Amino-Terminal Flanking Residues Determine the Conformation of a Peptide—Class II MHC Complex

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Amino-Terminal Flanking Residues Determine the Conformation of a Peptide–Class II MHC Complex

Scott B. Lovitch,2 Zheng Pu,2 and Emil R. Unanue3

The peptide spanning residues 48–62 of hen egg white lysozyme presented by I-Ak molecules is selected as families, with a common core of nine residues occupying the binding groove of the MHC molecule, and flanks of variable length extending from the open ends of the groove. The energy of peptide binding derives from conserved hydrogen bonds between the peptide backbone and nonpolymorphic residues of the MHC molecule, as well as from interactions between side chains of the bound peptide, most notably residues P1, P4, P6, and P9, and specificity pockets formed by polymorphic residues within the binding groove (for reviews, see Refs. 1 and 2).

Importantly, the plasticity of peptide–class II MHC interaction permits formation of multiple conformational isomers by the same peptide and MHC molecule. We have investigated this phenomenon through our studies of two T cell subsets, referred to as type A and type B, primed by the dominant 48–62 epitope of hen egg white lysozyme (HEL)3 (3) presented by I-Ak molecules. Whereas type A T cells respond to both native HEL and exogenous peptide, type B T cells respond only to exogenous peptide and fail to respond to the identical epitope generated by processing of native HEL (3, 4). This phenomenon was first observed in studies of a so-called cryptic epitope (4) but has since been observed with regard to a variety of epitopes and MHC molecules and appears to be a general feature of class II MHC–peptide interaction (5–8). Self-reactive type B T cells escape negative selection under conditions in which type A T cells are deleted in the thymus, suggesting that they may play a role in autoimmunity (9, 10). Furthermore, a subset of type B T cells recognize peptides as short as seven residues; we speculate that these may be involved in host defense, responding to short peptides of microbial origin (11).

We recently determined the pathways by which the conformers recognized by type A and B T cells are generated in APCs, using liposome-mediated Ag delivery to determine the intracellular sites of formation of the two conformers (12). Whereas peptides delivered to early endosomes could form both conformers, peptides delivered to late endosomes only formed the type A conformer. This segregation was fully dependent on the action of the class II–like accessory molecule H2-DM (the murine equivalent of HLA-DM in humans; herein abbreviated DM), because APCs lacking DM could form the type B conformer in late endosomes. Furthermore, DM abolished the type B conformer in vitro; peptide–MHC complexes exposed to DM stimulated only type A T cells, while complexes not exposed to DM stimulated both T cell subsets. We concluded that DM edits the conformation of the complex, eliminating the type B conformer in late endosomes and promoting presentation of the most stable conformer, i.e., type A. As a result, processing of native protein (which requires denaturation and proteolysis in late endosomes) only results in formation of the type A conformer, whereas exogenous peptide, which can form complexes in the absence of DM by peptide exchange in recycling endosomes, generates both conformers.

DM acts at multiple stages in the presentation of peptides by class II MHC: it releases the invariant chain–derived CLIP peptide from newly synthesized class II molecules (13, 14); functions as a general catalyst of peptide dissociation, accelerating release of weakly bound peptides and favoring presentation of those binding with high affinity (15–19); and chaperones class II, binding to and preventing degradation of molecules lacking bound peptide (20, 21). The mechanism underlying these disparate functions is under considerable discussion; one model suggests that DM catalyzes a conformational transition from a peptide-bound state to an open state from which the peptide rapidly dissociates, and subsequently stabilizes this open state (22). The interface of interaction between DM and class II, determined by Mellins and colleagues (23, 24) for HLA-DM and HLA-DR1 using random mutagenesis, is contained
within a single lateral face of each molecule; significantly, it also includes the amino terminus of the bound peptide, implying that this region of the peptide might be of particular importance for DM function.

Although several groups have investigated the role played by flanking residues in the catalytic mechanism of DM, their effects remain unclear. Although the intrinsic stability of a peptide-MHC complex clearly influences the efficacy of DM catalysis (18, 19), early studies by Jensen and colleagues (16) showed no correlation between peptide length and DM-mediated acceleration of peptide release. More recently, Mellins and colleagues (25) examined several MHC-peptide complexes and found that amino-terminal flank length did not correlate with DM susceptibility, although they did observe a weak correlation with the length of the carboxyl-terminal flank. Wiley and coworkers (26) specifically examined the role of the peptide amino terminus in DM function and found that attachment of a DR1-bound peptide to DM by placement of a cysteine residue at its amino terminus resulted in enhanced catalysis of DM-mediated release from DR1, whereas attachment at the carboxyl terminus did not. In a subsequent study, modification of the amino terminus to prevent formation of hydrogen bonds enhanced the ability of DM to catalyze dissociation of the peptide; this was not the case with modifications of the core or carboxyl terminus (27).

