–13) with modification. All samples were assayed in duplicate. For detection of IgG reacting with proteins, enzyme-linked immunosassay/radioimmunoassay plates (EIA/RIA, Corning Inc.) were coated with 100 µl of 4 µg/ml T4Hsp10, T4Hsp10dLIG, T4Hsp10d8, or T4Hsp10d8C. For the detection of IgG reacting with T4Hsp10 peptides, Reacti-Bind NeutAvdIn-coated plates (Pierce) were incubated with 100 µl of 2 µg/ml individual biotinylated T4Hsp10 peptides. Plates incubated with PBS, pH 7.4, alone were used as a background control. After 1 h at room temperature, the plates were washed five times with 0.1% Triton-PBS, pH 7.4. Using a plate washer (Dynatech Laboratories) and then blocked with 4% w/v in 0.5% Tween, PBS, pH 7.4, for 1 h at room temperature. Blocking buffer was removed, and 100-µl aliquots of serum dilutions were incubated in plate wells at room temperature for 1 h. For some assays, the 1:100-diluted serum had been preincubated (blocked) for 1 h at room temperature with PBS or with one of the proteins (T4Hsp10, T4Hsp10dLIG, T4Hsp10d8, or T4Hsp10-d8C) at a concentration of 0.4 mg/ml in PBS. After washing thoroughly as before, the plate wells were incubated with 100-µl aliquots of 1:2000 diluted peroxidase-labeled goat anti-mouse IgG (γ) (Kirkegaard & Perry Laboratories) at room temperature for 1 h. Finally, the plates were washed and developed by the addition of 100-µl aliquots of substrate (0.1 M sodium acetate, pH 6.0, 0.2 mg/ml 3,3',5,5'-tetramethylbenzidine, 0.01% H₂O₂). After 3 min at room temperature, the reactions were stopped by adding 100-µl aliquots of 1 M phosphoric acid. The plates were read immediately at a wavelength of 450 nm by an ELISA reader (Dynatech MR5000). Statistical Analysis—Pearson correlation coefficients were calculated using the facility implemented in Excel (Microsoft) and InStat (GraphPad). Significance tests for correlations were by analysis of variance. B-factors for amide nitrogen atoms were obtained from the Protein Data Bank (code 1G31, subunit A).

RESULTS

T-helper Cell Immunogenicity in Sequences Adjacent to Disordered Loops

Splenocyte proliferative responses to T4Hsp10 were mapped for five CBA/J and five C57BL/6J mice. Immunization was by three intranasal infusions of soluble antigen into one nostril and mLT into the other nostril at 1-week intervals. This protocol most likely preserves the native antigen structure prior to processing by antigen presenting cells. Control mice received only mLT. The panel of 19 15-mer peptides and one 16-mer peptide used for restimulation spanned T4Hsp10 residues 1–15, 6–21, etc. Splenocytes from all immunized mice proliferated strongly in response to several peptides as well as the native T4Hsp10 (Fig. 1). Proliferative responses were expressed as the SI, defined as the quotient of cpm in stimulated wells divided by cpm in medium-only control wells (1.9 × 10³ cpm average for CBA/J mice and 0.8 × 10³ cpm average for C57BL/6J mice). Mice in which splenocytes were stimulated with SI > 4 were regarded as having a positive response. By this criterion, each mouse responded to an average of 3 ± 1 (CBA/J) or 6 ± 2 (C57BL/6J) peptides. Only a minor fraction of the 20 peptides was stimulatory. Six peptides stimulated splenocytes from at least one immunized CBA/J mouse, and none of these peptides stimulated control CBA/J mice. Eight peptides stimulated splenocytes from at least one immunized C57BL/6J mouse. Two of these peptides (6 and 19) also stimulated splenocytes from one control mouse. For peptide 6, the
average SI in the immunized group was much higher than the average in the control group (10 versus 3.0); and thus, peptide 6 was considered stimulatory in C57BL/6J mice. For peptide 19, the average SI values were comparable in immunized and control mice (1.9 versus 2.0); thus, peptide 19 was not considered stimulatory in C57BL/6J mice.

