

Presentation of a Self-Peptide for In Vivo Tolerance Induction of CD4⁺ T Cells Is Governed by a Processing Factor That Maps to the Class II Region of the Major Histocompatibility Complex Locus

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Summary

Self-proteins are regularly processed for presentation to autoreactive T cells in association with both class I and class II major histocompatibility complex (MHC) molecules. The presentation of self-peptides plays a crucial role in the acquisition of T cell repertoire during thymic selection. We previously reported that the self-MHC class I peptide L^d 61-80 was immunogenic in syngeneic B10.A mice (H-2^a). We showed that despite its high affinity for self-MHC class II molecules, L^d 61-80 peptide failed to induce elimination of autoreactive CD4⁺ T cells, presumably due to incomplete processing and presentation in the B10.A's developing thymus (cryptic-self peptide). In this report, we showed that the cryptic phenotype was not an intrinsic property of the self-peptide L^d 61-80 since it was found to be naturally presented and subsequently tolerogenic in BALB/c mice (H-2^d) (dominant self-peptide). In addition, the self-peptide L^d 61-80 was found to be immunogenic in different H-2^a mice while it was invariably tolerogenic in H-2^d mice regardless of their background genes. We observed that L^d 61-80 bound equally well to H-2^d and H-2^k MHC class II molecules. Also, no correlation was found between the quantity of self-L^d protein and the tolerogenicity of L^d 61-80. Surprisingly, L^d 61-80 was not naturally presented in (H-2^d × H-2^a) F1 mice, indicating that the H-2^a MHC locus contained a gene that impaired the presentation of the self-peptide. Analyses of T cell responses to the self-peptide in several H-2 recombinant mice revealed that the presentation of L^d 61-80 was controlled by genes that mapped to a 170-kb portion of the MHC class II region. This study shows that (a) endogenously processed self-peptides presented by MHC class II molecules are involved in shaping the CD4⁺ T cell repertoire in the thymus; (b) The selection of self-peptides for presentation by MHC class II molecules to nascent autoreactive T cells is influenced by nonstructural MHC genes that map to a 170-kb portion of the MHC class II region; and (c) the MHC locus of H-2^a mice encodes factors that prevent or abrogate the presentation by MHC class II molecules of the self-peptide L^d 61-80. These findings may have important implications for understanding the molecular mechanisms involved in T cell repertoire acquisition and self-tolerance induction.

Processed foreign antigens are presented to T lymphocytes in the form of peptides associated with self-MHC molecules at the surface of APCs (1, 2). There is a body of evidence that demonstrates that autologous proteins are also regularly processed for presentation to self-reactive T cells in association with either class I or class II MHC molecules (3, 4). Furthermore, elution of the peptides bound to surface MHC molecules revealed that the vast majority of MHC peptide-binding grooves were filled with a variety of protein fragments of self-origin (5, 6). The self-peptides presented in the thymus during ontogeny play a key role in shaping the T cell repertoire. High affinity recogni-

tion of self-peptide/MHC complexes by developing T cells results in their deletion/inactivation (negative selection) (7, 8). Alternatively, T cells engaged in low avidity interaction with self-peptides presented by self-MHC presumably survive and undergo differentiation (positive selection; 9). The presentation of self-peptides is therefore an essential element in the establishment of a T cell repertoire.

Dissection of the different antigen processing pathways is crucial for understanding the mechanisms that govern the selection of autologous peptides for presentation to self-reactive T cells. It has become clear that determining the ability of peptides to bind to self-MHC molecules is neces-

sary but not sufficient to predict their presentation by APCs. The set of peptides selected for display also depends on other factors, including the enzymatic machinery of the APC, the route of antigen processing, and the presence of several protein cofactors. Among them, the MHC-encoded proteasome subunits, LMP-2 and -7 (10, 11), and the transporter proteins, TAP-1 and -2 (12–14) are known to be specific participants in the processing of endogenous cytosolic proteins and their presentation in peptide form by MHC class I molecules. Similarly, the invariant chain (Ii)¹ (15), heat shock proteins (16), and the recently described *Ma* and *Mb* gene products (17, 18) are specific cofactors in the presentation of exogenous foreign proteins by MHC class II molecules. The endogenous (class I) and exogenous (class II) antigen-processing pathways have always been considered distinct. However, considerable evidence suggesting that there is cross-talk between the two antigen processing pathways has recently been provided. Thus, endogenously synthesized proteins can also represent a source of peptides for class II presentation (19, 20). Supporting this view, a large variety of peptides eluted from MHC class II molecules has been found to be derived from endogenous self-proteins (6). Therefore, examination of the mechanisms underlying the MHC class II endogenous processing pathway may provide insight into the principals of thymic selection of CD4⁺ T cells.

We and others reported that certain self-peptides were immunogenic in syngeneic animals (21, 22). Using mouse self-MHC class I protein as a model antigen, we showed that the self-peptide L^d 61–80 elicited the proliferation of CD4⁺ class II-restricted T cells in syngeneic B10.A mice (H-2^a). This result demonstrated that although the self-peptide L^d 61–80 bound with high affinity to self-class II restriction elements, it failed to reach the threshold of presentation that ensures T cell tolerance during development (21, 23) (cryptic self-peptide). Another recent study by Loss et al. indicated that BALB/c (H-2^d) APCs naturally processed and presented the self-peptide L^d 61–80 in vitro in association with MHC class II molecules (24). This prompted us to determine whether, in contrast to B10.A mice, continuous presentation of the self-peptide L^d 61–80 in BALB/c mice had occurred in vivo during thymic selection and had indeed resulted in tolerance induction of the corresponding autoreactive T cells (dominant self-peptide).

In this article, our aim was to identify the factors that control the selection of the self-peptide L^d 61–80 for in vivo presentation to T cells. To address this question, we analyzed the class II-restricted presentation of the self-peptide L^d 61–80 to CD4⁺ autoreactive T cells in different mouse strains. We first showed that crypticity or dominance of L^d 61–80 was not an intrinsic property of this self-peptide since L^d 61–80 was found tolerogenic (dominant) in BALB/c mice while it was immunogenic (cryptic) in B10.A mice. Further immunogenetic analysis revealed that

the self-peptide L^d 61–80 was cryptic in different H-2^a mice, but consistently dominant in mice expressing the H-2^d MHC haplotype, regardless of their background genes. To map the gene that controlled L^d 61–80 presentation, we compared the MHC class II presentation of the self-peptide in a panel of H-2 recombinant mouse strains. This led us to identify a 170-kb region of the MHC locus that encodes a factor(s) responsible for differential processing and presentation of the self-L^d peptide in MHC class II context.

