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Insertion of the Dibasic Motif in the Flanking Region of a Cryptic Self-Determinant Leads to Activation of the Epitope-Specific T Cells

Hui Zhu,* Kechang Liu,* Jan Cerny,* Taiji Imoto,† and Kamal D. Moudgil2*

Efficient induction of self tolerance is critical for avoiding autoimmunity. The T cells specific for the well-processed and -presented (dominant) determinants of a native self protein are generally tolerized in the thymus, whereas those potentially directed against the inefficiently processed and presented (cryptic) self epitopes escape tolerance induction. We examined whether the crypticity of certain determinants of mouse lysozyme-M (ML-M) could be attributed to the nonavailability of a proteolytic site, and whether it could be reversed to immunodominance by engraftment of a novel cleavage site in the flanking region of the epitope. Using site-directed mutagenesis, we created the dibasic motif (RR or RK; R = arginine, K = lysine), a target of intracellular proteases, in the region adjoining one of the three cryptic epitopes (46–61, 66–79, or 105–119) of ML-M. Interestingly, the mutated lysozyme proteins, but not unmutated ML-M, were immunogenic in mice. The T cell response to the altered lysozyme was attributable to the efficient processing and presentation of the previously cryptic epitope, and this response was both epitope and MHC haplotype specific. In addition, the anti-self T cell response was associated with the generation of autoantibodies against self lysozyme. However, the results using one of three mutated lysozymes suggested that the naturally processed, dibasic motif-marked epitope may not always correspond precisely to the cryptic determinant within a synthetic peptide. This is the first report describing the circumvention of self tolerance owing to the targeted reversal of crypticity to dominance in vivo of a specific epitope within a native self Ag. The Journal of Immunology, 2005, 175: 2252–2260.

Both self and foreign Ags possess T cell determinants that are either well processed and presented (dominant determinants) or poorly processed (cryptic determinants) from whole (native) protein (1–10). The dominant epitopes of a foreign Ag are immunogenic, whereas those of a self Ag are generally tolerogenic (4, 5, 11–15). However, cryptic epitopes of both self and foreign Ags are potentially immunogenic in the preprocessed (peptide) form (2, 3). It has been shown in various experimental models that the T cells against dominant self epitopes are readily tolerized, whereas those against cryptic epitopes escape tolerance induction and are available in the mature T cell repertoire (1–9, 11–13, 15–17).

Self tolerance is crucial for protection against autoimmune diseases. Up-regulation of the processing and presentation of cryptic epitopes of a self Ag under appropriate immune stimulating conditions (e.g., inflammation following infection) has been implicated in the priming of ambient self-directed T cells, and thereby, the initiation and/or propagation of autoimmunity (2, 9, 11, 16, 18–20). Although this association has been documented in different experimental models of autoimmunity (9, 16, 20, 21), there is meager experimental evidence showing that deliberate unmasking of a specific preselected cryptic epitope within a native self Ag can induce epitope-specific immune response and overcome self tolerance in vivo. In addition, crypticity of antigenic determinants has been attributed to multiple factors (2, 4, 6, 19, 22–28), but sufficient experimental evidence has been gathered to date only for a limited number of these proposed mechanisms. Further defining the mechanisms underlying the crypticity of T cell epitopes is critical for better understanding of the role of cryptic self-determinants in the induction of autoimmunity (2, 4, 9, 11, 16, 19, 20), graft rejection (29), and antitumor immunity (30–32).

In our previous work using mouse lysozyme-M (ML-M) as the model self Ag (3, 15), we observed that mice of diverse MHC haplotypes were tolerant to native (unmutated) ML-M, but raised potent T cell response to the preprocessed (peptide) form of potential (cryptic) epitopes. We hypothesized that crypticity of these epitopes is not an inherent structural characteristic of these determinants; instead, it is attributable in part to inefficient processing of the epitope, owing either to the absence of a proteolytic cleavage site adjacent to that determinant, or to the inaccessibility to the proteolytic enzyme(s) of an existing cleavage site within that region of the molecule. In either case, the provision of a new proteolytic cleavage site adjacent to a cryptic determinant should permit scission of the Ag at that site, making the previously cryptic epitope region available for binding to the appropriate MHC molecule, and subsequent presentation to specific T cells of that determinant as a neodominant epitope on the APC surface. To test this proposition, we exploited the dibasic motif (consisting of two contiguous basic amino acid residues, e.g., arg-arg (RR) or arg-lys (RK)) (33–36) that has been shown by others to serve as a proteolytic cleavage site in the physiological conversion of precursor polypeptides into bioactive end products (e.g., of a prohormone to...
a hormone (33–36). The targeted regions within ML-M included 46–61, 66–79, and 105–119, which contain cryptic epitopes for mice of the H-2b, H-2d, and H-2d haplotype, respectively (3, 15) (Table I and Fig. 1).

