Utility of Azapeptides as Major Histocompatibility Complex Class II Protein Ligands for T-Cell Activation

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Major histocompatibility complex class II (MHC II) protein binding and antigen specific activation of $CD4^+$ "helper" T cells are demonstrated with peptides composed of the antigenic hen egg ovalbumin 325−339 peptide (OVA) substituted with azaamino acids. AzaAla and azaGly substitutions were made at 10 sequential peptide positions (326Ala−335Asn) that lie in the binding groove. The peptide positions substituted with azaamino acids encompass almost the entire binding groove, including positions where the identity of the amino acid side chain is known to have the most significant effect on MHC binding and the least effect on T-cell recognition. In addition, the T-cell contact 333Glu was substituted with azaGlu to generate a partial agonist ligand for the 3DO-54.8 T-cell hybridoma. Binding to MHC II protein was assayed by measuring the kinetic stability of complexes formed between detergent-solubilized MHC II $I\text{-}A^d$ protein and fluorescein-labeled OVA peptides using a fluorescence-HPLC assay. T-cell activation was also evaluated for aza-substituted peptides with azaamino acid substitutions at the peptide positions known to interact with the MHC II protein. All aza-substituted peptides showed detectable MHC binding, and some were found to show T-cell activation potency equal to the native peptide. Several of these were also found to be weak or partial agonists. Our results demonstrate that azaamino acids substituted into an antigenic peptide cause a subtle, global effect on peptide conformation that can be used to design altered peptide ligands (APL) as T-cell partial agonists. These may have potential as T-cell epitopes for synthetic vaccines and therapeutic agents for autoimmune diseases.

Introduction

$CD4^+$ "helper" T cells play a pivotal role in the initiation, amplification, and memory function of the mammalian acquired-immune response. Antigen-specific activation of T cell subpopulations takes place at the interface between the T cell and specialized antigen-presenting cells that display class II proteins of the major histocompatibility complex, class II (MHC II). Short, antigenic peptides of diverse, degraded protein origins bind promiscuously to MHC II molecules and are, in effect, sequentially examined by the T-cell antigen receptor (TCR). If the peptides are of bacterial or viral origin, the T cell may become activated and set in train the cascade of events leading to protective immunity. The endogenous or "self" peptides presented by MHC II proteins in any one individual constitute the immunological "self" at the molecular level. Endogenous peptides are ignored except in the case of immune dysfunction when a response is directed against the body's own tissues. The breakdown of T-cell tolerance to these "self" peptides is one of the hallmarks of numerous autoimmune diseases.[1]

There is now a growing body of evidence that the T-cell response is more sophisticated than just a simple on/off activation state. Different states of activation can result in the secretion of unique sets of cytokines, which are potent signaling molecules that can produce widely varying outcomes. The cytokines regulate further T-cell activation and the actions of other cells of the immune system. It is the concerted actions of the T cells and other cells that determine the outcome of an immune response. It has been shown that substitution of one or more of the amino acids in an antigenic peptide bound to an MHC II protein can elicit a T-cell activation state different than when stimulated with the native peptide. Some of these substituted peptides or "altered peptide ligands" (APL) have been shown to produce partial activation or even deactivation of T cells both in vitro and in vivo.[2] This can lead to anergy, an unresponsive state where the T cells are refractory to further antigenic stimulation and their ability to support an immune response becomes muted. This may be a passive mechanism for the production of specific immunological tolerance toward exogenous stimuli.[3] Some APL have also been shown to induce "regulatory" T cells that apparently mediate an active and sustained suppression of an immune response.[4,5] Vaccination with APL has produced remarkable realignments of the immune system, leading to temporary and permanent remission from the symptoms of a rodent model for multiple sclerosis.[3]

Such results with altered peptide ligands have spurred new research into the use of APL as therapeutic agents for the treatment of autoimmune diseases such as multiple sclerosis and type I diabetes. Some candidate peptides are in clinical trials.[6,7] Similarly, the prospect and progress of synthetic vaccines against pathogens and tumors are based on altering the set of presented peptides observed by T cells to directly manipulate T-cell activation such that the immune system can be "edu-

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cated" toward a desired outcome. Thus, the MHC II protein is a potential target for modulating the immune response. The minimal requirements for such MHC-based therapeutic agents are twofold; they must be able to bind to MHC II proteins and simultaneously be able to interact with the T-cell receptors that can recognize the native peptide. However, the pathways leading to peptide presentation by MHC II proteins are tightly regulated and peptides suffer from poor bioavailability and rapid degradation and clearance. These constraints indicate the need for peptide mimetics that are stable and synthetically accessible while affording structures that retain the salient features of peptides when bound to MHC proteins. Furthermore, such molecules presumably must display side chain functionality with a diversity at least as great as that of amino acids in order to permit "fine tuning" of T-cell recognition.