Materials and Methods

Peptides. The peptides used in this study were synthesized in the Norris Cancer Center Microchemistry Laboratory (USC) with a peptide synthesizer (model 430A; Applied Biosystems, Inc., Foster City, CA) using modified Merrifield chemistry as described (21). The amino acid sequence of the MHC class I-derived peptide L^d 61–80 was ERITQIAKGGQEQWFRVNLRT. The overlapping MHC class I peptides were synthesized using the pin synthesis technique. The procedure was modified as described in detail elsewhere (25) so that the peptides could be cleaved from the pins.

Mice and Immunizations. The mice used in this study were obtained from The Jackson Laboratory (Bar Harbor, ME) and were housed at UCSF animal facilities. The mutant mouse strain BALB/c-dm2 (dm2) does not express the MHC class I L^d protein because of a deletion of the relevant 3' flanking region of the L^d gene. B10.GD mice that do not express E^d class II MHC molecules were a generous gift from Dr. E. E. Sercarz (University of California at Los Angeles, Los Angeles, CA). Mice of either sex were used at 3–8 mo of age. The mice were immunized in their hind footpads with 20 µg of the MHC peptide emulsified in complete CFA (Difco Laboratories, Detroit, MI).

Lymph Node and Spleen T Cell Proliferation Assays. Popliteal lymph node and spleen cells were obtained 9–10 d after immunization, and they were used in antigen-induced proliferation assays. Suspensions of 5 × 10⁵ lymph node and 10⁶ spleen cells were prepared and washed in serum-free HL-1 medium (Ventrex, Portland, ME). The cells were then cultured in either 0.2 ml of HL-1 medium containing 2 mM glutamine alone, in the presence of the self-MHC class I peptide L^d 61–80 (50 µg/ml), or with a control peptide in 96-well culture dishes for 4 d. Antigen-induced proliferation was assessed by determining the incorporation of 1 µCi [³H]thymidine during the last 18 h of culture.

Immunofluorescence Analyses. Splenocytes and thymocytes from normal mice were collected and washed in Dulbecco's PBS containing 2% FCS and 0.1% sodium azide (Sigma Immunochemicals, St. Louis, MO). The cells (2–5 × 10⁵ cells per tube) were then incubated for 30 min on ice in the presence of serial concentrations of the FITC-conjugated anti-mouse H-2L^d-reactive mAb, 30-5-7 (26). In all experiments, cells from L^d-deficient BALB/c-dm2 (dm2) mice were used as a negative control. Non-specific fluorescence was also measured using an irrelevant isotype-matched FITC-conjugated mouse mAb (IgG2a) (PharMingen, San Diego, CA). Fluorescence analyses were performed on viable nucleated cells using a FACSort[®] flow cytometer (Becton Dickinson & Co., Mountain View, CA), and the data were displayed as mean channel fluorescence.

T Cell Lines. The CD4⁺ T cell lines P69.2 (E^d-restricted, L^d 61–80-specific) and B10.A-P11.2 (E^k-restricted, L^d 61–80-spe-

¹ Abbreviations used in this paper: dm2, BALB/c-dm2; HEL, hen egg white lysozyme; Ii, invariant chain.

cific) were obtained from BALB/c-dm2 and B10.A mice, respectively. Mice were immunized in their hind footpads with the peptide L^d 61-80 (20–50 µg) emulsified in CFA. 9 d later, popliteal lymph node cells were aseptically collected and cultured (5 × 10⁶ cells/ml) in DME (ICN, Costa Mesa, CA) supplemented with 2 mM glutamine, 2 × 10⁻⁵ M 2-ME, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% FCS (Gemini Bio-products, Inc., Calabasas, CA). T cell lines were stimulated every other week with either antigen (peptide at final concentration 20 mM), syngeneic irradiated splenocytes (2 × 10⁶ cells/ml) as feeders, and 10 U/ml of human rIL-2 (Genzyme Corp., Cambridge, MA) or IL-2 alone (25 U/ml). CD4⁺ phenotype of the T cell lines was shown by two-color fluorescence analysis using anti-CD4-FITC (RM4-4) and anti-CD8-PE (53.6.7) mAb conjugates (PharMingen). The proliferation of the T cell line B10.A-P11.2 to the peptide L^d 61-80 was found to be inhibited by anti-E^k (17-3-3S), but not anti-A^k (10.2.16) mAb, indicating that B10.A-P11.2 recognizes this peptide in association with E^k MHC class II molecules. The dm2-derived T cell line P69.2 recognized the peptide L^d 61-80 in the context of E^d restriction element since B10.GD splenocytes (lacking E^d) could not stimulate the proliferation of this cell line in the presence of the specific peptide. For T cell line proliferation assays, rested T cells were placed into microwells (3–5 × 10⁴ cells per well) with 1–5 × 10⁵ irradiated splenocytes in the presence of the relevant peptide and incubated for 60 h. Proliferation was measured by the incorporation of [³H]thymidine (1 µCi per well) during the last 12 h of culture.

Competition for MHC Binding. 1E1 T hybridoma cells that recognize the λ repressor peptide 12-26 in association with I-E^d were used in MHC binding experiments (10⁵ cells per well). T cells were cocultured for 24 h with the BALB/c-derived A20 B cell lymphoma (10⁵ cells per well) used as APCs, in the presence of the relevant peptide (λ rep 12-26, 10 µM) and serial concentrations of the self-MHC class I peptide L^d 61-80. The hen egg white lysozyme (HEL) 105-120 peptide known for its binding to E^d was used as a positive control in the competition experiments. Each 96-well microplate was then centrifuged and 100 µl of each culture supernatant transferred to a new microtiter tray. IL-2 levels in culture supernatants were assessed by monitoring the [³H]thymidine incorporation of the IL-2-dependent T cell line CTLL-20. Briefly, 0.04 ml of each culture supernatant was further incubated with 10⁴ CTLL-20 cells for 24 h in a total volume of 0.2 ml HL-1 medium. Incorporation of 1 µCi [³H]thymidine was determined during the last 4 h of culture. Binding of the peptide L^d 61-80 to E^k MHC class II molecules was performed as described earlier (27).