For this study, we reasoned that the amino terminus of the peptide might play a critical role in determining the conformation of the complex. Like most peptides selected for binding by class II molecules, the 48–62 epitope of HEL contains several amino-terminal flanking residues that contribute to peptide-MHC binding and enhance the stability of the complex (28, 29). The crystal structure of the 48–62/α1β2 complex revealed an extensive network of contacts between the amino-terminal flank of the peptide and the MHC molecule, although flanking residues distal to P(−2) were not resolved (30). Many type B T cells respond preferentially to peptides lacking the amino-terminal flank, suggesting that this region of the peptide disproportionately influences conformation (11). Here, we demonstrate that the amino-terminal flank of the peptide is specifically required for editing by DM. (In this paper, “editing” refers to loss of the type B conformer, as evidenced by the lack of reactivity of type B T cells; the term does not imply a particular mechanism of action.) Further, we show that this requirement is mediated through an interaction between the side chain of the P(−2) residue of the peptide and the MHC molecule. Thus, the peptide amino-terminal flank plays a previously unappreciated role in determining the conformation of peptide-MHC complexes and their susceptibility to editing by H2-DM.

**Materials and Methods**

**Mice**

B10.BR mice were purchased from The Jackson Laboratory, DM−/− (H-2d) mice (31) were a generous gift from Dr. Elizabeth Bikoff (Wellcome Trust Centre for Human Genetics, Oxford, U.K.). All mice were maintained under specific pathogen-free conditions at the Washington University School of Medicine in accordance with all institutional animal care guidelines. Peritoneal macrophages were elicited from mice of either sex between ages 6 and 20 wk.

**Peptide synthesis**

All peptides were synthesized using fluorenylmethoxycarbonyl (Fmoc) chemistry on a Symphony Multiplex peptide synthesizer (Protein Technologies), purified to homogeneity by C18 reverse-phase HPLC, and verified by mass spectrometry before use in assays. 125I-labeled peptide was prepared using the chloramine-T method (32).

**Liposome preparation and assays**

Dioloylphosphatidylcholine (DOPC), dioloylphosphatidylethanolamine (DOPE), and dioloylphosphatidylserine (DOPS) were purchased from Avanti Polar Lipids and dissolved in chloroform. Cholesterol hemisuccinate (CHEMS) was purchased from Sigma-Aldrich and dissolved in methanol. All lipids were stored under nitrogen at −20°C.

Encapsulation of peptide in liposomes, and assays testing presentation of liposome-encapsulated peptide, were conducted as previously described (12, 33). Briefly, DOPC/DOPS or DOPE/CHEMS were combined at a molar ratio of 4:1 (10 μmol total lipid); the mixture was dried under nitrogen, lyophilized to remove residual solvent, and reconstituted in 1 ml 0.2% PBS plus 0.2 mM EDTA containing 60–100 μM peptide, with a trace amount of 125I-labeled peptide added to measure incorporation. Liposomes were rehydrated at 42°C for 30 min, freeze-thawed 5 times in dry ice/ethanol, passed 11 times through a Liposofast extruder (Avestin) with a 200-nm-pore-size polycarbonate filter, treated with 0.2 mg/ml Pronase E (Roche) for 2 h at 37°C to degrade any unencapsulated peptide, and dialyzed overnight against PBS plus 0.2 mM EDTA. Efficiency of encapsulation (generally 8–10% of input peptide) was determined by quantitating recovery of radiolabeled peptide using gamma scintillation counting (Wallac); the concentration of encapsulated peptide was calculated from this value, the amount of input peptide, and the final volume after dialysis. Liposomes were used immediately in assays or stored at 4°C for a maximum of 1 wk. To assay presentation, peritoneal exudate cells were elicited by intraperitoneal injection of B10.BR or DM–/– mice with 100 μg Con A (Sigma-Aldrich) in pyrogen-free saline. Mice were sacrificed 24 h after injection, and cells were harvested by peritoneal lavage with DMEM plus 5% FCS. Cells (106/well) were transferred to flat-bottom 96-well plates (Costar) and adhered for 1 h at 37°C and 5% CO2. Liposomes diluted in DMEM plus 5% FCS were added, and cells were incubated for 2 h at 37°C, then fixed in 1% paraformaldehyde and 0.2 M lysine and washed extensively. Hybridoma cells (5 × 103/well) were added and incubated for 18 h, and supernatants were assayed for IL-2 content by CTLR proliferation (2 × 103/well), with [3H]thymidine added for the final 8 h of a 24-h culture. Incorporation was measured by beta scintillation counting (Wallac).