We generated three mutant ML-M proteins (MLM-A42R, MLM-Y62R, and MLM-D101K) by inserting a basic amino acid residue (R/K) adjacent to a pre-existing arginine residue (R), creating the dibasic motif (RR/RR) in the region flanking the corresponding cryptic ML-M determinant 46–61, 66–79, and 105–119, respectively, with each protein having only one site of mutation (Table I and Fig. 1). We then tested the immunogenicity of these proteins by measuring the Ag/epitope-directed T cell and Ab response in the appropriate mouse strains. Our results provide experimental evidence for one of the mechanisms underlying the crypticity of antigenic epitopes, namely, the nonavailability of a proteolytic cleavage site. Furthermore, these results furnish direct evidence for the role of up-regulation of cryptic epitopes in overcoming self tolerance in vivo and, thereby, in the priming of an anti-self autoimmune response.

Materials and Methods

Mice

Inbred C3H/HeJ (C3H) (H-2b), C57BL/6 (B6) (H-2d), and BALB/c (H-2d) female (5–6 wk) mice were purchased from The Jackson Laboratory and maintained in our animal facility at the University of Maryland School of Medicine, following the guidelines of the Institutional Animal Care and Use Committee.

Antigens

Peptides (14–16-mers) of ML-M were obtained from Macromolecular Resources and Global Peptide Services, and all peptides tested in this study contained the amino acid sequence of the unmutated self lysozyme (3, 15). Hen egg white lysozyme (HEL) and keyhole limpet hemocyanin were obtained from Sigma-Aldrich.

Cloning and expression of the ML-M gene

ML-M gene (393 bp) was constructed in the M13 phage mp19 vector (37). Full-length ML-M gene was amplified from M13-ML-M ssDNA using the appropriate upstream (27-bp) and downstream (29-bp) primers (both obtained from Sigma-Genosys) by PCR, and then cloned as a BamHI-HindIII fragment into the pRSETa vector (Invitrogen Life Technologies) (pRSETa-MLM). The ML-M gene sequence was confirmed by DNA sequencing (Biopolymer Core Facility, University of Maryland, Baltimore, MD). The purification of ML-M was conducted using probond His.tag nickel column (Invitrogen Life Technologies). Any endotoxin contaminating the recombinant protein was removed using Acetilene Extopep kit (Sterogene Bioseparations), and its depletion was validated using a Limulus Amebocyte kit (BioWhittaker). The expressed protein was characterized by SDS-PAGE, Western blot analysis using anti-His.tag mAb (Invitrogen Life Technologies), and the Lyso-protease assay. The bioassay is based on the lysis by lysozyme of dead Micrococcus lysodeikticus (Sigma-Aldrich) in agar (3, 38). The diameter of the clear zone (circle) of bacterial lysis around the sample wells is proportional to the bioactivity of lysozyme.

Site-directed mutagenesis of the ML-M gene, and generation and characterization of mutated ML-M proteins

Oligo-directed mutagenesis of the plasmid pRSETa-MLM was performed using the QuickChange Mutagenesis kit (Stratagene). Three pairs of sense- and antisense-specific mutagenesis primers were designed for creation of the dibasic motif (R41R42, R61R62, and R100R101) within ML-M (Table I and Fig. 1). Each of the three individual mutations was confirmed by DNA sequencing (data not shown). The mutated proteins were overexpressed in BL21 Escherichia coli cells, purified using the His.tag nickel column, and further characterized, as described above. The enzymatic activity of mutated ML-M proteins as measured by a lyso-plate assay (38) was comparable to that of unmutated ML-M (Fig. 1), suggesting that the overall folding of the major domains (39) of each of the mutated lysozymes was essentially preserved.

Lymph node cell (LNC) proliferation assay

Mice were immunized s.c. either with recombinant (unmutated/mutated) ML-M protein or with a peptide of ML-M (1 mg/ml each), emulsified in CFA (Invitrogen Life Technologies) (1:1, v/v). After 8 or 9 days, the draining LNC (5 × 10⁶/well) of these mice were tested in a proliferation assay using the appropriate peptides following the method described elsewhere (3). Purified protein derivative (PPD) (Mycos Research) was used as a positive control. The incorporation of radioactive [³H]thymidine was assayed by liquid scintillation counting (3). The results were expressed either as cpm or as a stimulation index (stimulation index = cpm with recall Ag/cpm with cells in medium alone).