Results

The Cryptic Phenotype Is Not an Intrinsic Property of the Self-Peptide L^d 61-80. We have previously shown that the self-MHC class I peptide L^d 61-80 induced potent MHC class II-restricted T cell responses in syngeneic B10.A mice. This self-peptide was cryptic in that despite its high binding affinity for self-MHC class II molecules, it had not been presented efficiently enough to ensure tolerance in the developing thymus of B10.A mice (21). Also, recent work by Loss et al. suggested that L^d 61-80 was regularly presented in vitro at the surface of BALB/c APCs in association with self-MHC class II molecules (24). Here, we observed that in contrast to B10.A mice (Fig. 1 A), the pep-

ptide L^d 61-80 was nonimmunogenic in BALB/c mice (Fig. 1 B), suggesting that continuous presentation of the self-peptide in BALB/c mice in vivo had resulted in deletion/inactivation of the corresponding autoreactive T cells.

We compared the immunogenicity of the self-MHC class I peptide L^d 61-80 in mouse strains differing either by their background or MHC genes. As shown in Table 1, A/J mice, which express the same MHC haplotype as B10.A mice (H-2^a) but with different background genes, mounted vigorous T cell responses to the self-peptide L^d 61-80. On the other hand, B10.D2 mice that display the same background as B10.A mice (B10), but distinct MHC allelic forms (H-2^d), failed to respond to the peptide L^d 61-80. Furthermore, the peptide L^d 61-80 was nonimmunogenic in two other H-2^d mice, BALB/c and DBA/2, differing in their background genes. The self-peptide L^d 61-80 was therefore cryptic in H-2^a mice while it was consistently dominant in all H-2^d strains, regardless of their background. We conclude that the phenotype of the self-peptide L^d 61-80, whether cryptic or dominant, is associated with genes located in the MHC locus.

In addition, the following results provided direct evidence that BALB/c APCs continuously present the self-peptide L^d 61-80 in association with MHC class II molecules: (a) The peptide L^d 61-80 was immunogenic in dm2 mutant mice, a strain that does not express the L^d MHC class I antigen (Fig. 1 C). This result demonstrated that, when seen as a foreign antigen, the peptide L^d 61-80 bound to H-2^d class II molecules (E^d) with sufficient affinity to elicit in vivo T cell responses. (b) The peptide L^d 61-80 bound with high affinity to E^d class II molecules, as shown by its capacity to competitively inhibit the response of the E^d-restricted T cell hybridoma 1E1 to its specific peptide, λ repressor 12-26 (Fig. 2 B). Furthermore, the peptide L^d 61-80 bound equally well to E^k class II molecules (Fig. 2 A), showing that differential binding to E^d and E^k was not responsible for L^d 61-80 phenotype.

We then investigated the presentation of the self-peptide L^d 61-80 in (B10.A × B10.D2) F1 mice, expressing both nontolerant H-2^a and tolerant H-2^d MHC haplotypes. F1 mice were immunized with the peptide L^d 61-80, and their T cell responses were determined after in vitro challenge with the priming peptide. As shown in Fig. 1 D, the self-peptide L^d 61-80 was found immunogenic and therefore cryptic in hybrid F1 mice. Contrary to our expectations, the presentation of the L^d 61-80 peptide by E^k molecules was not restored.

We conclude that the self-peptide L^d 61-80 is not naturally presented in vivo (cryptic and immunogenic) in B10.A mice, while it is efficiently processed and continuously presented (dominant and tolerogenic) in BALB/c mice. The phenotype of the self-peptide L^d 61-80, whether cryptic or dominant, therefore does not represent an intrinsic property of this peptide. It does however, suggest that selection of the self-peptide L^d 61-80 for presentation to autoimmune T cells is determined by factors encoded in the MHC locus that control the processing of L^d self-protein by APCs.

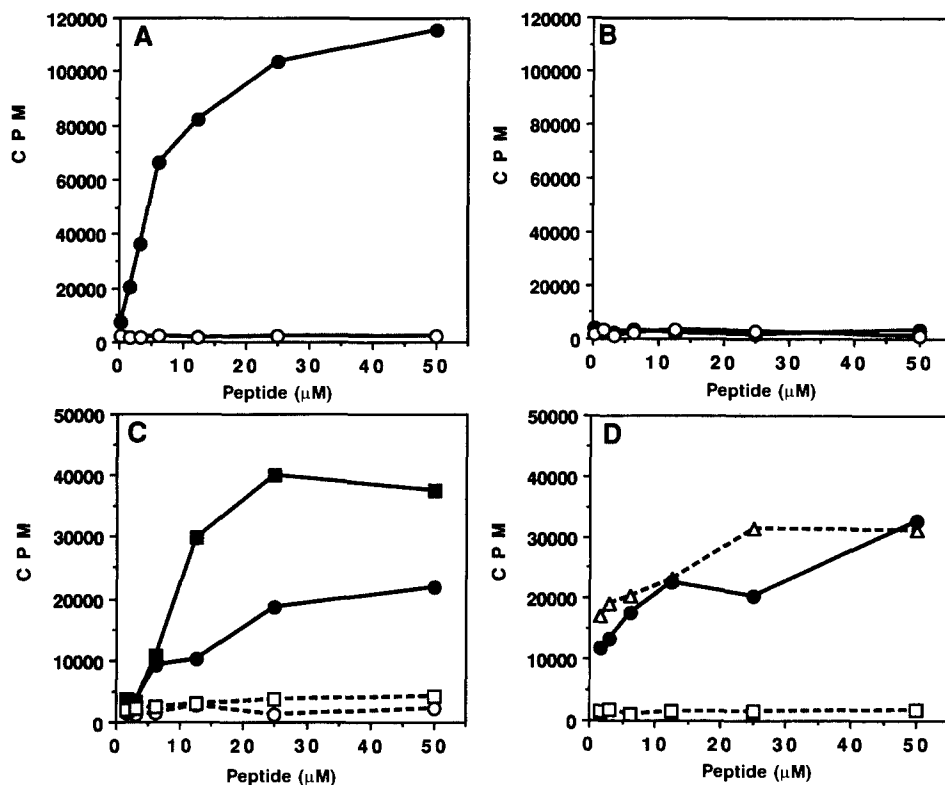


Figure 1. T cell proliferative responses to L^d 61-80 self-peptide in different mouse strains. Results are expressed as cpm recorded with lymphoid cells from mice immunized with L^d 61-80 peptide. Lymph node cells from B10.A (A) and BALB/c (B) mice restimulated in vitro with the immunizing L^d peptide (●) or a control HEL peptide (○). The data shown here are representative of five separate experiments, each including three to five mice tested individually. The background values (cells without antigens) ranged from 3,540 to 5,910 cpm. BALB/c mice immunized in the same assay with control antigen HEL mounted strong proliferative responses to HEL 105-120 peptide (95,610 ± 5,200 cpm). (C) Results are expressed as cpm recorded with lymph node (circles) and spleen (squares) cells from BALB/c (dashed lines) and BALB/c-dm2 (solid lines) mice restimulated in vitro with the immunizing L^d peptide. The data shown here are representative of three experiments in which three mice of each group were tested individually. No T cell responses could be detected after restimulation of lymph node cells with an irrelevant HEL peptide. The background values ranged from 2,710 to 3,640 cpm. (D)

Results are expressed as cpm recorded with lymph node cells from B10.A (△), B10.D2 (□), and (B10.A × B10.D2) F1 (●) mice challenged in vitro with the immunizing L^d peptide. The background values ranged from 1,850 to 4,870 cpm.