Sequences corresponding to stimulatory peptides are presumed to contain at least one epitope and thus are identified as "immunogenic sequences" (Fig. 2). Some sequences were immunogenic in both strains of mice, whereas others were unique to one strain. The sequence corresponding to peptide 4 was immunodominant in both strains of mice. Peptide 4 stimulated proliferation with the highest average SI for five mice (24 in CBA/J and 28 in C57BL/6J mice), and it stimulated the largest number of mice with SI > 4, four out five of both strains. Peptides 5 and 12 stimulated at least one mouse of the different mouse strains. Other peptides stimulated one or more mice of only one strain, including peptides 8, 19, and 20 in CBA/J mice and peptides 6, 7, 17, and 18 in C57BL/6J mice.

The relationship of immunogenicity to structural disorder, and presumably also to proteolytic sensitivity, was investigated by correlating epitope frequency with crystallographic B-factors in T4Hsp10. Other work showed that the mobile loop contains the most proteolytically sensitive sites in Hsp10s (8), and the mobile loop exhibits the highest B-factors in the protein (4). Epitope frequencies were assigned to each residue in the sequence according to the frequency that the peptide stimulated either CBA/J or C57BL/6J mice. Because the peptides overlap, it is possible for a residue to have an epitope frequency greater than the number of mice in the experiment. The cumulative epitope frequencies from both strains of mice emphasize the influence of T4Hsp10 structure on epitope selection and de-emphasize the influence of peptide affinity for the MHC class II protein.

Pearson correlation coefficients for epitope frequencies with B-factors were sampled for a series of offsets ranging from -8 to +8 residues to relate immunogenicity to disorder in adjacent sequences (Fig. 2F, squares). In previous studies, epitope frequencies in hen egg lysozyme (2) and the outer domain of HIV gp120 (14) were found to correlate best with B-factors for residues located six and eight residues N-terminal (−6 and −8), respectively. Thus, epitopes tended to be on the C-terminal flanks of disordered loops in those antigens. However, in T4Hsp10, the correlation was at a maximum ($r_{max} = 0.60$) when the offset was +2. In the profiles of epitope frequency and B-factors, epitopes occur on both sides of loops. One peak lies on the N-terminal side of the mobile loop, one straddles the roof β-hairpin, and one lies on the C-terminal flank of the peripheral loop (Fig. 2E).

Immunogenic sequences of T4Hsp10 that were identified in the different mouse strains tend to coincide with each other despite potential sequence discrimination by different MHC alleles. Aside from sequences noted above that were immunogenic in both strains, additional immunogenic sequences were located adjacent to each other. The sequence corresponding to peptide 8 was immunogenic in CBA/J, and the sequence corresponding to peptides 6–7 was immunogenic in C57BL/6J. The sequences corresponding to peptides 19–20 were immunogenic in CBA/J, and the sequence corresponding to peptides 17–18 was immunogenic in C57BL/6J. A strong correlation ($r_{max} = 0.79$) between epitope frequencies in CBA/J and C57BL/6J mice was obtained with offset equal to 0, and thus epitopes in the two strains were coincident or narrowly distributed to the N- and C-terminal sides of each other (Fig. 2F, triangles).

**Modulation of T-helper Epitope Immunogenicity by Mobile Loop Deletions**

The location of the immunodominant sequence adjacent to the mobile loop in T4Hsp10 could be due to proteolytic sensitivity in the mobile loop, which may favor presentation of the
Peptides 9 and 11 were newly stimulatory, but they share mouse responded to previously identified peptides 5, 8, 12, and 4). A majority of mice responded to peptide 4, and at least one similar to those obtained in the first mapping experiment (Fig. 2). T-helper epitope mapping results obtained for T4Hsp10 were mice as indicated by the production of IgG antibody (see below).