T cell proliferation assay using paraformaldehyde (PF)-fixed APC

Briefly, thymocytes of naive mice were used as APC. APC were fixed by incubation with 0.5% PF (Sigma-Aldrich) for 10 min at room temperature either before or after pulsing with Ag (40). Naive unfixed APC were used as a control for fixed APC. Ag-primed T cells were purified from LNC and spleen of Ag-challenged mice using a nylon wool column (Polysciences) (41), and then cultured (1.5 × 10⁶/well) with fixed/unfixed APC (3.75 × 10⁶/well). APC plus T cells without Ag, and T cells with Ag only (no APC) served as additional controls. The results were expressed as cpm or stimulation index, as described above.

Measurement of the cytokine levels

LNC of Ag-primed mice were restimulated with Ag in vitro for 48 h. Thereafter, the culture supernatants were collected and assayed by ELISA using kits for IFN-γ and IL-4 (BioSource International). The absorbance was read at 450 nm using MicroElisa autoreader (Molecular Devices). The results were expressed as Δ pg/ml (= cytokine secreted by LNC with Ag – cytokine in medium control). The Th1/Th2 ratio was derived from the levels of IFN-γ/IL-4, respectively.

Determination of the serum levels of Ag-specific Abs

Ab (total IgG, IgG1, and IgG2a) in sera tested at different dilutions were detected by ELISA using different Ag (0.1 μg/ml of a high binding ELISA plate (Greiner Bioscience)) and the appropriate HRP-conjugated secondary Ab against total Ig, or Ab specific for the IgG1 or IgG2a isotype (BD Pharmingen) (1:1000) following standard procedures. The results were expressed as OD (450 nm) units.

Table I. Creation of the dibasic motif in the flanking region of a defined cryptic epitope within ML-M

<table>
<thead>
<tr>
<th>Target ML-M Epitopea</th>
<th>Cryptic Epitope for Mouse Strain (H-2b)</th>
<th>Mutant ML-M Proteinb</th>
<th>Mutation Site (Nucleotide Change in ML-M gene)</th>
<th>Resulting Dibasic Motifc</th>
</tr>
</thead>
<tbody>
<tr>
<td>46–61</td>
<td>C3H/HeJ/H (H-2b)</td>
<td>MLM-A42R</td>
<td>A42R (GCT→CGT)</td>
<td>R41R42</td>
</tr>
<tr>
<td>66–79</td>
<td>C57BL/6/6 (B6) (H-2b)</td>
<td>MLM-Y62R</td>
<td>Y62R (TAC→CGC)</td>
<td>R61R62</td>
</tr>
<tr>
<td>105–119</td>
<td>BALB/c (H-2b)</td>
<td>MLM-D101K</td>
<td>D101K (GAT→AAG)</td>
<td>R100K101</td>
</tr>
</tbody>
</table>

a Each of these epitopes of ML-M is cryptic for the mouse strain of the indicated MHC haplotype (3, 15).

b The mutant lysosomes were generated such that a particular mutant protein had the dibasic motif targeted only to one of the three epitopes, and they were named according to the site of mutation. For example, MLM-A42R refers to ML-M, with mutation at position 42 causing alanine (A) to arginine (R) change. A single-letter code was used for the amino acid involved: A, alanine; R, arginine; Y, tyrosine; D, aspartic acid; and K, lysine.

c The first amino acid residue is the pre-existing arginine (R) within ML-M at the indicated position, whereas the second amino acid residue (R/K) change. A single-letter code was used for the amino acid involved: A, alanine; R, arginine; Y, tyrosine; D, aspartic acid; and K, lysine.
Results

We determined the impact on the processing and presentation of each of the three well-defined cryptic determinants of ML-M (epitope regions 46–61, 66–79, and 105–119) of the mutation creating the dibasic site, RR or RK, within the region immediately flanking the N-terminal end of that epitope (Table I and Fig. 1). These three targeted regions within ML-M contain cryptic epitopes flanking the N-terminal end of that epitope (Table I and Fig. 1).