**Expression of soluble I-Ak and DM**

The constructs used to express recombinant, soluble I-Ak–CLIP and H2-DM were described previously (12). I-Ak–CLIP contains an acidic leucine zipper and hexahistidine tag fused to the carboxyl terminus of the β-chain, a basic leucine zipper fused to the carboxyl terminus of the α-chain, and the CLIP peptide fused to the amino terminus of the β-chain; CLIP is a small peptide and the β-chain; CLIP is a small peptide. DM contains an enterokinase cleavage site, basic leucine zipper, and hexahistidine tag fused to the carboxyl terminus of the β-chain, such that cleavage with thrombin results in association of I-Ak–CLIP and DM through interaction of the leucine zippers on the two β-chains. Constructs were transfected into SF9 cells to generate recombinant baculoviruses, and proteins were expressed by infection of insect cells using standard protocols and purified over Ni-NTA-Agarose (Qiagen).

**Kinetic analysis of MHC-peptide dissociation**

To assess dissociation of peptide–I-Ak complexes, thrombin-treated I-Ak–CLIP (generally 1 mg) was incubated overnight at 37°C, pH 5.5, with 125I-labeled peptide. The pH was neutralized, MHC-peptide complexes were isolated by gel filtration, and bound radioactivity was quantitated. Samples were divided in two and, 200 nM H2-DM was added to one of the samples; both samples were readjusted to 150 mM NaCl, pH 5.5, and excess unlabeled peptide was added to prevent reassociation of labeled peptide (this time point was defined as t = 0). At various time points thereafter, aliquots were isolated, free peptide was removed by gel filtration, and the amount of radioactivity remaining bound to the complex was measured. Plots of remaining bound reactivity vs time were fitted to single- or double-exponential decay curves using Prism version 4.0 (GraphPad).

**T hybridoma assays using in vitro MHC-peptide complexes**

To assay persistence of the type A and B conformers over time, thrombin-treated I-Ak–CLIP (200 nM) was combined with 320 nM peptide and incubated overnight at 37°C in 150 mM NaCl, pH 5.5. Free peptide was then removed by buffer exchange using a CentriTec YM-30 concentrator (Millipore), and complexes were incubated at 37°C and pH 5.5 for the indicated lengths of time (0–7 days). Complexes were then neutralized, concentrated, centrifuged for 10 min at 10,000 rpm to remove aggregates, transferred to flat-bottom 96-well plates, adhered by incubation for 3–4 h at 4°C, and washed extensively. Hybridoma cells (1 × 105/well) were added...
and incubated overnight at 37°C and 5% CO₂, after which IL-2 production was assessed by CTLL assay.

For assays determining susceptibility to conformational editing by DM, thrombin-treated I-A<sup>δ</sup>-CLIP was incubated overnight with peptide in the absence of H<sub>2</sub>-DM as above; 200 nM DM was then added (or not), and the incubation continued for an additional 2 h at 37°C and pH 5.5. Complexes were then neutralized, concentrated, centrifuged, and transferred to 96-well plates, and hybridoma responses were assayed as above.

For all presentation assays, the data reported are representative of testing at least three cells of each type; these, in turn, were selected from a larger panel of hybridomas whose pattern of response has been established in multiple experiments (11). The hybridomas chosen are representative of the larger panel with respect to sensitivity and response to liposomal vs free peptide (Refs. 11, 12, and data not shown).

Results

Peptides lacking flanking residues are resistant to conformational editing

The full-length HEL (48–63) peptide (sequence DGSTDYGIL-QINSRWQ, with the underlined aspartic acid residue serving as the obligate P1 anchor; Ref. 34) contains four amino- and two carboxyl-terminal flanking residues. To evaluate whether these residues were necessary for conformational editing by DM, we tested the responses of type A and B hybridomas to a truncated peptide, 52–61 (DYGILQINSR), that lacks all flanking residues except for the arginine residue at P10. This peptide was encapsulated in DOPC/DOPS liposomes that deliver their contents to late endosomes, or in DOPE/CHEMS liposomes that open in early endosomes (12, 33, 35, 36), and its presentation, compared with free peptide, was tested to type A and B hybridomas. In our previous study, type A hybridomas responded to the free full-length peptide and to both forms of liposomal peptide, whereas type B hybridomas failed to respond to peptide delivered to late endosomes, responding only to free peptide and to peptide delivered to early endosomes (12).