Reductions in proteolytic sensitivity ranged from slight for T4Hsp10dLIG to dramatic for T4Hsp10d8C (8). Sensitivity to each of the four proteases, protease K, trypsin, protease V8, and cathepsin S, followed the progression: T4Hsp10dLIG, 0–6-fold; T4Hsp10d8, 4–36-fold. Each protein elicited an immune response in all five CBA/J mice, including the entire segment that forms a β-hairpin structure in the mobile loop (Fig. 3). Three residues were deleted to create variant the T4Hsp10dLIG. The resulting sequence places an isoleucine opposite a threonine on the other strand in the nascent hairpin, which is predicted to stabilize the hairpin on the basis of amino acid preferences for β-sheet secondary structure (15). T4Hsp10d8 and T4Hsp10d8C were constructed from T4Hsp10dLIG by deleting an additional eight residues. For T4Hsp10d8, four residues were removed symmetrically from each side of the nascent hairpin; and for T4Hsp10d8C, eight residues were removed from the C-terminal flank of the mobile loop, including the entire segment that forms an α-helix in the T4Hsp10 crystal structure. Each mobile loop deletion variant was expressed in E. coli and purified in a manner similar to that for T4Hsp10 (8). The biochemical and biophysical properties, including behavior on ion-exchange chromatography, circular dichroism spectroscopy, and chemical cross-linking were comparable with those of T4Hsp10. Each protein behaved as a well folded protein with native-like secondary and quaternary structure. Moreover, as expected for the removal of a disordered segment, each protein was more thermostable than T4Hsp10, exhibiting midpoints of thermal denaturation ranging from 3 to 7° higher than T4Hsp10.

Reductions in proteolytic sensitivity ranged from slight for T4Hsp10dLIG to dramatic for T4Hsp10d8C (8). Sensitivity to each of the four proteases, protease K, trypsin, protease V8, and cathepsin S, followed the progression: T4Hsp10 → T4Hsp10dLIG → T4Hsp10d8s → T4Hsp10d8C. Depending on the protease, sensitivity decreases were as follows: T4Hsp10dLIG, 0–30%; T4Hsp10d8, 10% to 6-fold; and T4Hsp10d8s, 4–36-fold. Each protein elicited an immune response in all five CBA/J mice as indicated by the production of IgG antibody (see below). T-helper epitope mapping results obtained for T4Hsp10 were similar to those obtained in the first mapping experiment (Fig. 4). A majority of mice responded to peptide 4, and at least one mouse responded to previously identified peptides 5, 8, 12, and 20. Peptides 9 and 11 were newly stimulatory, but they share sequence, and possibly all or part of the epitope, with peptides 8 and 12, respectively.

T-helper epitope mapping of the deletion variants utilized the T4Hsp10 peptides and therefore was restricted to epitopes shared with T4Hsp10. Nevertheless, these partial maps of the deletion variants deviate from that for T4Hsp10. The map for T4Hsp10dLIG retains peptides 4, 9, and 12 but acquires several newly stimulatory peptides: 2, 10, 13, 15, and 18. The map for T4Hsp10d8 retains peptides 11, 12, and 19 (which was stimulatory in the first experiment) but acquires peptides 3, 13, 16, and 17. The map for T4Hsp10d8C is least similar to that for T4Hsp10. It retains only peptide 4, albeit at low frequency, and acquires peptides 1–3, 13, and 14. As many as three mice re-
sponded to peptides corresponding to sequences altered by the deletions. Although these stimulatory peptides share partial sequences with the mobile loop deletion variants, it is not clear that the stimulation arises from an epitope that was present in T4Hsp10. Because epitopes unique to one deletion variant cannot be compared with epitopes in the other immunogens, responses to the altered peptide ligands were disregarded.

Variance in the intensity of proliferative responses from mouse to mouse is a well established phenomenon, even in inbred strains of mice (16). Because variance in presentation efficiency of the immunodominant epitope could be a source of variance in the response to the whole protein, dominance of a given epitope should be evident in a correlation of SI for the peptide with SI for the intact protein. SI values for peptides 4, 12, and 19 were tested for correlation with SI for the corresponding intact immunogen in the five mice of each group. SI for peptide 4 correlated well with SI for intact T4Hsp10 (r = 0.88, p = 0.05), less well with SI for intact T4Hsp10dLIG (r = 0.83, p = 0.08), and poorly with SI for intact T4Hsp10d8C. SI for peptide 12 correlated with SI for only intact T4Hsp10d8 (r = 0.97, p = 0.01). SI for peptide 19 marginally correlated with SI for intact T4Hsp10d8C (r = 0.86, p = 0.06).