MHC Locus of H-2^a Mice Encodes a Factor That Prevents the Presentation of the L^d 61-80 Peptide. The E^d-restricted CD4⁺ T cell line P69.2 (dm2 anti-L^d 61-80) was shown to proliferate and secrete IL-2 when incubated with BALB/c APCs in the absence of exogenously added peptide (Fig. 3 B). In contrast, addition of peptide L^d 61-80 was required to stimulate P69.2 T cell line in the presence of dm2 APCs. Similar in vitro studies were conducted on the B10.A-derived B10.A-P11.2 CD4⁺ T cell line, which recognizes L^d 61-80 peptide associated with E^k MHC class II molecules. As

shown in Fig. 3 A, the B10.A-P11.2 cell line was stimulated by syngeneic APCs only when the L^d 61-80 self-peptide was added to the culture. This indicated that, in contrast to BALB/c APCs, B10.A splenocytes did not naturally process the peptide L^d 61-80 efficiently enough to stimulate autoreactive T cells. Taken together, these results show that in contrast to their B10.A counterparts, BALB/c APCs regularly processed L^d 61-80 and efficiently presented the self-peptide to autoreactive T cells.

In vitro studies have revealed that, in contrast to the pa-

Table 1. The Peptide L^d 61-80 Is Immunogenic in H-2^a, but Not H-2^d Mice

Haplotype	Strain	L ^d 61-80*		HEL	
		Medium [‡]	L ^d 61-80	Medium	HEL
H-2 ^d	B10.D2	3,253 ± 1,324	4,810 ± 890	1,615 ± 299	89,905 ± 5,426
	BALB/c	4,228 ± 1,033	4,734 ± 392	5,034 ± 2,945	177,104 ± 5,128
	DBA/2	2,357 ± 865	4,515 ± 1,263	ND	ND
H-2 ^a	B10.A	4,104 ± 737	<u>72,442 ± 11,921</u>	2,232 ± 690	<u>104,082 ± 6,112</u>
	A/J	4,078 ± 986	<u>96,048 ± 7,472</u>	3,790 ± 1,140	<u>128,634 ± 9,111</u>

*Mice were immunized either with the peptide L^d 61-80 or HEL.

[‡]Lymph node cells from primed mice were restimulated in vitro either with the immunizing L^d peptide or HEL.

Results are expressed as cpm. The data are representative of three separate experiments, each including three mice tested individually. The values that are significantly over the background counts ± SD are underlined.

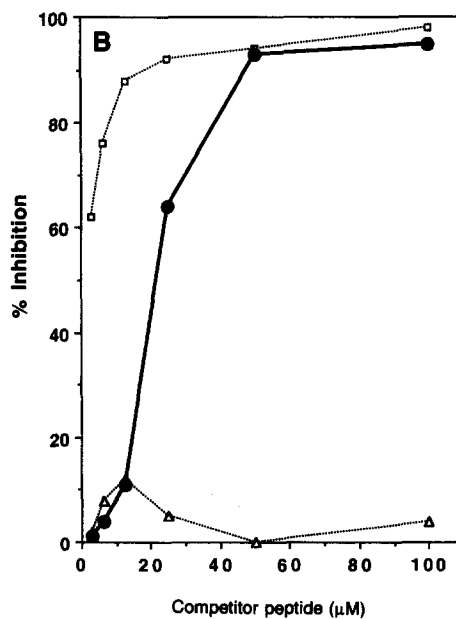
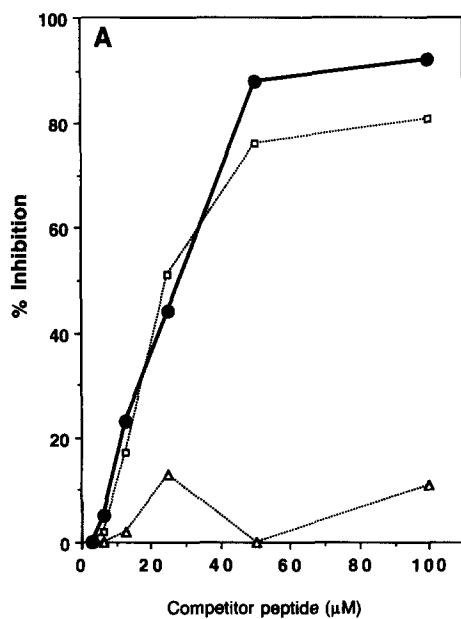


Figure 2. L^d 61-80 peptide competitively inhibits the E^k- and E^d-restricted responses of T cell hybridomas to their specific peptides. The following T cell hybridomas were tested in these inhibition assays: AOIT.13.1, specific for the HEL peptide 85-96 in association with I-E^k (A); and 1E1, specific for the λ repressor peptide 12-26 in association with I-E^d (B). Serial concentrations of the following peptides were tested: L^d 61-80 (●) in A and B; the known binder peptides (□): HEL 1-17 in A and HEL 105-120 in B; and the non-binder negative control peptides (Δ): P4 in A and L^d 61-75 in B. The responses of the T cell hybridomas AOIT.13.1 to pHEL 85-96 and 1E1 to λ repressor p12-26 ranged from 75,800 to 85,350 and from 38,420 to 46,990 cpm, respectively. Results are expressed as percentages of inhibition, i.e., 100 × cpm obtained with specific peptide + competitor peptides/cpm obtained with specific peptides.

rental B10.D2 APCs, (B10.A × B10.D2) F1 splenocytes could not mediate the proliferation of the E^d-restricted anti-L^d 61-80 T cell line in the absence of exogenously added peptide (Fig. 3 C). These results showed that F1 APCs could not spontaneously process and present the self-peptide L^d 61-80 either in association with E^k or even E^d class II molecules. This indicated that the presence of genes located in the MHC locus of H-2^a mice had impaired L^d 61-80 peptide presentation.