The type A response to the truncated peptide was identical to that reported with the full-length peptide; the type A hybridoma P1.1A1 recognized 52–61 whether delivered to early or to late endosomes or provided as free peptide (Fig. 1A). In contrast, the response of type B hybridomas to the truncated peptide differed from that observed with the full-length peptide. Type B hybridomas were fully capable of responding to the truncated peptide delivered to late endosomes, as shown for CP1.7 (Fig. 1B) and CP3.42 (Fig. 1C; see also Fig. 2, A–C). In fact, presentation of the type B conformer for these short peptides was enhanced upon delivery to late endosomes, as type B hybridomas responded to 52–61 encapsulated in DOPC/DOPS liposomes at doses 10<sup>4</sup>-to 10<sup>5</sup>-fold lower than were required for free peptide; with the full-length peptide, such enhancement was observed only for presentation of the type A conformer (12). Therefore, removing the flanking residues of the peptide rendered it resistant to conformational editing.

Editing depends on the length of the amino-terminal flank

The responses of type A and B hybridomas to a variety of peptides delivered to late endosomes were subsequently tested to determine which flanking residues were necessary for editing; these peptides shared the core sequence (52–61) but varied in the length of their flanks. The responses of type A hybridomas improved when peptides were delivered to late endosomes, regardless of the length of the amino-terminal flanks (Fig. 2A). However, the type B response varied with the length of the peptide. As expected from our previous results (12), the full-length 48–61 peptide was edited, i.e., the type B response to the peptide was absent upon delivery to late endosomes. Similarly, 49–61, containing three amino-terminal flanking residues, was edited. However, 52–61 was resistant to editing, as the type B response was maintained. Two peptides with flanks of intermediate length, 50–61 (two residues) and 51–61 (one residue), induced type B responses between those seen with the truncated and full-length peptides (Fig. 2B, C and D).

Testing presentation by peritoneal macrophages from DM-deficient mice (31) proved that these findings reflected the ability of DM to edit the conformation of the complex. In the absence of DM, the enhancement of presentation observed when peptides were delivered to late endosomes of wild-type APCs was lost; the sensitivity of the response to liposomal peptide was the same as for free peptide, both for type A (Fig. 2D) and for type B hybridomas (Fig. 2, E and F). Moreover, the type B response to liposomal and free peptide was no longer dependent on amino-terminal flank length, as both CP1.7 and CP3.42 responded similarly to each of the peptides (Fig. 2, E and F). Thus, enhancement of peptide loading by DM in late endosomes was universal, occurring irrespective of peptide length and for both the type A and, where applicable (i.e., 52–61), the type B conformers. In contrast, conformational editing by DM was highly dependent on amino-terminal flank length and specific for the type B conformer. This observation implies that the function of DM as a catalyst of peptide loading can be dissociated from its function as a conformational editor, a point that is addressed in detail below.

Adding residues to the carboxyl terminus of the core peptide did not restore its ability to be edited by DM; neither addition of one or both carboxyl-terminal tryptophan residues (52–62 and 52–63, respectively) to the 52–61 peptide nor addition of one or both carboxyl-terminal flanking residues (52–61 or 52–62, respectively) to the 52–61 peptide restored its ability to be edited by DM.

![FIGURE 1](https://example.com/figure1.png)

**FIGURE 1.** Truncated peptide lacking flanking residues is resistant to conformational editing by H2-DM. Synthetic 52–61 peptide was encapsulated in DOPC/DOPS (■) or DOPE/CHEMS (○) liposomes as described in Materials and Methods. Peritoneal macrophages were elicited from B10.BR mice, adhered to 96-well flat-bottom plates, and incubated in the presence of liposomal or free peptide (▲) for 2 h at 37°C, then fixed and washed. The type A hybridoma P1.1A1 (A) or the type B hybridoma CP1.7 (B) or CP3.42 (C) was then added at a density of 5 × 10<sup>4</sup> cells/well and incubated overnight at 37°C, after which supernatants were tested for IL-2 content as measured by CTL assay. Each hybridoma was tested at least twice in independent experiments, and each data point represents the mean cpm of triplicate wells; error bars indicate the SD. None of the hybridomas responded to liposomes lacking peptide (data not shown).
respectively), nor extension of the carboxyl terminus with up to four alanine residues, prevented formation of the type B conformer in late endosomes (Fig. 2, G–L). These results indicated that susceptibility to editing was specifically dependent upon the length of the amino-terminal flank.