FIGURE 1. A, Mutagenesis leading to creation of the dibasic motif in the flanking region of a defined cryptic determinant of ML-M. The amino acid sequence of unmutated ML-M is shown in the figure along with the position and type of mutation (shown in the box) in the flanking region of each of the three epitopes within ML-M, namely, 46–61, 66–79, and 105–119. These mutations were created separately such that each variant of mutant ML-M had only one site of mutation. The replacement of a particular amino acid in ML-M was achieved by altering the appropriate nucleotide: A42R (GCT→CGT), Y62R (TAC→CGC), and D101K (GAT→AAG), respectively. The resulting dibasic motif is shown in bold. For uniformity in description of the T cell epitopes of ML-M in different studies, the aa residue 48 (glycine, G; +) of ML-M was numbered as 47a (3, 15). B, The bioactivity of recombinant unmutated/mutated ML-M proteins. Three different mutated lysozymes (100 µg/ml each) were loaded separately (10 µl/well) into the wells of a lyso-plate containing dead M. lysodeikticus as the substrate (3, 38). Also tested in the assay were unmutated ML-M (positive control) and HEL. PBS served as the negative control. The level of enzymatic activity of each mutated protein was determined from the diameter of the zone of lysis and compared with that of the unmutated control (ML-M).

Table II. Insertion of the dibasic motif within the region flanking a defined cryptic epitope of ML-M leads to induction of response to that epitope, and thereby the circumvention of self tolerance

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Immunization with</th>
<th>T Cell Recall Response to</th>
<th>Ab Response to</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ML-M</td>
<td>Mutant ML-M</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>p46-61</td>
<td>p66-79</td>
</tr>
<tr>
<td>C3H (H-2k)</td>
<td>ML-M</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>MLM-A42R</td>
<td>(A42R)</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>MLM-Y62R</td>
<td>(Y62R)</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>MLM-D101K</td>
<td>(D101K)</td>
<td>–</td>
</tr>
<tr>
<td>B6 (H-2b)</td>
<td>MLM-A42R</td>
<td>(A42R)</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>MLM-Y62R</td>
<td>(Y62R)</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>MLM-D101K</td>
<td>(D101K)</td>
<td>–</td>
</tr>
<tr>
<td>BALB/c (H-2b)</td>
<td>ML-M</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>MLM-A42R</td>
<td>(A42R)</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>MLM-Y62R</td>
<td>(Y62R)</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>MLM-D101K</td>
<td>(D101K)</td>
<td>–</td>
</tr>
</tbody>
</table>

* Abs are reactive to both MLM-A42R and MLM-D101K, but not keyhole limpet hemocyanin.

C3H (H-2k) mice are tolerant to the unmutated ML-M, but respond well to the mutant lysozyme, MLM-A42R, with the response focused on epitope 46–61

Interestingly, C3H mice challenged with ML-M failed to respond to the immunogen and to various peptides of this self lysozyme, including peptide 46–61 (p46–61) (Fig. 2A and Table II), whereas those immunized with MLM-A42R raised a potent T cell response to this altered lysozyme as well as to p46–61 (Fig. 2B and Table II). These results demonstrate that the dibasic mutation (A42R) mediated an efficient processing of the epitope 46–61 from MLM-A42R within the APC, leading to activation of specific T cells, and thereby, it successfully altered the hierarchy of determinant 46–61 from cryptic (in ML-M) to dominant (in MLM-A42R). The effect of this mutation was epitope specific because there was no significant recall response to peptides representing the N-terminal, middle region, or C-terminal determinants of ML-M (Fig. 2B). Furthermore, the T cells primed by p46–61 could be restimulated in vitro with MLM-A42R, but not by ML-M or peptides from other regions of ML-M (Fig. 2C). These results further confirmed the efficient presentation by the APC of epitope 46–61 from MLM-A42R, but not ML-M to specific T cells, and also demonstrated the T cell cross-reactivity between synthetic p46–61 and the corresponding naturally processed epitope from MLM-A42R. Finally, unlike C3H (H-2k) mice, the control BALB/c (H-2b) mice immunized with MLM-A42R failed to raise a T cell response both to the immunogen as well as to p46–61 (Fig. 2D), ruling out a nonspecific or indiscrete effect of the dibasic mutation on the induction of epitope-specific response to MLM-A42R in C3H mice. Thus, the effect of the mutation A42R on the unmasking of determinant 46–61 of ML-M in C3H mice was both epitope and MHC haplotype specific.