**Antibody Immunodominance in the Mobile Loop**

* IgG Titer—Reactivity was compared at a serum dilution of 1:100, at which level the reaction was strong but not saturated (Fig. 5A). The reactions of sera raised against T4Hsp10 were consistently low, but none was less than 2-fold greater than the reaction without antigen (A_\text{450} = 0.05). The average titers for sera raised against T4Hsp10dLIG and T4Hsp10d8C were more than 10-fold higher than for sera raised against T4Hsp10, and the average titer for T4Hspd8 was 6-fold higher than for T4Hsp10 (Fig. 5B).

* Cross-reactivity—Cross-reactivity of the various antisera was analyzed by ELISA. Unexpectedly, the five sera raised against T4Hsp10 cross-reacted more strongly with T4Hsp10d8 and T4Hsp10d8C than with the immunogen (Fig. 5C), whereas sera raised against the deletion variants cross-reacted with T4Hsp10 only as well or less than they reacted with the immunogen.

* Cross-blocking—The unexpectedly strong cross-reaction of anti-T4Hsp10 with the mobile loop deletion variants suggested that T4Hsp10 epitopes were disrupted or sequestered upon binding of the protein to the microtiter plate. Because ELISA may not accurately reflect cross-reactivity in solution, the cross-blocking of sera from two mice of each group was analyzed by incubating the serum with a 4-fold excess of antigen (relative to the typical IgG concentration) prior to its reaction with the immobilized immunogen (Fig. 6). Reactions for sera blocked with antigens were normalized to the reaction of the same serum incubated without blocking antigen. As expected, the immunogen blocked each serum with equal or greater effectiveness than any other antigen. However, there were clear differences in the extent to which the sera cross-reacted. Whereas T4Hsp10 blocked more than 90% of the antibody raised against T4Hsp10, the deletion variants blocked an average of only 50%. In contrast, greater than 95% of the antibody raised against T4Hsp10d8C was blocked by each protein including T4Hsp10. Sera against T4Hsp10dLIG and T4Hsp10d8 exhibited intermediate levels of blocking by cross-reacting antigens.

**Continuous Antibody Epitope Mapping**—The stronger cross-reaction by sera raised against deletion variants suggested that the mobile loop contained one or more immunodominant epitopes that were eliminated by the deletions. Because the mobile loop is essentially unstructured, it should present continuous epitopes that can be recognized by antibody in the context of synthetic peptides. Sera from three mice immunized with T4Hsp10 were screened with peptides 3–20 corresponding to T4Hsp10. Only six peptides reacted with any of the sera, and each of the reactive peptides overlapped one of the three disordered loops (Fig. 7). Peptides 6 and 7 overlap the mobile loop, peptide 11 overlaps the roof β-hairpin, and peptides 15–17 overlap the peripheral loop.

**DISCUSSION**

* Helper T-cell Epitope Immunodominance—T4Hsp10 was used to study the relationship of antigen structure to helper T-cell epitope immunodominance because the flexibly disordered mobile loop provides a natural target for an initial endoproteolytic processing event (1). Immunodominant epitopes
had already been identified on the flanks of the mobile loop in mycobacterial Hsp10s, but the possibility of a mechanistic connection between the loop and the immunodominant epitope was not discussed (5–7). Although the amino acid sequences of heat shock proteins from various sources typically are very similar, the bacteriophage T4Hsp10 has very little sequence identity to other Hsp10s (17 and 19% with Mycobacterium tuberculosis and human Hsp10s, respectively). Thus, any similarity in immunology is likely to be related to similarity in three-dimensional structure. In addition, immune tolerance of the mouse Hsp10 probably does not limit the repertoire of T-cells specific for T4Hsp10 sequences. Peculiar immune responses to other heat shock proteins have been attributed, in part, to their peptide-binding function (17). However, this is not likely to be an issue for Hsp10, which is thought to bind only to the Hsp60 chaperonin and only in the presence of ATP (18).