We also tested the responses of type A and B T cells to soluble I-Aκ molecules loaded with peptide in vitro, then incubated for 2 h in the presence or absence of DM, in a protocol identical to that reported previously (12). This system eliminates the possibility of peptides becoming resistant to editing through removal of flanking...
residues by endosomal aminopeptidases, which we suspect accounts for the intermediate type B responses observed upon delivery of amino-terminal flank variant peptides to late endosomes (Fig. 2, B and C). The results of these experiments are displayed in Fig. 3 and summarized in Table I. In brief, all peptides interacted and complexed with I-A^K molecules, as indicated by direct binding assays (tested previously; data not shown) and by stimulation of the type A hybridoma 3A9 (Fig. 3, A–D). In the absence of DM, all peptides stimulated the type B hybridoma CP1.7; as expected from our previous study (12), treatment of the 48–61/I-A^K complex with DM for 2 h abolished presentation to the type B hybridoma CP1.7. Thus, two amino-terminal flanking residues were required for DM to edit the conformation of peptides whose amino-terminal flanks had been replaced by polyalanine. The peptide containing two amino-terminal alanine residues (AAA-52–61) was not edited (i.e., was resistant to editing by DM). To investigate possible side-chain interactions, we tested presentation of peptide variants of 50–61 with substitutions at the P(−2) position. All peptides induced a type A response, regardless of the presence of DM, as assessed by CTL assay. Data points represent the mean cpm of triplicate wells, and error bars reflect SD; each hybridoma was tested at least twice with each peptide in independent experiments. E–H, Two amino-terminal peptide flanking residues are required for elimination of the type B conformer in vitro by DM. Assay conditions were the same as in A–D, but presentation to the type B hybridoma CP1.7 was tested. Note that in H the two curves are superimposed.

The finding that 50–61, with two amino-terminal flanking residues, was the shortest of the peptides edited by DM could reflect the paramount importance of length per se (i.e., of backbone interactions) or a specific interaction involving the P(−2) residue. To distinguish these possibilities, we assessed the susceptibility to editing of peptides whose amino-terminal flanks had been replaced by polyalanine. The peptide containing two amino-terminal alanine residues (AA-52–61) was resistant to editing by DM (i.e., was presented to CP1.7), whereas the wild-type 50–61 peptide (ST-52–61) was edited (i.e., its presentation was abolished by DM). Extension of the peptide amino terminus with three or more alanine residues conferred susceptibility to editing by DM (Table I). Therefore, susceptibility to editing could be conferred through sequence-dependent (i.e., side-chain) interactions involving the P(−2) residue, as well as through length-dependent interactions involving the peptide backbone.

To investigate possible side-chain interactions, we tested presentation of peptide variants of 50–61 with substitutions at the P(−2) position. All peptides induced a type A response, regardless of the presence of DM, as assessed by CTL assay. Data points represent the mean cpm of triplicate wells, and error bars reflect SD; each hybridoma was tested at least twice with each peptide in Table I. Each peptide was tested at least twice in independent experiments.

Table 1. Formation and editing of the type B conformer by amino-terminal flank variant peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Formation of Type B Conformer</th>
<th>Editing of Type B Conformer by DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-52–61</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>ST-52–61 (51–61)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GST-52–61 (49–61)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DGST-52–61 (48–61)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>A-52–61</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>AA-52–61</td>
<td>+</td>
<td>−</td>
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<td>AAAA-52–61</td>
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<td>+</td>
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<tr>
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</tr>
<tr>
<td>TT-52–61</td>
<td>+</td>
<td>+</td>
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<tr>
<td>CT-52–61</td>
<td>+</td>
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</tr>
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<td>FT-52–61</td>
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<td>+</td>
</tr>
<tr>
<td>YT-52–61</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* Formation of the type B conformer was assessed by measuring the response of the type B hybridoma CP1.7 to I-A^K-peptide complexes formed in vitro with the indicated peptides, as described in Materials and Methods. Editing was assessed by measuring the response of the same hybridoma to complexes pulsed with DM for 2 h prior to introduction of the hybridoma; + indicates complete abolition of the type B response; +/− indicates partial elimination (at least threefold), and − indicates a response indistinguishable from that observed in the absence of DM. All peptides formed the type A conformer, regardless of the presence of DM, as assessed by stimulation of the type A hybridoma 3A9 (not shown). Each peptide was tested at least twice in independent experiments.

a* Cf. Fig. 3.

b* Cf. Fig. 4.

c N/A, not applicable.
of exposure to DM (Fig. 4, A–F). The wild-type 50–61 peptide, with serine at the P(−2) position, was edited, as expected (Fig. 4G), as was a peptide with the conservative substitution of threonine for serine at P(−2) (Fig. 4H). However, replacement of the wild-type serine with cysteine, which substitutes a sulfhydryl group for the γ-hydroxyl group (Fig. 4I), or with alanine, which replaces it with a hydrogen atom (Fig. 4J), prevented editing, as DM could no longer abolish the type B response. Other substitutions at P(−2) resulted in partial editing, with reduction, but not complete elimination, of the type B response in the presence of DM (Table I); this may reflect the competing effects of stabilizing interactions involving this side chain with destabilizing steric effects. Besides serine and threonine, the only other residue at P(−2) that conferred full susceptibility to editing by DM was proline; its