To determine whether the effect of the dibasic motif on revealing the previously cryptic epitope 46–61 was dependent on the processing of native MLM-A42R within the APC, we performed an assay using PF-fixed APC. (PF treatment of APC interferes with processing of a native Ag, but generally not of a peptide.) The T cells primed by p46–61 could be restimulated in vitro by APC that had been incubated with MLM-A42R before fixation with PF, but not by APC pulsed with the same mutant protein, but after fixation with PF (Fig. 2E, left panel). However, in both situations, efficient restimulation of the peptide-specific T cells by p46–61 was observed. In comparison, as expected, both MLM-A42R and p46–61 could restimulate p46–61-primed T cells when Ag-pulsed, unfixed (naive) APC were used in the assay (Fig. 2E, right panel).
To further characterize and compare the epitope 46–61-specific T cell response induced by MLM-A42R vs p46–61 of ML-M in C3H mice, we determined the pattern of cytokine secretion. Both MLM-A42R and the corresponding p46–61 induced a similar cytokine response directed against epitope 46–61. Furthermore, the restimulation of MLM-A42R-primed T cells by p46–61 in vitro, and vice versa, also yielded a comparable cytokine profile. Additional results validate that the T cell response of C3H mice to a challenge with MLM-A42R correlated well with that induced by p46–61. Thus, the introduction of the dibasic motif upstream of the cryptic determinant 46–61 facilitates the induction of T cell response to this epitope in C3H mice.

**FIGURE 2.** Introduction of the dibasic motif upstream of the cryptic determinant 46–61 facilitates the induction of T cell response to this epitope in C3H mice. A–D, LNC proliferation assay. A group (n = 4–6) each of C3H (H-2k) mice were immunized with unmutated ML-M (A), MLM-A42R (B), or p46–61 (C) (50 μg/mouse, each emulsified in CFA). A group of BALB/c mice immunized with MLM-A42R/CFA (D) served as controls. After 9 days, the draining LNC of these mice were harvested and tested in a proliferation assay using a panel of Ag. The optimal concentrations of the Ag used were determined in pilot experiments. The results were expressed as cpm (mean ± SEM). The cpm value for the positive control, PPD, ranged between 40,000 and 80,000 for A–D. In both B and C, the level of response to MLM-A42R was significantly different (*, p < 0.05; Student’s t test) from that to ML-M, whereas response to p46–61 was significantly different (*, p < 0.05) from that to control ML-M peptides, namely, p1–15, p19–33, p66–79, and p105–119. E, Proliferation assay using purified T cells and PF-fixed APC. Ag-primed T cells (T) were purified from lymph nodes and spleen of C3H mice immunized with p46–61, and then cultured with naive syngeneic APC that had been fixed with 0.5% PF (left panel) either before or after Ag (MLM-A42R, p46–61, pulse). Naive APC without fixation (right panel) were used as controls. The level of proliferative T cell recall response to MLM-A42R using APC fixed after pulse with Ag ((APC + Ag) fix + T cells) was significantly (*, p < 0.05) higher than that with APC fixed before an Ag pulse (Fix APC + Ag + T cells). F, Cytokine secretion by MLM-A42R/p46–61-reactive T cells. Proliferation assays using LNC of immunized mice in groups (i and ii) were set up, as described in B and C of the figure, respectively. After 48 h, the culture supernatants were harvested and tested for IFN-γ (■) and IL-4 (□). The results were expressed as Δ pg/ml (mean ± SEM). The Th1/Th2 (IFN-γ/IL-4) ratios in response to MLM-A42R and p46–61 were 4.85 and 5.02, respectively, for i, and 4.94 and 4.69, respectively, for ii.
with MLM-D101K did not induce any significant T cell response to p105–119 (Fig. 4D). In addition, the effect of the dibasic motif on epitope 105–119 of MLM-D101K was dependent on Ag processing (Fig. 4E), and the T cells primed by either the naturally processed epitope region 105–119 from MLM-D101K (Fig. 4Fi) or the corresponding synthetic peptide, p105–119 (Fig. 4Fii), had a comparable cytokine profile. Additional results shown in Fig. 3 (bottom panel, D–F) further corroborate the comparable nature of T cell response induced by the mutated lysozyme vs the corresponding synthetic peptide of ML-M.

The mutant protein MLM-Y62R failed to reveal epitope 66–79 in B6 (H-2b) mice

B6 mice immunized with either ML-M or MLM-Y62R failed to raise a significant T cell response to p66–79, and the overall response profile using the two Ags was comparable (Table II, and data not shown). Therefore, unlike the successful display of the dibasic motif-targeted cryptic epitopes as dominant determinants from MLM-A42R (in C3H mice) and MLM-D101K (in BALB/c mice) described above, a similar mutation in MLM-Y62R failed to reverse the hierarchy of the cryptic epitope 66–79 in B6 mice. Thus, the effect of the dibasic motif on the induction of T cell response to the targeted epitopes within ML-M is not universal; instead, it is both position and MHC haplotype dependent.