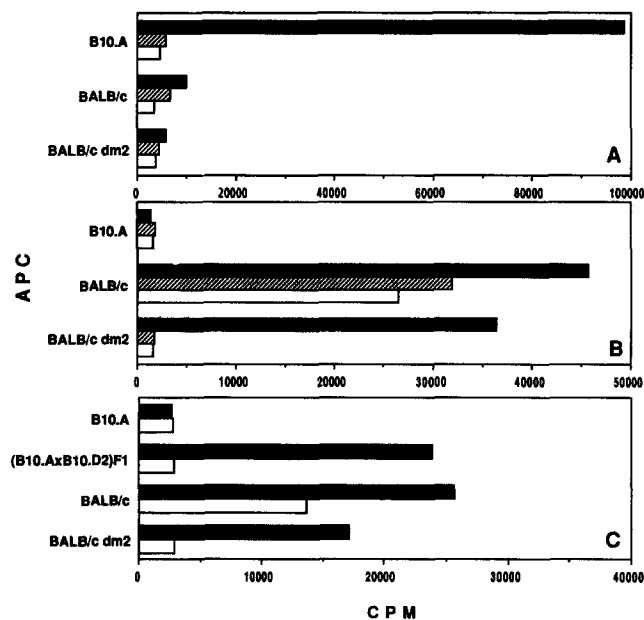


Figure 3. BALB/c APCs continuously process and present the self-peptide L^d 61-80, but not B10.A and (B10.A × B10.D2) APCs. Different APCs were tested for their ability to induce the proliferation of the anti-L^d 61-80 T cell lines B10.A-P11.2 (A) and dm2-P69.2 (B) either in the absence of exogenously added peptide (open bars) or in the presence of their specific peptide L^d 61-80 (solid bars) or of a control HEL peptide (dashed bars). The results are representative of three separate experiments.

H-2^k and H-2^d MHC Class II-restricted T Cells Recognize a Similar Determinant on the Self-Peptide L^d 61-80. It was possible that H-2^d- and H-2^k-restricted T cell responses were directed towards distinct determinants on the long 20-mer L^d 61-80. In recent work, we mapped the T cell determinant recognized by H-2^k class II-restricted T cells on the peptide L^d 61-80. We showed that T cells from L^d 61-80-primed B10.A mice were specific for a determinant located in the middle area of the molecule whose core region (residues shared by all immunogenic peptides) contained residues 66-74 (27). Here, we examined the fine structure of the determinant recognized in vivo by T cells on the peptide L^d 61-80 presented in association with H-2^d MHC class II molecules. dm2 mice were immunized with the peptide L^d 61-80 and were challenged in vitro with a series of overlapping 12 mers that progressed along the L^d 61-80 sequence by single residue steps. As shown in Fig. 4, dm2 lymph node T cells responded to a series of five consecutive peptides describing a determinant whose core region was 69-76. We conclude that after immunization of B10.A and dm2 mice with the self-peptide L^d 61-80, both H-2^k and H-2^d class II-restricted T cell responses were directed to a similar determinant on the self-peptide.

The Self-Antigen L^d Is Expressed at Similar Levels in Tolerant and Nontolerant Mice. Several reports indicate that the extent of T cell tolerance to self-determinants often correlates with quantitative expression of the self-protein (28, 29). To test this possibility, we compared the quantity of L^d ex-

No spontaneous proliferation of a control BALB/c anti-HEL 105-120 peptide T cell line was observed under the same experimental conditions (data not shown). (C) Different APCs expressing A^d/E^d class II molecules were tested for their ability to induce the proliferation of the E^d-restricted anti-L^d 61-80 T cell line dm2-P69.2 either in the absence of exogenously added peptide (open bars) or in the presence of the peptide L^d 61-80 (solid bars). B10.A APCs were used as control APCs.

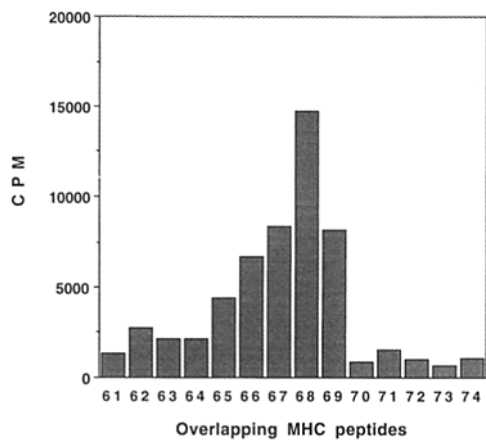


Figure 4. The proliferative response of BALB/c-dm2 lymph node cells to overlapping L^d MHC peptides after immunization with the peptide L^d 61-80. Results are expressed as cpm obtained with in vivo L^d 61-80-primed BALB/c-dm2 spleen cells restimulated in vitro with a series of MHC L^d singly overlapping peptides (12 mers). The peptide number shown at the bottom of each panel corresponds to the position of the amino-terminal residue of the peptide in the MHC class I sequence. The average non-MHC peptide background responses of six to eight wells \pm SD were $1,220 \pm 610$ cpm.

pressed at both messenger RNA and surface protein levels in tolerant (BALB/c, B10.D2) versus nontolerant (B10.A, A/J) mice. The level of expression of L^d mRNA in tolerant and nontolerant mice was identical as determined using an L^d-specific probe (30) in a quantitative RNase protection assay (data not shown). Next, we used an anti-L^d mAb (30-5-7) (26) to compare the level of L^d protein cell surface expression in L^d 61-80-tolerant and -nontolerant mice. The 30-5-7 mAb was fluoresceinated and used in direct binding assays to detect the surface expression of L^d by cytofluorometry. As shown in Fig. 5, BALB/c splenocytes expressed significant levels of L^d surface antigen, whereas no L^d protein was detected after exposure of control dm2 (L^d-deficient) cells to 30.5.7 mAb. No quantitative differences in the expression of L^d could be detected on both spleen and thymus cells of tolerant (BALB/c) and nontolerant (A/J) mouse strains tested. Similar results were obtained with B10.D2 and B10.A mice (data not shown). Collectively, these results show that similar amounts of L^d mRNA and protein are expressed in both L^d 61-80-tolerant and -nontolerant mice. We therefore conclude that low expression of the autoantigen does not account for the crypticity of its determinant L^d 61-80 in H-2^a mice.