FIGURE 4. Susceptibility to conformational editing depends on the identity of the P(−2) side chain. A–F, Substitutions at the P(−2) position of 50–61 do not affect formation of the type A conformer in vitro. I-Aκ–CLIP (200 nM) was incubated overnight with the indicated peptide (320 nM) in the absence of DM, then incubated in the presence of DM (200 nM; □ or in its absence (■) for an additional 2 h. Samples were then neutralized, concentrated, treated with enterokinase to remove bound DM, and coated onto 96-well plates; cells of the type A T cell hybridoma 3A9 were added (10⁵ cells/well), and responses were measured by CTL assay. G–L, Substitutions at the P(−2) position of 50–61 confer resistance to editing by DM in vitro and/or prevent formation of the type B conformer. Assay conditions were the same as in A–F, but presentation to the type B hybridoma CP1.7 was tested.
unique structure may position the amino nitrogen such that it can interact with the MHC molecule.

Most strikingly, peptides with basic residues (lysine or histidine) at P(−2) failed to stimulate CP1.7 regardless of exposure to DM, indicating that these substitutions prevented formation of the type B conformer (Fig. 4, K and L). These peptides are recognized by T cells and bind with high affinity to I-Ak (Ref. 28 and data not shown). Because all type B T cells recognize the 52–61 peptide, this residue cannot be a TCR contact for these cells (11). Furthermore, the hypothesis that the P(−2) residue might directly contact the TCR is contradicted by biochemical data indicating that this residue contributes to peptide-MHC affinity and stability (28, 29) and by the crystal structure of the complex, in which the P(−2) residue is oriented downward and contacts the MHC molecule (30). Instead, it appears that peptides with basic amino-terminal flanking residues bind to I-Ak but do not adopt a type B conformation, in contrast to all other peptides tested, which interact with I-Ak to form a complex recognized by either type A or B hybridomas.

We concluded that interactions involving the P(−2) side chain determine the conformation of the complex and its susceptibility to editing. The abolition of editing by replacement of serine with cysteine suggests that these interactions might involve hydrogen bonds, because the sulfhydryl group of cysteine is less prone to form hydrogen bonds than the hydroxyl group of serine. The failure of peptides with lysine or histidine at P(−2) to form the type B conformer indicates that charge-mediated interactions are critical at this position as well.

Amino-terminal flanking residues determine the stability of the type B conformer

We also examined the ability of DM to accelerate the dissociation from I-Ak of peptides with amino-terminal flanks of varying length or composition; we reasoned that this assay would serve as an indicator of the ability of DM to interact with the complex, independent of its function as a conformational editor. The rates of dissociation of 48–61, 49–61, and 50–61 were all accelerated by DM by ~60-fold (Fig. 5, A–C; and Table II). The dissociation of 51–61 (Fig. 5D) and of 52–61 (Fig. 5E) was accelerated to a lesser extent; however, these peptides also have rapid intrinsic dissociation rates (Table II), bind to I-Ak with low affinity, and are poorly SDS stable (28). Thus, it was not unexpected that these peptides would show reduced susceptibility to catalysis of dissociation by DM (25).

To eliminate the confounding effects of differences in intrinsic dissociation rates, we compared the ability of DM to accelerate the dissociation of 50–61, which was susceptible to conformational editing by DM, and its variant with cysteine at P(−2). Both peptides bound I-Ak with high affinity (data not shown) and formed long-lived complexes in the absence of DM (Fig. 6, filled symbols), and DM increased their dissociation to a similar degree (Fig. 6, open symbols).

Dissociation half-lives of amino-terminal flank variant peptides bound to I-Ak in the presence and absence of H2-DMa

Table II. Dissociation half-lives of amino-terminal flank variant peptides bound to I-Ak in the presence and absence of H2-DMa

<table>
<thead>
<tr>
<th>Peptide</th>
<th>t1/2 (−DM) (h)</th>
<th>t1/2 (+DM) (h)</th>
<th>Fold Acceleration by DM</th>
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<tbody>
<tr>
<td>48–61</td>
<td>724.5</td>
<td>15.26</td>
<td>47.5</td>
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<td>49–61</td>
<td>688.4</td>
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<td>52.70</td>
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<td>3.95</td>
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<td>52–61</td>
<td>8.28</td>
<td>4.50</td>
<td>1.84</td>
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<tr>
<td>50–61 (WT)</td>
<td>442.7</td>
<td>5.93</td>
<td>74.6</td>
</tr>
<tr>
<td>50–61 (C50)</td>
<td>138.5</td>
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*Half-lives were calculated from single-exponential and, where appropriate, double-exponential fits of the fraction of radiolabeled peptide remaining bound to I-Ak after incubation for varying lengths of time at 37°C and pH 5.5 (cf Fig. 5). For complexes whose half-lives exceeded the duration of the assay (125 h), values were extrapolated from the fitted curve. These data fit best to a double-exponential curve. The half-life given is that for the first exponential, as our previous studies indicate that this represents the complexes susceptible to DM activity (12). Dissociation of these peptides was measured separately from the others (cf Fig. 5). WT, wild type.
open symbols). Therefore, replacement of the P(2) serine with cysteine, although conferring resistance to conformational editing (cf Fig. 4), did not prevent DM-MHC interaction.