Mice immunized with a mutant lysozyme generate autoantibodies that are cross-reactive with unmutated ML-M

We next examined whether the induction of an anti-self T cell response by mutant lysozymes described above also facilitated the production of Ag-specific autoantibodies. C3H mice were immunized (with Ag/CFA s.c. with either ML-M or MLM-A42R, whereas BALB/c mice were challenged with either ML-M or MLM-D101K). Thereafter, all mice were boosted i.v. twice with the corresponding soluble Ag, and their sera (collected at weekly intervals) were tested for Ag-specific Abs. The results show that neither C3H (Fig. 5A and Table II) nor BALB/c (Fig. 5E and Table II) mice immunized with unmutated ML-M developed any Abs against ML-M or mutant ML-M variants. However, the same mouse strains challenged with an altered lysozyme produced Abs reactive against both mutated lysozyme and unmutated ML-M (Fig. 5, B and F, and Table II). Also shown in the figure are end point titrations of 4-reactive Abs of the IgG1 or IgG2a isotype in sera of mice challenged with MLM-A42R (C3H mice) (Fig. 5, C and D) or MLM-D101K (BALB/c mice) (Fig. 5, G and H). Taken together, these results demonstrate that the dibasic motif-mediated induction of T cell response to a previously cryptic epitope of ML-M also facilitated the induction of autoantibodies against native ML-M. However, the Ab response to mutated lysozymes was not epitope specific.

Discussion

In this study, we have shown that mice are tolerant to the native (unmutated) form of self lysozyme (ML-M), but the same mouse strains raise a vigorous T cell response to an altered ML-M protein containing the dibasic motif (RR or RK) created within the region flanking a previously cryptic epitope of ML-M. The induction of a T cell response to mutant lysozyme was attributable to the dibasic motif-mediated enhanced processing and presentation (by the APC) of the previously cryptic determinant as a neodominant epitope. This T cell response generated by the mutant ML-M protein was not only epitope specific, but was also MHC haplotype specific. These results exclude any general (nonspecific) or indiscr   e effect of the single amino acid mutation on the enhancement of the processing and presentation of a given cryptic determinant.
Furthermore, the T cells primed by the mutant protein were cross-reactive (as measured by T cell proliferation and cytokine profile) with the synthetic peptide containing that epitope, and vice versa. These findings rule out the activation of nonoverlapping T cell subsets by the naturally processed vs the synthetic peptide version of the immunogen (42). In addition to the T cell response, mice immunized with mutated lysozyme, but not those challenged with unmutated ML-M, raised anti-lysozyme Abs that were also cross-reactive with unmutated ML-M. These results suggest that unlike T cell response, the B cell response induced by mutated lysozymes was not epitope specific. The influence of the dibasic site within mutated lysozymes on the induction of T cell response to defined cryptic epitopes is evident from the findings that mice challenged with a mutated ML-M protein do not give a recall T cell response to unmutated ML-M, and vice versa. These results also show that T cell tolerance to native self lysozyme is being maintained, and