Mapping of the MHC Locus Region That Controls the MHC Class II-restricted Presentation of the Self-Peptide L^d 61-80. To identify the region of the MHC locus that contains the gene(s) involved in the processing and presentation of the self-peptide L^d 61-80, we analyzed the T cell response to this peptide in a series of H-2 recombinant strains. As shown in Table 2, the peptide L^d 61-80 represents a self-component for all the mice selected in this study since they either express the L^d class I molecule or the L^a molecule

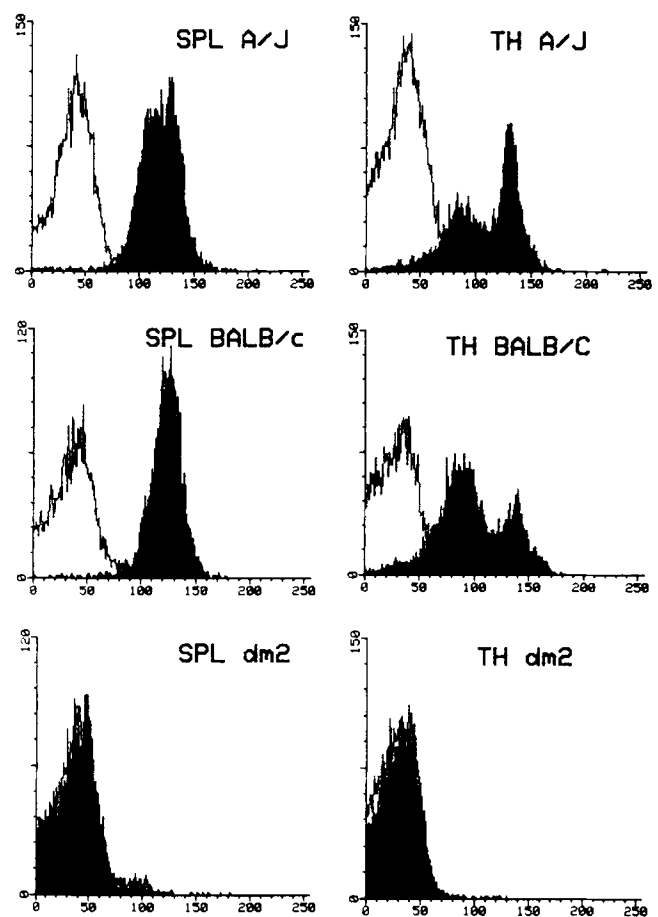


Figure 5. Flow cytometry analysis of L^d cell surface expression in different mouse strains. Both spleen and thymus cells from A/J, BALB/c, and L^d-deficient BALB/c-dm2 mice were stained with FITC-conjugated anti-L^d mAbs, 30.5.7 at saturating concentrations and analyzed by flow cytometry (solid profiles). Open profiles were obtained after staining of the same cells with a nonspecific, isotype-matched mAb.

whose sequence is identical to L^d for residues 61-80. L^d 61-80 self-peptide was found immunogenic in B10.AKM mice (Table 2). Nontolerant B10.A and B10.AKM mice differ from tolerant B10.D2 mice in the portion of the H-2 region that is centromeric to the *D* gene, suggesting that the MHC gene(s) that govern(s) self-peptide L^d 61-80 processing is located in this region (Table 2). On the other hand, the L^d 61-80 self-peptide was cryptic in A.TL and B10.MBR mice while it was dominant in the parental H-2^s and H-2^b mice. Although unlikely, the absence of E molecules could have contributed to the nonresponder phenotype in the parental strains. A.TL and B10.MBR mice differ from A/J and B10.AKM mice, respectively, at the H-2 K locus (Table 2), thus excluding the involvement of this area in the processing of L^d 61-80 peptide. It is noteworthy that A.TL, B10.D2, and BALB/c mice share Qa1, Qa2, and Tla alleles, thus ruling out the contribution of this region to L^d 61-80 phenotype. Together, these results suggest that the region of the MHC locus of H-2^a mice flanked by

Table 2. Mapping of the H-2 Region Gene(s) That Control(s) the Presentation of L^d 61-80

Strain	H-2	K	A	E	L	D	Medium	L ^d 61-80
BALB/c	d	d	d	d	d	d	2,410 ± 380	2,890 ± 450
BALB/c dm2	dm2	d	d	d	-	d	2,740 ± 1,520	<u>36,120 ± 4,660</u>
B10.D2	d	d	d	d	d	d	6,120 ± 320	<u>5,410 ± 910</u>
DBA/2	d	d	d	d	d	d	2,130 ± 380	3,870 ± 820
B10.A	a	k	k	k	d	d	1,220 ± 210	<u>98,630 ± 5,140</u>
A/J	a	k	k	k	d	d	1,720 ± 440	<u>72,140 ± 2,710</u>
A.TL	tl	s	k	k	d	d	9,210 ± 2,990	<u>88,220 ± 3,110</u>
B10.AKM	m	k	k	k	q	q	6,330 ± 990	<u>63,620 ± 4,150</u>
B10.MBR	bq1	b	k	k	q	q	1,100 ± 280	<u>14,130 ± 2,680</u>

Results are expressed as cpm recorded with lymph node cells from different H-2 recombinant mouse strains immunized with the peptide L^d 61-80. Lymph node cells from primed mice were tested for [³H]thymidine incorporation after in vitro restimulation with the immunizing L^d 61-80 peptide or in the absence of peptides. The values that are significantly over the background counts ± SD are underlined.

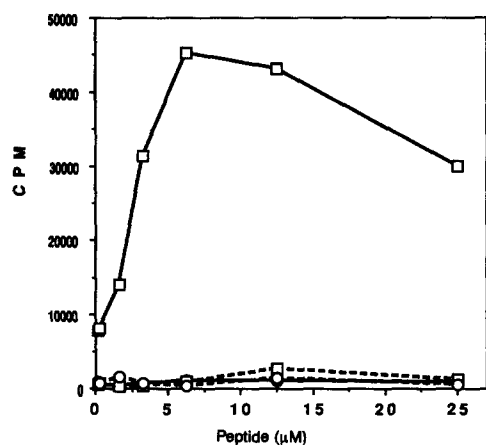


Figure 6. The self MHC class I peptide L^d 61-80 is nonimmunogenic in syngeneic B10.A(5R) mice. Results are expressed as cpm recorded with lymph node cells from B10.A(5R) mice immunized with the peptide L^d 61-80 (dashed lines) or the HEL control antigen (solid lines) and restimulated in vitro with different doses of the L^d peptide (○) or a control HEL peptide (□). The data shown here are representative of five mice tested individually. The background values (cells without antigens) ranged from 2,980 to 7,450 cpm.

the two class I genes *K* and *D* contains the gene(s) whose product(s) prevent(s) the processing and presentation of the self-peptide L^d 61-80 to T cells.