We also measured presentation of the type B conformers of the various peptides after long periods (>72 h) of incubation. Presentation of peptides after brief pulses of incubation with DM reflects conformational editing, whereas presentation following long-term incubation reflects the stability of the type B conformer and, if DM is present, its ability to accelerate dissociation. With the exception of 52–61, all peptides showed no diminution of their ability to stimulate type A T cells after 7 days at 37°C; the type A response to 52–61 was reduced by ~75% after 48 h. By comparison, the ability of the peptides to stimulate type B hybridomas decreased over time, regardless of length or composition (Fig. 7). (The differences in kinetics as measured by dissociation of radiolabeled peptide and by T cell stimulation reflect the divergent end points being tested. The former assay measures physical dissociation of the peptide from the MHC molecule, whereas the latter measures loss of a particular conformer; this could occur by means short of outright dissociation, such as conversion to a third conformer not recognized by either T cell subset.) In either case, our results agree with previous findings with the 48–61 peptide (12) and indicate that the type B conformer is inherently short-lived, whereas the type A conformer is highly stable.

These conclusions are best illustrated by the 50–61 peptides bearing a serine or a cysteine at P(2). Both peptides remained bound to I-Ak for long periods, and their ability to stimulate type A hybridomas was unaffected by prolonged incubation at 37°C (data not shown). Presentation of the type B conformer of the 50–61 peptide with serine at P(2) (i.e., wild-type) was eliminated by incubation at 37°C; after 3 days, little stimulatory activity remained (Fig. 8A). Moreover, addition of DM accelerated the loss of the type B conformer, with most eliminated within 24 h (Fig. 8B). The 50–61 peptide with cysteine at P(2) also lost its type B conformation with time, albeit significantly more slowly than did the wild-type peptide (Fig. 8C). Incubation in the presence of DM caused only a slight reduction in presentation of the type B conformer at 24 h (in contrast to the wild-type peptide) but completely eliminated its presentation after 3 days, just as for the wild-type peptide (Fig. 8D). Thus, DM was unable to rapidly edit the conformation of the cysteine-substituted peptide; nevertheless, it accelerated the elimination of the type B conformer, most likely by promoting peptide-MHC dissociation.

In sum, peptides with long amino-terminal flanks, or favorable residues at P(2), formed relatively short-lived type B conformers that were edited by DM. In contrast, peptides lacking such residues also formed short-lived complexes, but these resisted editing. The presence of basic residues at P(2) prevented formation of the type B conformer, irrespective of DM. Thus, the effects of DM were reflected by two distinct end points: first, DM (as many have shown) accelerated the physical dissociation of the peptide-MHC complex and, second, DM acted as a conformational editor, rapidly
eliminating the unstable type B conformer. These end points were not absolutely correlated, as exemplified by the 50–61 (C50) peptide, whose dissociation was accelerated by DM although it resisted editing.

Discussion
This study demonstrates that amino-terminal flanking residues of an MHC class II–bound peptide profoundly influence the conformation of the complex. Editing of the 48–62/I-Ak complex by DM required the amino- but not the carboxyl-terminal flank of the peptide, and depended in particular on the identity of the amino acid at the P(−2) position. Replacement of the P(−2) residue with cysteine or alanine prevented formation of the type B conformer. Furthermore, modifications of the amino-terminal flank did not prevent DM from accelerating dissociation of the complex, implying that the conformational effects of amino-terminal flanking residues were mediated not through interaction with DM but, rather, through interactions intrinsic to the peptide-MHC complex. By rearranging the hydrogen-bond network stabilizing the complex, DM modulates the association of the peptide with the MHC molecule. This action typically manifests as an accelerated rate of physical dissociation, depending on the intrinsic affinity of the peptide, but more subtle rearrangements alter the conformation of the complex. Our findings suggest that contacts involving amino-terminal flanking residues determine the equilibrium between the various conformations, resulting in the phenomenon we have termed conformational editing, i.e., elimination of relatively unstable (type B) conformers in the presence of DM. Although these conclusions stem from analysis of the response of individual T cells, the cells tested are representative of a much larger panel (11); moreover, all results were confirmed by testing additional hybridomas (not shown), confirming that our results accurately reflect display of the peptide by the APCs.