**FIGURE 4.** The mutation creating the dibasic motif in the region flanking the cryptic determinant 105–119 of ML rendered this epitope dominant in BALB/c mice. A–D, LNC proliferation assay. Four groups of mice (n = 4–6 each) were immunized with Ag in CFA, as follows: BALB/c/ML-M (A), BALB/c/MLM-D101K (B), BALB/c/p105–119 (C), and C3H/MLM-D101K (D). After 9 days, the draining LNC of these mice were tested in a proliferation assay. The cpm value for PPD ranged between 23,000 and 56,000 for A–D. In both B and C, the level of response to MLM-D101K was significantly higher (*, p < 0.05) than that to ML-M, whereas response to p105–119 was significantly higher (+, p < 0.05) than that to each of the control ML-M peptides: p1–15, p19–33, p46–61, and p66–79. E, Proliferation assay using purified T cells and PF-fixed APC. The T cells (=T) primed by p105–119 were cultured with naive APC that had been fixed with 0.5% PF (left panel) either before or after Ag (MLM-D101K, ■; p105–119, □) pulse. Unfixed naive APC (right panel) served as controls. The level of proliferative T cell recall response to MLM-D101K using APC fixed after pulse with Ag was significantly (*, p < 0.05) higher than that with APC fixed before Ag pulse. F, Cytokine profile of T cells primed by MLM-D101K or p105–119. Proliferation assays were set up for mice in groups i and ii as in B and C of the figure, respectively. After 48 h, the culture supernatants were collected and tested for cytokines IFN-γ (■) and IL-4 (□). The Th1/Th2 (IFN-γ/IL-4) ratios in response to MLM-D101K and p105–119 were 1.98 and 1.81, respectively, for i, and 2.69 and 2.20, respectively, for ii.
The crypticity of T cell epitopes within a native Ag has been attributed to multiple factors, including competition between determinants for binding to the same or different MHC molecule within the APC (2), hindrance in optimal interaction of the determinant with the MHC or the TCR by amino acid residues flanking the core epitope (22), destruction of the epitope within the APC owing to the presence of a cleavage site within the core epitope (6), modulation of the presentation of an antigenic epitope by Abs bound to native Ag (19, 23), a low frequency of epitope-specific T cells (25), the influence of DM (H2-M) on epitope selection and presentation by the APC (26, 27), etc. Importantly, the results of this study highlight a novel mechanism that could render a potential self-determinant cryptic, namely, the absence of a proteolytic site in the region flanking the core epitope and/or poor accessibility to intracellular proteases of a naturally existing cleavage site. However, under the existing experimental conditions, we cannot distinguish between these two possibilities, as there is no reliable algorithm to identify with certainty the physiologically relevant diverse proteolytic sites within an Ag that are most likely to be cleaved by endosomal/lysosomal or cytosolic enzymes within the APC for the generation of a defined epitope. In addition, the results demonstrating that a particular epitope region was cryptic in unmutated ML-M, but dominant in the mutant lysozyme, have also provided experimental support to our above-mentioned proposition that crypticity is not an innate structural feature of a determinant; instead, it is the result of inefficient processing of the determinant. Furthermore, we (22) and others (28, 44–46) have previously shown that amino acid residues flanking a potential T cell epitope can significantly influence the immunogenicity of that epitope. The results of this study provide a new perspective on the role of flanking residues in constituting a potential proteolytic site adjacent to a core epitope, and consequently, in determining the hierarchy (dominance/crypticity) of that epitope within a native Ag in vivo.

The role of the dibasic site in mediating the in vivo display of a potentially immunogenic cryptic epitope observed in our study is further corroborated by results of an elegant study in which the influence of a similar mutation introduced within the foreign lysozyme, HEL, on the in vitro stimulation of defined HEL peptide-specific T cell hybridomas was studied (48). The dibasic mutation within HEL facilitated the processing and presentation of the targeted subdominant determinant by the APC in vitro, leading to activation of specific T cell hybridomas. Furthermore, the effect of the mutation within HEL was epitope specific. However, HEL being a foreign Ag, any speculation regarding the likely ability of the mutated HEL to overcoming self tolerance in vivo in wild-type mice would not be applicable to the physiological setting of a self Ag. In comparison, our study is based on self lysozyme (ML-M), and it is the first report addressing in vivo both the change in the hierarchy of a targeted determinant from cryptic to dominant owing to the implanted dibasic motif within the Ag, and its impact on circumvention of self tolerance.

The identity of the precise proteolytic enzyme activity involved in the processing and presentation of cryptic epitopes from the mutated lysozyme in this study remains to be determined. Several members of the family subtilisin proprotein convertases, including furin, are known to recognize the dibasic (RR/RK) motif (49–51). Furthermore, these enzymes are found predominantly in the early endosomes (having a mildly acidic pH) and the late endosomes (52–55). In this regard, the display of the targeted cryptic epitopes

![Figure 5](http://www.jimmunol.org/)
from mutant lysozymes MLM-A42R and MLM-D101K might involve processing and presentation preferentially via the nonconventional nonendocytic route in the absence of invariant chain and the DM (H2-M) molecule, with a relatively minor contribution of the conventional endosomal/lysosomal pathway. The lack of response to epitope 66–79 within MLM-Y62R in B6 mice might be an indirect indication to the predominant participation of the former pathway; Aβ2 molecules are highly dependent on invariant chain and DM for trafficking and peptide loading (56, 57), and the nonendocytic compartment is deficient in these accessory molecules. Interestingly, TGF-β not only up-regulates its own converting enzyme, furin, but it also serves as a substrate for furin, creating a novel activation/regulation cycle (58). This finding may be of physiological relevance in regard to facilitation of the presentation of certain cryptic self-determinants under conditions associated with the availability of TGF-β.

Our results provide a convincing proof of concept for the idea that efficient processing and presentation (in this case, mediated by the dibasic motif) of a previously cryptic epitope may lead to the circumvention of self tolerance in vivo. Enhanced presentation of the dibasic motif) of a previously cryptic epitope may lead to the recognition of self MHC class II restricted T cells leading to self-tolerance. J. Immunol. 154: 554–555.