The design of an APL is facilitated by the existence of numerous crystal structures of peptide−MHC II complexes.[8] The proteins bind peptides (typically 10−20 amino acid residues) promiscuously, yet they do so in a highly conserved manner. The peptides bind in a groove that is open at both ends, and they are constrained in an extended conformation similar to a polyproline II helix. An array of hydrogen bonds between the peptide backbone and highly conserved amino acid residues of the MHC extends along the length of the binding groove. The peptide backbone structure and number of associated hydrogen bonds are almost identical in all published crystal structures of MHC II molecules, and we reason that successful artificial ligands will mimic these striking features. Following the precession of the helical twist are suitably positioned pockets that align with the side chains of peptide residues oriented toward the MHC (Figure 1b). The pockets may offer further favorable binding interactions with peptide "anchor" residues or accommodate unfavorable interactions. The peptide residues that direct their side chains away from the MHC (TCR contacts) have the most direct effect on TCR binding and the subsequent events following ligation.

The conservation of peptide backbone hydrogen bonds in MHC II complexes is reflected in the structure of an MHC II protein bound by a large, diverse pool of endogenous peptides.[9] The backbone electron density is well-defined despite the presence of thousands of different peptides. This observation has led to the suggestion that the peptide backbone hydrogen bonds are critical determinants for stable binding.[9−11] This has been confirmed by site-directed mutagenesis studies where deletion of critical residues from the hydrogen bond array leads to MHC−peptide complexes that are up to 3 orders of magnitude less stable.[12] The effects of peptide backbone perturbations on T-cell activation might be unpredictable given the large diversity of TCRs. Each TCR displays a low affinity for its cognate MHC−peptide (on the order of micromolar) and the window of sensitivity is an extremely narrow one: a 10-fold difference in affinity spans the full range of agonism through antagonism to inactivity.[13,14] With this in mind, it can be argued that ligands deviating only minimally from the optimal conformation are required for a general