For this work, we have identified flexible regions of Hsp10 using crystallographic B-factors for backbone amide nitrogen atoms. B-factors weight the contribution of atoms in the back-calculation of electron density from the model structure (19). High B-factors can result from time-averaged structural fluctuations or static inhomogeneity. Thus, B-factors should provide a measure of the ease with which a protein segment can be molded to fit into a protease active site, and B-factor maxima in protein loops predict protease nick sites quite well (20).

Helper T-cell epitopes in T4Hsp10 were located in three regions associated with flexible loops (Fig. 8). On the N-terminal flank of the mobile loop, peptide 4 stimulated the most consistent and intense responses in both CBA/J and C57BL/6J mice. The immunodominance of the peptide 4–5 sequence also was evident in a correlation between the SI for peptide 4 and the SI for the protein. A similar correlation was reported for the immunodominant epitopes of diphtheria toxoid and tetanus toxoid in a collection of human subjects (21). These correlations suggest that a large portion of the variance in individual immune responses is due to variance in the processing of the immunodominant epitope(s). Peptides 8 and 12 were the second most consistently immunogenic in CBA/J mice. Peptide 8 lies on the C-terminal flank of the mobile loop, and peptide 12 overlaps the roof β-hairpin. Although the roof β-hairpin is much smaller than the mobile loop, five residues in the roof β-hairpin have higher than average crystallographic B-factors (Fig. 2), and the corresponding loop in human Hsp10 is highly flexible (22). The roof β-hairpin also was able to present a continuous epitope to antibodies. To date, we have not identified products of cleavage in the roof β-hairpin after limited proteolysis in vitro. Nevertheless, a low level of proteolytic processing may be sufficient for priming in vivo. Other immunogenic sequences also were associated with disordered loops. Peptides 6, 7, and 9 overlap the mobile loop, peptide 11 overlaps the roof β-hairpin, and peptides 17–20 overlap the peripheral loop. The peripheral loop is nearly as large as the mobile loop, although it is much less flexible, which may account for the weak immunogenicity of these sequences.

The relationship of disordered loops to helper T-cell epitope immunodominance was confirmed in a residue-by-residue correlation of epitope frequency with crystallographic B-factors in adjacent sequences. We summed epitope frequencies in two strains of mice to emphasize the MHC protein-independent contribution to immunodominance, in effect focusing on the “promiscuous” epitopes (23). The strong correlation of epitope frequencies in CBA/J and C57BL/6J mice justifies this procedure. The coefficient of determination ($r^2$) indicates that structural disorder predicts epitopes two residues N-terminal to the extent of 36%. For comparison, $r^2_{max}$ was 26% for lysozyme and 40% for the outer domain of HIV gp120. These values indicate the minimum possible influence of antigen structure on immunodominance. This analysis attempts to correlate epitopes with a single optimum distance from peaks in B-factor, but this obviously does not take into account the fine tuning in agretope selection by MHC protein or the possibility that exopeptidases eliminate agretopes prior to MHC protein binding. Nevertheless, these correlations are highly significant. The probability that epitope frequencies are not correlated with B-factors in T4Hsp10 is very low ($p < 0.00001$). In a similar qualitative analysis, Diethelm-Okita et al. (21) found that sequences with high relative B-factors were included in and/or flanked one or both ends of all of the “universal” epitopes in diphtheria toxoid and tetanus toxoid.