References


Our results provide a convincing proof of concept for the idea that efficient processing and presentation (in this case, mediated by the dibasic motif) of a previously cryptic epitope may lead to the circumvention of self tolerance in vivo. Enhanced presentation of a cryptic determinant has been observed under different conditions, for example, increased uptake of the Ag by APC (19, 23, 24), under the inflammatory milieu (19, 20), the treatment of APC by cytokines such as IFN-γ or IL-6 (29, 59), etc. These conditions may in turn facilitate the activation of cryptic epitope-specific T cells, which are available in the mature T cell repertoire of a healthy individual (1, 3, 7, 8). These activated T cells could then target and damage cells/tissues displaying the previously cryptic self-determinant, whose presentation might be favored under those same conditions, leading to the initiation of autoreactivity and autoimmunity. The above-mentioned series of events have also been implicated in the progression and chronicity of the acute phase of an autoimmune disease via epitope spreading involving cryptic epitopes of a self Ag (9, 20). Furthermore, a similar process targeted to disease-regulating cryptic determinants may be of significance in the control of autoimmunity (60). Our results offer a novel experimental approach for modulation of the display of specific dibasic motif-targeted cryptic antigenic determinants in vivo in studies relating to the pathogenesis and immunoregulation of autoimmunity.

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Disclosures

The authors have no financial conflict of interest.

References


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tease and enkephalin precursor processing: cleavage at dibasic and monobasic

37. Mine, S., S. Tate, T. Ueda, M. Kainosho, and T. Imoto. 1999. Analysis of the
relationship between enzyme activity and its internal motion using nuclear mag-
netic resonance: 15N relaxation studies of wild-type and mutant lysozyme. J.

38. Osserman, E. F., and D. P. Lawlor. 1966. Serum and urinary lysozyme (mur-
amicidase) in monocytic and monomyelocytic leukemia. J. Exp. Med. 124:
921–952.


in Immunology. A. M. K. J. E. Coligan, D. H. Margulies, E. M. Shevach, and

quantitation of a single immunodominant foreign epitope are presented as large
diversed compared with those generated by intracellular processing. J. Immunol.
156: 2365–2368.

erated by the binding of free peptides to class II MHC molecules are antigenically
diverse compared with those generated by intracellular processing. J. Immunol.
156: 2365–2368.


44. Kim, B. S., and Y. S. Jang. 1992. Constraints in antigen processing result in
cleavage motif into a determinant flanking region of hen egg lysozyme results in


overcoming the crypticity of a viral T cell determinant by insertion into a

47. Rudensky, A., P. Preston-Hurlbut, B. K. al-Ramadi, J. Rothbard, and

48. Schneider, S. C., J. Ohlen, L. Fosdick, B. Gladstone, J. Guo, A. Ametani,
cleavage motif into a determinant flanking region of hen egg lysozyme results in


31–39.

Proprotein convertases in tumor progression and malignancy: novel targets in

52. Boshart, H., J. Humphrey, E. Deignan, J. Davidson, J. Drazba, L. Yuan,
V. Oorschot, P. J. Peters, and J. S. Bonifacino. 1994. The cytoplasmic domain
mediates localization of furin to the trans-Golgi network en route to the endo-

1999. Sorting of furin at the trans-Golgi network: interaction of the cytoplasmic
274: 8199–8207.

54. Thomas, G. 2002. Furin at the cutting edge: from protein traffic to embryogenesis

in HLA-DM expression influences conversion of MHC class II αβ-class II-as-
sociated invariant chain peptide complexes to mature peptide-bound class II αβ

56. Blachette, F., P. Rudd, F. Gronid, L. Attisano, and C. M. Dubois. 2001. In-
volvevment of Smads in TGFβ1-induced furin (fur) transcription. J. Cell. Physiol.
188: 264–273.

P. Beverley, and B. Chain. 1998. In vivo priming of T cells against cryptic
determinants by dendritic cells exposed to interleukin 6 and native antigen. Proc.

cytoplasmic domain serines in intracellular trafficking of furin. Mol. Biol. Cell 15:
2884–2894.

1998. The phenotype of H-2M-deficient mice is dependent on the MHC class II

receptor recognition of MHC class II-bound peptide flanking residues enhances
immunogenicity and results in altered TCR V region usage. Immunity 7:
387–399.

61. Rudensky, A., P. Preston-Hurlbut, B. K. al-Ramadi, J. Rothbard, and
C. A. Janeway. 1992. Truncation variants of peptides isolated from MHC class

62. Schneider, S. C., J. Ohlen, L. Fosdick, B. Gladstone, J. Guo, A. Ametani,
cleavage motif into a determinant flanking region of hen egg lysozyme results in