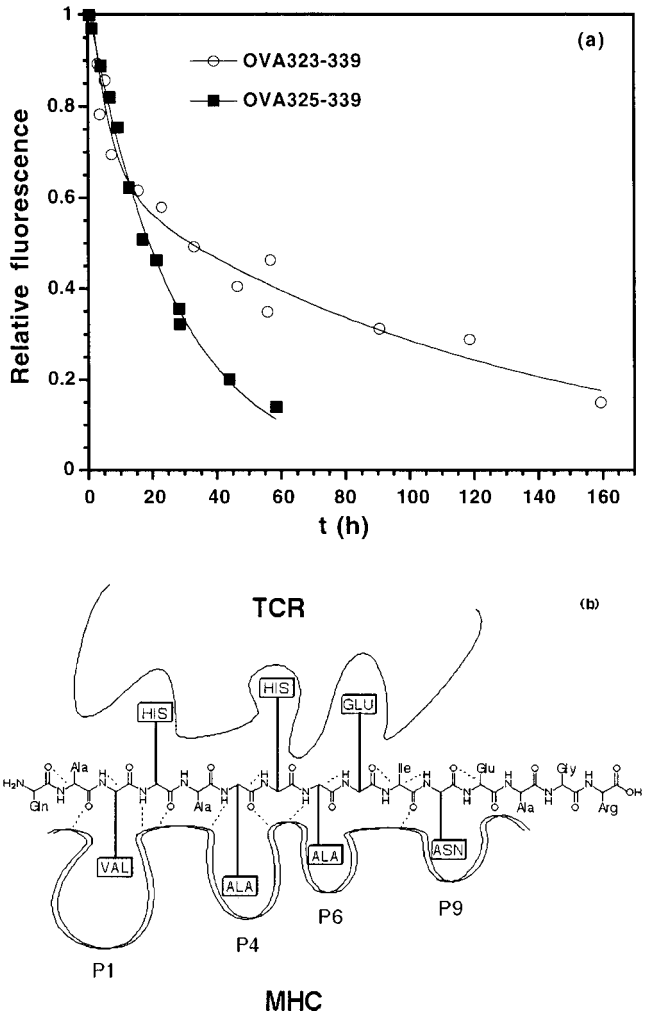


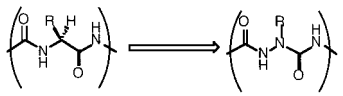
Figure 1. (a) Data for dissociation of OVA(323−339) and OVA(325) peptides from the I-A^d MHC II protein. The peptides are fluorescein-labeled at the N-terminus, and the dissociation of peptide bound to protein was monitored by high-performance size exclusion chromatography with a fluorescence detector. (b) Schematic of the Ova(325−339) peptide bound to the I-A^d protein. Dashed lines indicate conserved hydrogen bonds between protein and the peptide backbone.

observance of T-cell activation, and this poses an even greater challenge for the design of peptide-mimetic TCR ligands.

Several peptide mimetics have been investigated as potential MHC ligands, but the majority of these have been directed at MHC class I molecules, which have a less restrictive binding motif than class II proteins. The class I proteins bind 8- or 9-mer peptides, and these are firmly anchored only at the N- and C-termini with few constraints for the peptide midriff. Potential MHC II ligands that have been evaluated include pseudopeptides (peptide bond reduction),[15] peptoids[16] (*N*-alkylglycines), oligocarbamates, and D-amino acids.[17,18] In general, these backbone-modified peptide mimetics fail to bind MHC II proteins.[19] Pyrrolinone−peptide hybrids[20] and peptides with other embedded spacers[21] display MHC binding capacity and, thus, the ability to inhibit antigen-specific activation of cells in vitro, but they do not afford a facile means of manipulating the primary sites of T-cell recognition. Similarly, N-methylated peptides,[22] proline homologues,[23] other *N*-alkyl-amino acids, and ethylene-linkage replacement of the

amide bond[24] variously show protease resistance, MHC binding, and T-cell stimulation capacities. However, these require stereospecific synthesis and are limited by their application to residues where the backbone amide is solvent-exposed (i.e., not involved in MHC binding) and/or not required for T-cell recognition. This highlights the problem that any given site on the peptide backbone may be required for MHC and/or TCR interactions and that designing a peptide mimetic that meets these requirements is not a trivial problem. To our knowledge, there are no reports of peptide mimetics that successfully meet all of the general and specific requirements for presentation by MHC and activation of T cells as detailed above.

Azapeptides arise when the asymmetric C_α carbon of an amino acid is replaced by nitrogen.



The residue in an oligomer is thus a monoalkyl-diacyl hydrazine with the peptidic "ψ" angle constrained by the energetics of the amide bond in a trisubstituted urea moiety. In most of the X-ray crystal structures of small-molecule azapeptides, the relevant heavy atoms usually lie in a plane corresponding to a "ψ" angle of 180°, showing a rotation of up to ±30° from this angle.[25−27] This is consistent with the steric strain known to exist in polysubstituted ureas.[28] Experimental data and ab initio calculations commonly report the N−N dihedral "ϕ" angle as twisted by approximately ±90°,[26,27,29−34] but this seems less energetically constrained because in diformyl and diacetyl hydrazine this angle has been reported as 0°/180° but 45°/135° in the hydrated crystal.[35] The nitrogen atoms frequently assume an sp^2 hybridization state but also appear able to adopt asymmetric *R*- or *S*-configurations, especially when in a cyclic azaproline structure.[25,36] There are numerous literature examples of the use of azapeptides as peptide mimetics, particularly as protease inhibitors and β-turn-inducing substitutions[25,37] but also as enzyme active-site titrants,[38−43] Leu-enkephalin,[44] eledoisin,[45] RGD mimetics,[46] and renin inhibitors.[27,47] Studies have demonstrated azapeptide resistance to amino- and carboxypeptidases, and they have also been used as prodrugs.[48]