In lysozyme and gp120, T-helper epitopes tended to be located six to eight residues C-terminal from the center of disordered loops, as opposed to two residues N-terminal for T4Hsp10. In T4Hsp10, the most immunogenic sequence is located 10 residues N-terminal from the center of the mobile loop. Thus, the statistical correlation probably was weakened by the N-terminal position of the single most immunogenic epitope. However, this is a limitation of the analysis and not the model. We have proposed that preferential cleavage in disordered sites relieves conformational restraints and promotes presentation of the flanking sequences. It is not clear why the C-terminal flank should be presented more frequently, although one can imagine mechanisms that would create such a bias, such as a high ratio of carboxy- to amino-peptidase activity or a bias against C termini in the MHC peptide-binding site. Immunodominance of the N-terminal flank of the mobile loop may simply indicate the presence of compensating factors that over-

![Image 83x634 to 263x729](http://www.jbc.org/)

![Image 99x401 to 247x542](http://www.jbc.org/)

![Image 144x741](http://www.jbc.org/)
come this bias and lead to selection of the N-terminal flank instead of the C-terminal flank.

The strongly immunogenic T-helper regions of Hsp10s are conserved along with the three-dimensional structure. The three promiscuously immunogenic regions in T4Hsp10 correspond to the stimulatory peptides observed in BALB/C mice upon immunization with the M. leprae or M. tuberculosis Hsp10s (Fig. 8). T4Hsp10 shares less than 20% sequence identity with either of the mycobacterial sequences. Thus, the immunodominance pattern transcends mouse strains and diverse antigen sequences. In addition, peptide 8, which was strongly immunogenic in CBA/J mice, corresponds to a peptide that contains the immunodominant epitope in humans exposed to M. leprae (5, 24). The observations suggest that immunodominance caused by antigen three-dimensional structure can supersede peptide selectivity by the MHC protein.

Deletions in the mobile loop caused shifts in epitope immunodominance, and the magnitude of the shifts correlated with reduction in proteolytic sensitivity. We considered only the peptide sequences that are shared by deletion variants and native T4Hsp10. The proteolytic sensitivity of T4Hsp10dLIG was almost unaffected by the three-residue deletion (8), and sequences flanking the mobile loop (peptides 4 and 9) retained strong immunogenicity in this protein. As in T4Hsp10, SI for the immunodominant peptide 4, but not peptide 12 or 19, correlated with SI for intact T4Hsp10dLIG. In contrast, removal of an additional eight residues from the mobile loops in T4Hsp10dLIG and T4Hsp10d8C rendered these proteins much more resistant to proteolysis (8). In T4Hsp10d8C, both flanks of the mobile loop were changed by the deletion, and thus their immunogenicity cannot be compared. Nevertheless, the sequence corresponding to peptides 11–13 became more immunogenic, and peptide 12 became immunodominant, as indicated by a strong correlation of its SI with the SI for intact T4Hsp10dLIG. In T4Hsp10d8C, the sequence corresponding to peptide 4 became much less immunogenic, and the SI for peptide 19, but not 4 or 12, correlated with SI for intact T4Hsp10dLIG. Thus, sequences beyond the mobile loop became immunodominant in the protease-resistant variants, and, in T4Hsp10d8C, the previously immunodominant sequence was encrypted.

We propose that the reduced immunogenicity of peptide 4 in T4Hsp10d8C results from reduced proteolytic cleavage in the adjacent sequence. However, other explanations cannot be excluded. The creation of a new epitope spanning the rejoined sequence in the mobile loop could block presentation of the original epitope by determinant capture (25). Alternatively, two epitopes flanking the now shorter mobile loop could simultaneously engage MHC proteins, and the failure to proteolytically separate them interferes with their transport to the cell surface. Such a circumstance recently was described for two epitopes of hen egg lysozyme (26). On a different level, B-cells acting as antigen-presenting cells could change the T-helper epitope map because mobile loop deletions affect the B-cell epitope immunodominance pattern (see below). Antigen-specific B-cells could diversify (27–30) or restrict (28) presentation. Further work will be necessary to distinguish these possibilities for any particular epitope. Nevertheless, the overall patterns of immunodominance in this and other antigens suggest that local variation in proteolytic sensitivity is an important influence on epitope presentation.