Specifically, we propose a model in which peptide amino-terminal flanking residues participate in contacts with the MHC molecule (including, but not necessarily limited to, hydrogen bonds) that stabilize the type A conformer. As a result, the gain of stability upon formation of this conformer (ΔG_A) is significantly greater than that for other, more flexible conformers that lack these interactions (ΔG_B) (Fig. 9A). By accelerating the rearrangement of hydrogen bonds and allowing the complex to sample multiple conformations, DM assures that the complex will ultimately assume its optimal conformation, i.e., type A. In contrast, peptides unable to form these contacts will have little difference in energy between the type A conformer and other, less stable conformers. Because DM does not affect the equilibrium between conformers, but merely accelerates its achievement, both conformers will persist in the late endosome and eventually reach the cell surface (Fig. 9B). One attractive feature of this model is that it does not invoke ad hoc any novel function for DM. In fact, we argue that the mechanism of DM-mediated editing of the repertoire of MHC-bound peptides and the mechanism of DM-mediated editing of peptide-MHC conformation are fundamentally the same: just as DM favors binding of high-affinity peptides to class II MHC by accelerating release of weakly bound peptides, it also favors formation of the lowest-energy conformer of a peptide-MHC complex by accelerating dissociation of suboptimal conformers.

The particular dependence of conformation on the P(−2) residue supports this model, because this residue, including its side chain, is a key participant in the hydrogen-bonding network that stabilizes the complex (30). Although stabilizing hydrogen bonds between the peptide and MHC molecule occur across the length of
the peptide (37), those involving the amino-terminal region of the peptide are particularly critical (38), and even minor alterations of this network result in weak binding and rapid release of bound peptides (39–42). Hydrogen bonds have been implicated as a substrate of DM activity (27), and small organic molecules that interfere with hydrogen bonding can mimic the function of DM (43, 44). Furthermore, the I-Aβ molecule contains several basic residues in close proximity to the P(−2) side chain, including arginine residues at α52 and α53, a histidine residue at β81, and a lysine residue at β84a (30). Thus, although polar side chains capable of serving as hydrogen-bond donors can stabilize the complex, basic residues at P(−2) likely participate in repulsive interactions, preventing peptides from forming the type B conformer regardless of the presence of DM.

Although our study focused solely on the 48–62/I-Aβ complex, we anticipate that the role of the amino-terminal flank in determining conformation will prove generalizable to other complexes. Participation of amino-terminal flanking residues in the hydrogen-bonding network stabilizing the complex has been observed for multiple peptide–class II MHC complexes from both human and mouse, and it appears to be a universal feature of this interaction (1). The conformational flexibility of the amino terminus of a class II MHCI–bound peptide correlates inversely with the stability of the complex, suggesting that interactions involving this region of the peptide can “lock” the complex into a rigid, stable (i.e., type A) conformation (45). In addition, contacts involving the peptide amino-terminal flank, located near the nexus of interaction between DM and the complex, should be particularly susceptible to DM-catalyzed breakage and rearrangement (although we do not discount the possibility that the conformational effects of DM may “ripple” across the complex and affect the core and carboxy-terminal regions of the peptide). We have observed that the minor epitope of HEL spanning residues 20–35 does not prime type B T cells (our unpublished observations). The amino-terminal flank of the peptide contains a glycine residue at P(−2) and a basic residue (arginine) at P(−3). Whether this is the cause of its failure to prime type B T cells remains to be demonstrated; nevertheless, this observation raises the possibility that the sequence of the amino-terminal flank of a peptide may determine its ability to prime T cells specific for multiple conformations and, conversely, that the MHC residues surrounding the amino terminus of the peptide may determine its range of conformational possibilities, such that some alleles may be more prone to others to prime conformation-specific T cells.

Finally, our findings carry implications for the in vivo priming of type B T cells. We have hypothesized that type B T cells are primed when peptide–MHC complexes are generated through DM-independent pathways, such as processing in recycling endosomes (46–48) or via the action of extracellular proteolytic enzymes expressed by APCs that are up-regulated during acute inflammation (49, 50). The results of this study indicate that in vivo generation of the type B conformer can occur in the presence of DM, so long as the peptide lacks amino-terminal flanking residues. Endogenous processing preferentially generates peptides with long amino- and carboxy-terminal flanks, because these residues stabilize MHC-peptide complexes (28, 29) and, once bound to the MHC molecule, are protected from further enzymatic trimming (51). These peptides would be subject to conformational editing by DM, preventing presentation of the type B conformer. In contrast, during inflammation, increased proteolysis (both intra- and extracellular) may favor the generation of peptides that lack flanking residues and, as such, stimulate type B T cells regardless of the presence of DM. This may explain why many type B T cells primed by peptide immunization preferentially recognize peptides lacking amino-terminal flanking residues (11). Presentation of autologous epitopes in this manner could prime self-reactive type B T cells, resulting in autoimmunity.

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Disclosures

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References