To further constrict the location of the gene(s) involved in the presentation of L^d 61-80, we then examined the immunogenicity of the self-peptide in B10.A(5R) H-2 recombinant mice. This strain was of particular interest because although the telomeric portion of their MHC locus is common with other H-2^a mice, it has an additional recombination site located in the class II *E^bβ* gene (referred to as *E^b*) (see Fig. 8). As shown in Fig. 6, no T cell proliferation was detected after immunization of B10.A(5R) mice with the peptide L^d 61-80. Importantly, in contrast to B10.A APCs, B10.A(5R) splenocytes could stimulate the proliferation of the E^k-restricted B10.A anti-L^d 61-80 T cell line (Fig. 7). C57BL/6 (H-2^b) and (C57BL/6 × BALB/K) F1, (H-2^b × H-2^k) F1 APCs expressing A^b and E^b, did not stimulate B10.A-P11.2, thus excluding the hypothesis of an alloreactive response (data not shown). Taken together, the lack of immunogenicity of L^d 61-80 and its continuous presentation at the surface of APCs show that L^d 61-80 represents a dominant self-peptide in B10.A(5R) mice.

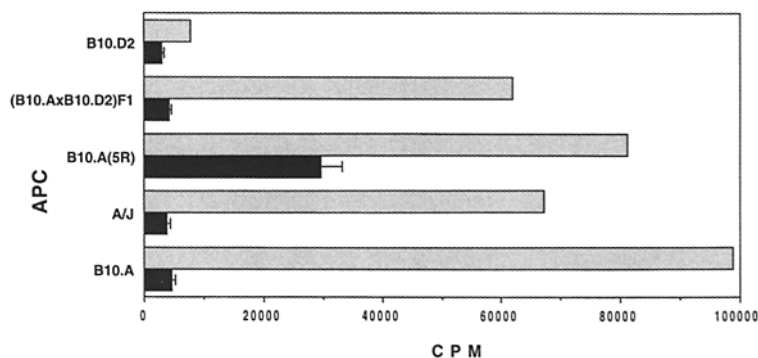


Figure 7. B10.A(5R) APCs present spontaneously the self-peptide L^d 61-80 to the T cell line B10.A-P11.2. Different APCs expressing A^k/E^k class II molecules (irradiated splenocytes from [B10.A × B10.D2] F1, A/J, and B10.A[5R] mice) were tested for their ability to induce the proliferation of the E^k-restricted anti-L^d 61-80 T cell line, B10.A-P11.2, either in the absence of exogenously added peptide (solid bars) or in the presence of their specific peptide L^d 61-80 (shaded bars). B10.D2 APCs were used as control APCs.

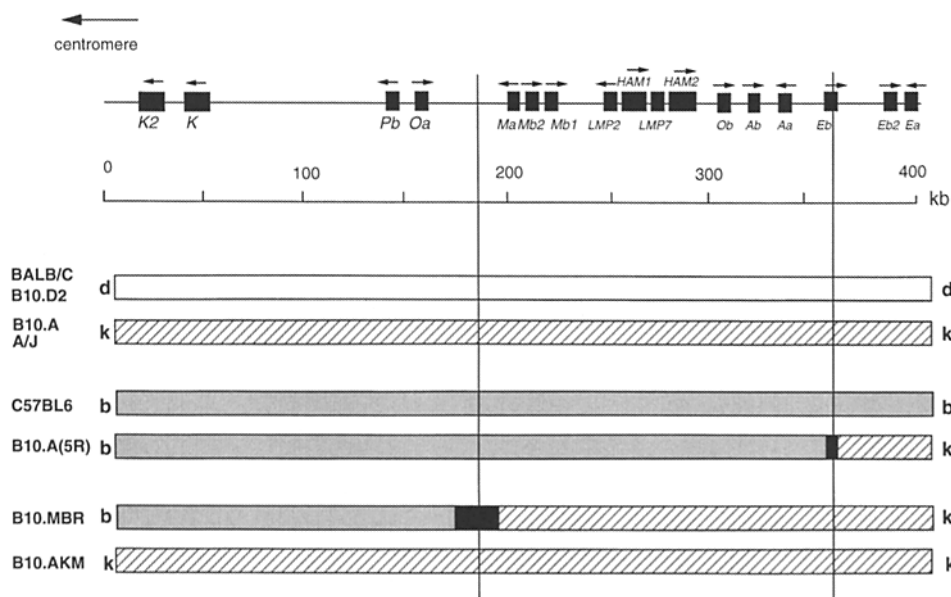


Figure 8. Schematic picture of the murine MHC class II region including the recombination regions of the mouse strains used in this study. Letters flanking the “recombination bars” indicate the MHC haplotypes. The arrows indicate the direction of gene transcription.

We conclude that the gene(s) responsible for the phenotype of the peptide L^d 61-80 must reside centromeric of the *Eβ* gene and telomeric of the K locus. Furthermore, the observation that L^d 61-80 was dominant in B10.A(5R) and cryptic in B10.MBR allowed us to refine the mapping of the gene(s) controlling L^d processing to a region of ~170 kb delimited by the intrarecombinational points in these two mouse strains (Fig. 8).

Discussion

Recent studies have revealed that certain self-peptides (cryptic self) do not reach the threshold of presentation that ensures tolerance induction of developing T cells (21, 22). Meanwhile, there is increasing evidence that circumstances which lead to the presentation of cryptic self-determinants in adults result in the initiation of an autoimmune process (27, 31, 32). It is therefore crucial to determine which factors control the *in vivo* processing and presentation of self-antigens. In this paper, the self-MHC class I peptide L^d 61-80 was used as a model antigen to elucidate the mechanisms that govern the presentation *in vivo* of self-peptides for T cell tolerance induction.

The self-peptide L^d 61-80 was previously reported to be immunogenic in syngeneic B10.A mice while here we observed that it could not induce any *in vivo* T cell responses in BALB/c mice. We have accumulated much evidence that associates lack of tolerance in B10.A mice to L^d 61-80 with poor or incomplete processing of this self-peptide. In this report we show that, in contrast to B10.A APCs, the self-peptide L^d 61-80 was naturally processed and continuously presented in association with MHC class II by BALB/c APCs; a phenomenon that ensured the deletion/inactivation of L^d 61-80-reactive T cells during development. Therefore, the cryptic phenotype of L^d 61-80 we previously reported in B10.A mice does not represent an intrinsic

property of this self-peptide as it is dominant in another mouse strain, BALB/c.