**Antibody Epitope Immunodominance**—Mobile loop deletions produced large increases in antibody titer. A trivial explanation is that the proteins contained different levels of a contaminating mitogen such as lipopolysaccharide. However, all four proteins stimulated similar levels of splenocyte proliferation, and the highest level of proliferation for a given antigen was obtained when splenocytes had been primed against that antigen (data not shown). Thus, the level of any immune-activating contaminants must be comparable in the four preparations. Another possible explanation is that the deletion variants bypassed immune tolerance of the structurally similar (but sequence dissimilar) mouse Hsp10. If T4Hsp10 were challenging tolerance, then we expected to see cross-priming of T-helper responses against the endogenous mouse Hsp10. Cross-priming of self-reactive responses has been associated with exposure to well conserved heat shock proteins (31). As part of a study on cross-priming, we have observed that E. coli Hsp10 and a hybrid T4-mouse Hsp10 each cross-prime T-helper responses specific for mouse Hsp10; but we have not observed cross-priming of mouse-Hsp10 responses by native T4Hsp10 (data not shown). Thus, we do not think that tolerance affected the antibody titers observed here. Our favored explanation for the increased antibody titers for the deletion variants is that they resulted from increased breadth in the specificity of both B- and T-cell responses.

![Alignment of Hsp10 sequences from M. leprae (M.l.), M. tuberculosis (M.t.), and bacteriophage T4 (T4) illustrating the three promiscuously immunogenic regions (dashed-line boxes) associated with disordered loops (highlighted in gray).](image-url)
The location of the immunodominant antibody epitopes can be inferred from the patterns of cross-blocking. Antisera raised against T4Hsp10 were only partially blocked by the mobile loop deletion variants, indicating that a large fraction of these antibodies recognized epitopes that are absent from the deletion variants. Likewise, antisera raised against T4Hsp10dLIG and T4Hsp10d8 were only partially blocked by the other three antigens. Because each immunogen most effectively blocked the corresponding serum, T4Hsp10, T4Hsp10dLIG, and T4Hsp10d8 have uniquely dominant epitopes, most likely within the mobile loop. ELISA using immobilized peptides confirmed the presence of antibodies against mobile loop epitopes. The exceptional cross-reactivity of T4Hsp10d8C antibodies created a mechanism for pathogens to evade immune surveillance could have selected features in antigen three-dimensional structure that increase epitope immunodominance and therefore increase the probability that a minor helper T-cell epitope, resulting in a failure to nodominance and therefore increase the probability that a minor helper T-cell epitope, resulting in a failure to

Modulation of Epitope Immunodominance by Mobile Loop Deletions

Acknowledgments—We are grateful to Ken Bost, John Clements, and James Robinson and their co-workers for technical assistance and critical discussion, and we thank John Clements for mLT.