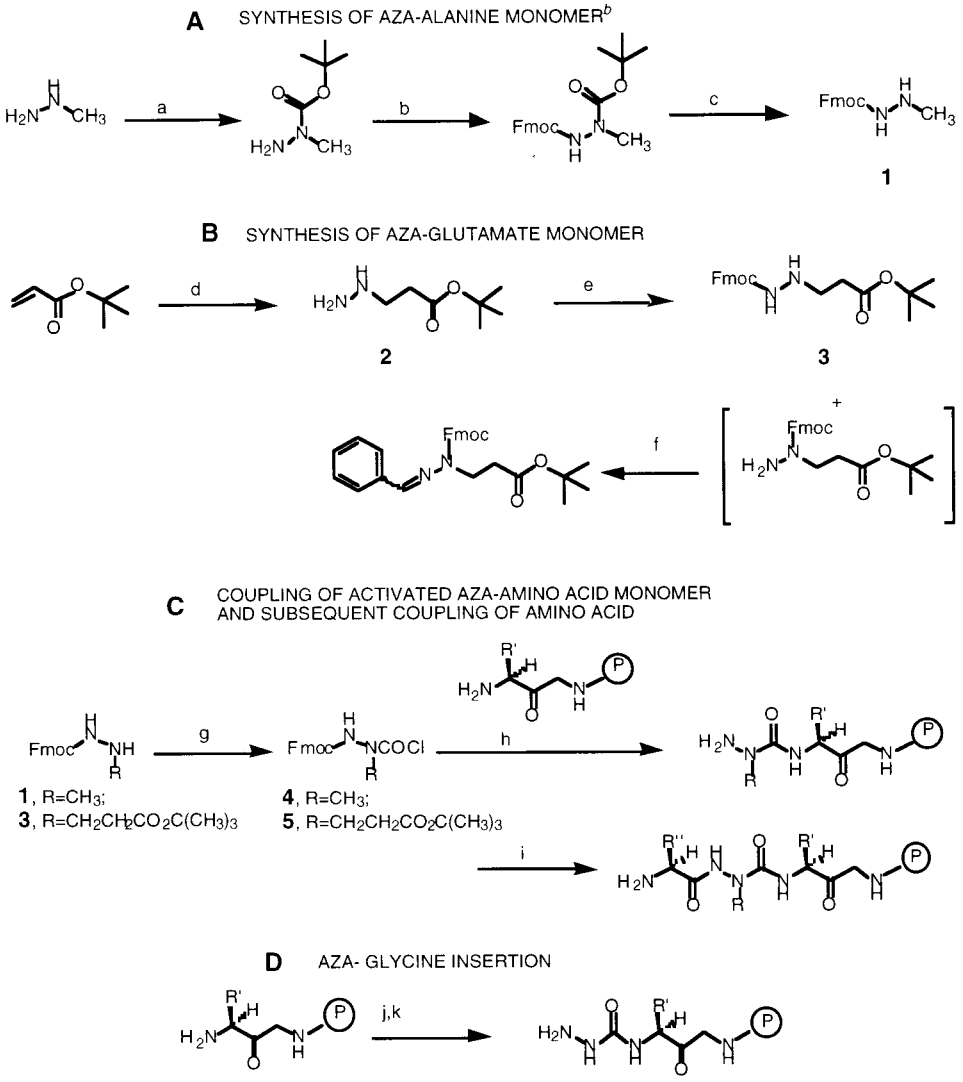
We report here the synthesis and evaluation of azapeptides as potential TCR ligands when bound to MHC II proteins. To test the effect of azapeptide conformational bias, azaAla residues were singly substituted at each position of the hen-egg ovalbumin 325−339 (OVA) peptide that is encompassed within the MHC II I-A^d binding site. To discriminate between the azapeptide backbone conformational bias and steric effects due to a simple N-alkyl side chain, each position was also substituted with an azaGly residue. The kinetic stabilities of complexes between the substituted peptides and I-A^d were determined. The kinetic stabilities were examined in the context of the I-A^d crystal structure, known sensitivities of binding to disruption of hydrogen bonds, and the structural biases of azapeptides. A subset of the aza-substituted peptides was tested for T-cell stimulation with an OVA-specific T-cell hybridoma cell line. Antigen-specific activation was measured as the amount of secreted T-cell growth cytokine interleukin-2 (IL2). It is shown that the azapeptide conformational bias is not disruptive to the binding of the MHC II protein or to T-cell activation. It is concluded that azapeptide analogues have the potential to be incorporated in the design of functional APL and peptide-mimetic vaccines.