The quantity of self-protein available for presentation to autoimmune T cells plays a critical role in the process of tolerance induction to self-determinants present on this protein (28, 29). Here, we showed that APCs derived from tolerant and nontolerant mouse strains expressed similar amounts of the L^d self-antigen at both mRNA and protein levels. It was, however, possible that the presentation of the L^d 61-80 peptide was rather dependent on the extracellular level of L^d protein. Supporting this hypothesis, Stockinger et al. showed that T cell tolerance induction to peptides derived from the fifth component of the complement (C5) was merely dependent on the presence of the self-protein in mouse serum (33). Here, two lines of evidence indicate that circulating L^d protein does not contribute to the presentation of the peptide L^d 61-80 by APCs: (a) Recent evidence has been provided that L^d 61-80 peptide is exclusively processed from intracellular L^d protein (24); (b) No correlation between L^d protein serum levels in H-2^a and H-2^d mice and tolerance to the self-peptide L^d 61-80 was found (data not shown). Therefore, defective presentation of L^d 61-80 in B10.A mice does not result from insufficient expression of the self-protein, but rather from the B10.A APC's inability to process this self-peptide.

Analyses of the autologous peptides bound to MHC molecules, as well as determination of the molecular and cellular events involved in antigen processing, have begun to shed light on the mechanisms by which determinants on self-protein are selected for presentation to T cells. Recent studies using genetically engineered antigen processing-deficient mice showed the contribution of different processing cofactors to antigen presentation *in vivo* and suggested their involvement in the acquisition of T cell repertoire during development (34, 35). However, it remains unclear whether allelic polymorphism of the MHC genes encoding

these processing cofactors influences the selection of the T cell repertoire of normal mice. Here, we observed that tolerogenicity or immunogenicity of the self-peptide L^d 61-80 was a result of differential processing of the self-L^d protein in different mouse strains. This finding gave us a unique opportunity to investigate the contribution of antigen processing to *in vivo* T cell tolerance induction in normal mice.

The results presented in this paper indicate that continuous presentation of L^d 61-80 peptide by APCs results in tolerance induction of autoreactive T cells in BALB/c and other H-2^d mice. Interestingly, Loss et al. showed that intracellular, endogenous, L^d protein represented the unique source of self-antigen for continuous processing and presentation of the peptide L^d 61-80 by BALB/c APCs (24). This study demonstrated sole involvement of the endogenous processing pathway in the presentation of L^d 61-80, by MHC class II molecules. Together with our results, this indicates that self-peptides processed from intracellular self-proteins play an important role in the elimination/inactivation of class II-restricted CD4⁺ developing autoreactive T cells.

MHC class II-restricted presentation of L^d 61-80 peptide from internally synthesized L^d protein segregates from both exogenous and endogenous presentation pathways (24). We therefore hypothesized that either class I or class II processing-associated cofactors could play a role in this "non-classical" antigen processing pathway. Here, we showed that class II presentation of the self-peptide L^d 61-80 for tolerance induction of corresponding CD4⁺ autoreactive T cells was controlled by gene(s) which maps to a 170-kb portion of the class II region of the MHC locus. *Ma* and *Mb* genes located in this class II region of MHC locus have recently been shown to play an important role in class II-restricted processing. H-2M molecules presumably promote antigen presentation by "freeing" MHC class II peptide-binding grooves from Ii peptides in the endosomes thus allowing the binding of other antigen peptides to MHC class II molecules (17, 18). Alternatively, the factor(s) involved in L^d 61-80 processing mediates its function by disrupting the presentation of the peptide. H-2M molecules and the L^d 61-80 processing-associated factor, therefore, seem to be distinct. Another molecule, the Ii, also contributes to the selection of class II-restricted peptides by targeting class II molecules to selected endosomal subcompartments (15). However, Ii is encoded outside the MHC locus, and a class II-restricted L^d 61-80-specific T cell hybridoma could be stimulated in the presence of both Ii-positive and -negative APCs (24). We conclude that Ii does not control the presentation of the L^d peptide.

Among the genes associated with MHC class I processing, TAP genes are unlikely to be responsible for the phenotype of L^d 61-80. First, L^d 61-80 presentation by class II molecules does not require a functional TAP-1 protein (24). Second, although limited allelic polymorphism in the mouse transporter genes has been found, it does not seem to have a major impact on the selection of the pool of pep-

tides available for presentation (36, 37). Alternatively, the LMP-7 subunit of the proteasome, despite its documented involvement in the class I processing pathway, could represent a possible candidate for regulating the presentation of L^d 61-80. Supporting this view, H-2^k and H-2^d mice express different LMP-7 alleles (38, 39). The LMP-7 allelic form expressed by H-2^a, but not H-2^d mice, by cleaving the L^d molecule at a particular site could destroy the L^d 61-80 determinant and therefore prevent its presentation in MHC class II context to autoreactive thymic T cells. Work to address this possibility is in progress in our laboratory.

The lack of spontaneous presentation of the self-peptide L^d 61-80 in H-2^a, but not H-2^d, mice results in incomplete tolerance induction of corresponding autoreactive T cells. Our results show that a factor encoded within a 170-kb portion of the H-2^a, but not H-2^d, MHC locus prevents the presentation of the self-peptide L^d 61-80. This factor presumably mediates its function by disrupting the processing of the antigen peptide. It is possible that this processing factor cleaves L^d protein within the sequence 61-80 thus destroying the determinant. Another alternative is that this factor cleaves L^d protein outside the sequence 61-80. This would create another determinant that cannot bind to MHC class II molecules or that could trigger another set of T cells. Finally, this factor could mediate its effect by binding L^d 61-80 peptide, thus preventing its association with E^k MHC class II restriction elements. It is important to note that B10.A APCs do not apparently have a major defect in antigen processing since they can present a large variety of endogenously and exogenously processed peptides of self- and foreign origin at their surface. Allelic variation of processing factors may control the selection of certain self-peptides thus contributing to the acquisition of the T cell repertoire in a given strain.

In conclusion, we have shown that while tolerance induction of CD4⁺ T cells is generally attributed to exogenous processing and presentation of circulating self-proteins, endogenously processed self-peptides presented in association with MHC class II molecules also contribute to the selection of developing CD4⁺ T cells in the thymus. In addition, we observed that MHC class II-restricted presentation of the endogenously processed self-peptide L^d 61-80 for T cell tolerance induction is controlled by a factor encoded within a 170-kb portion of the class II region of the MHC locus. Apparently, this factor mediates its function by preventing the presentation of the self-peptide in H-2^a but not H-2^d mice and therefore seems to segregate from previously described antigen processing cofactors. This suggests that allelic polymorphism in antigen processing cofactor genes, by contributing to the selection of self-peptides available for presentation by self-MHC molecules, may play an essential role in tolerance induction and acquisition of the T cell repertoire during ontogeny. Therefore, identification of the antigen processing factors and elucidation of their functions may be crucial for understanding genetic susceptibility to certain autoimmune diseases and the mechanisms that initiate self-reactive T cell responses.

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