REFERENCES

1. Landry, S. J. (1997) ImmunoL. Today 18, 527–532
2. Landry, S. J. (2000) J. Theor. Biol. 203, 189–201
3. Landry, S. J., Taher, A., Georgopoulos, C., and van der Vies, S. M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 11622–11627
4. Hunt, J. F., van der Vies, S. M., Henry, L., and Deisenhofer, J. (1997) Cell 96, 175–177
5. Kim, J., Sette, A., Rodda, S., Southwood, S., Siegel, P. A., Mehra, V., Ohmen, J. D., Oliveros, J., Appella, E., Higashimoto, Y., Rea, T. H., Bloom, B. R., and Maddin, R. L. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 925–930
6. Hussain, R., Dockrell, H. M., Shahid, F., Zafar, S., and Chiang, T. T. (1998) Clin. Exp. Immunol. 114, 204–209
7. Chaulktra, B., Ivanji, J., Hille, A., Thiele, J., Moreno, C., and Vordermeier, H. M. (1998) Immunology 93, 64–72
8. Carmiche, S., Dai, G., Steede, N. K., and Landry, S. J. (2002) J. Biol. Chem. 277, 155–160
9. van der Vies, S. M., Gatenby, A. A., and Georgopoulos, C. (1994) Nature 368, 654–656
10. Cardenas-Freytag, L., Cheng, E., Mayeux, P., Demer, J. E., and Clements, J. D. (1999) Infect. Immun. 67, 836–833
11. Ball, J. M., Rushlow, K. E., Issel, C. J., and Montelaro, R. C. (1992) J. Virol. 66, 752–764
12. Moore, A. M., Murphy-Corb, M., and Montelaro, R. C. (1992) AIDS Res. Hum. Retroviruses 8, 1153–1164
13. Moore, J. P., Sattentau, Q. J., Wyatt, R., and Sodroski, J. (1994) J. Virol. 68, 469–484
14. Dai, G., Steede, N. K., and Landry, S. J. (2001) J. Biol. Chem., in press
15. Richardson, A., van der Vies, S. M., Keppel, F., Taher, A., Landry, S. J., and Georgopoulos, C. (1999) J. Biol. Chem. 274, 52–58
16. Mougdil, K. D., Wang, J., Yeung, V. P., and Sercarz, E. E. (1998) J. Immunol. 161, 6046–6053
17. Srivastava, P. K., and Amato, R. J. (2001) Vaccine 19, 2590–2597
18. Mouslim, A. M., Murphy-Corb, M., and Montelaro, R. C. (1992) AIDS Res. Hum. Retroviruses 8, 1153–1164
19. Hubbard, S. J., Beynon, R. J., and Thornton, J. M. (1998) Protein Eng. 11, 359–359
20. Ringo, D., and Petuko, G. A. (1996-Methods Enzymol. 313, 389–433
21. Hubbard, S. J., Beynon, R. J., and Thornton, J. M. (1998) Trends Biochem. Sci. 23, 138–141
22. Dethlefsen-Oika, K. B., Okita, D. K., Banaszak, L., and Conti-Fine, B. M. (2000) J. Infect. Dis. 181, 1001–1009
23. Landry, S. J., Steede, N. K., and Masokos, K. (1997) Biochemistry 36, 10975–10986
24. Panina-Bordignon, P., Tan, A., Termijtelen, A., Demotz, S., Corradin, G., and Lanzevezica, A. (1989) Eur. J. Immunol. 19, 2257–2262
25. Chua-Intra, B., Peersaporn, S., Davey, N., Jurcevic, S., Busson, M., Vordermeier, H. M., Pirayavaraporn, C., and Ivanji, J. (1998) Infect. Immun. 66, 4903–4909
26. Mougdil, K. D., Deng, H., Nanda, N. K., Grewal, I. S., Ametani, A., and Sercarz, E. E. (1996) J. Autoimmun. 9, 237–245
27. Castellino, F., Zappacosta, F., Coligan, J. E., and Germain, R. N. (1999) J. Immunol. 161, 4048–4057
28. Capin, L., deAlba, V. B., Casrione, A., Cabaniols, J. P., Kourilsky, P., and Kanellopoulos, J. (1998) J. Immunol. 160, 1555–1564
29. Siminosk, P. D., Campbell, D. G., Lanzevezica, A., Fairweather, N., and Wats, C. (1995) J. Exp. Med. 181, 1957–1963
30. Manula, M. J., and Janeway, C. A., Jr. (1993) Immunol. Today 14, 151–152
31. Ozaki, S., and Berzofsky, A. J. (1987) J. Immunol. 138, 4133–4142
32. Cohen, I. R. (1996) in Stress Proteins in Medicine (van Eden, W., and Young, D. B., eds) pp. 93–102, Marcel Dekker, New York
33. Wang, H., and Eckels, D. D. (1999) J. Immunol. 162, 4177–4183
34. Boudhoud, L., Villain, P., Merzouki, A., Arella, M., and Couture, C. (2000) J. Biol. Chem. 275, 2121–2130
35. Kemp, D. J., Coppel, R. L., and Anders, R. F. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 151–155
36. Jones, S., and Robinson, J. M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 13–20
Structural Basis for Helper T-cell and Antibody Epitope Immunodominance in Bacteriophage T4 Hsp10: ROLE OF DISORDERED LOOPS
Guixiang Dai, Stephanie Carmicle, N. Kalaya Steede and Samuel J. Landry

J. Biol. Chem. 2002, 277:161-168.
doi: 10.1074/jbc.M102259200 originally published online October 15, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M102259200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 34 references, 16 of which can be accessed free at
http://www.jbc.org/content/277/1/161.full.html#ref-list-1