Results

The OVA(323−339) peptide has the sequence ISQAVHAAHAEINEAGR and binds to the MHC II protein I-A^d with high affinity ($K_d \approx 40$ pM). Like most peptide−MHC II complexes, it is extraordinarily stable (dissociation half-time $t_{1/2} = 46$ h, pH 7.4, 37 °C), although its dissociation is characterized by at least double-exponential kinetics (Figure 1a). A crystal structure of the OVA(323−339) peptide covalently tethered to the I-A^d shows the canonical binding mode with a polyproline II peptide backbone conformation and with the first 10−12 residues from the N-terminus lying in the binding groove.[49] Subsequent studies have shown that the OVA(323−339) peptide can bind in at least two registers that differ in the placement of side chains into the binding pockets.[50] The register observed in the crystal structure places the Val327 side chain in a pocket near the center of the binding groove (the P4-pocket). The second register places the Val327 residue in the N-terminal P1-pocket (Figure 1b). To eliminate the P4-pocket register, which does not stimulate 3DO-54.8 T cells, we chose to use the shorter OVA(325−339) sequence Q*AVHAAHAEIN*EAGR, which is known to bind only in the P1-pocket register (the residues encompassed within the binding groove of I-A^d are italicized).[50] The OVA(325−339) peptide bound to I-A^d activates the 3DO-54.8 T-cell hybridoma and also dissociates with monophasic kinetics, $t_{1/2} = 18$ h, pH 7.4, 37 °C (Figure 1a).

Peptides with azaAla and azaGly residues singly substituted at 10 sequential positions (326−335) of the OVA(325−339) peptide were prepared by solid-phase synthesis (Scheme 1). The "fmoc-azaAla" (**1**) was prepared according to literature methods.[46] Acylation of a native amino acid N-terminus with a phosgene-activated fmoc-azaAla (**4**) was relatively efficient with typical yields of about 90% (Scheme 1C). The azaAla N-terminus exposed upon fmoc deprotection is weakly nucleophilic, requiring HATU/HOAt for efficient coupling of the next amino acid. To insert azaGly, the N-terminus of the peptide was first derivatized with disuccinimidyl carbonate followed by hydrazine to generate the desired azaGly residue (Scheme 1D). The N-terminal hydrazide was again acylated using HATU activation of the next fmoc-amino acid. For comparison purposes natural Ala and Gly residues were singly substituted at the same positions where they do not normally occur in the native peptide. The effect of aza-substitution of a TCR contact residue was evaluated by substitution of an azaGlu at position 333, a known TCR contact for the 3DO-54.8 T cell. The "fmoc-azaGlu" (**3**) was readily synthesized by Michael addition of hydrazine to *tert*-butyl acrylate and reaction of the purified hydrazine (**2**) with fmoc-*O*-succinimide. The undesired regioisomer was eliminated by reaction with benzalde-

Scheme 1[a]



[a] Reagents and conditions: (a) dibutylpyrocarbonate, THF, −70 °C; (b) Fmoc-OSu, methanol; (c) 50% TFA/dichloromethane; (d) hydrazine, 2-propanol, 10 min; (e) Fmoc-OSu, methanol; (f) benzaldehyde, toluene, 70 °C, 15 min; (g) phosgene, dioxane; (h) NMP, 24 h, then 20% piperidine in NMP, 0.5 h; (i) 5 equiv of Fmoc-AA-OH/HATU/HOAt in NMP, 24 h, then 20% piperidine in NMP, 0.5 h; (j) 0.2 M disuccinimidyl carbonate in NMP, 4 h; (k) 5% hydrazine in NMP, 12 h. [b] See ref 46.

hyde to give the hydrazone that was readily removed by column chromatography. Activation with phosgene and coupling via intermediate **5** was achieved in the same manner described above. All of the peptides were fluorescein-labeled at the N-terminus using the *N*-hydroxysuccinimidyl ester of 5(6)-carboxyfluorescein prior to cleavage from the solid phase. The resulting crude peptides were purified by HPLC, and the identity was confirmed by electrospray mass spectrometry and purity of >95% by analytical HPLC (Table 1 and Supporting Information).

The binding reactions of peptides with MHC II proteins are complex.[51,52] When devoid of peptide, the MHC II protein rapidly converts to a state that is unable to bind peptide.[53,54] Reversion to a peptide-receptive state is exceedingly slow. The MHC II proteins obtained either from detergent lysis of mammalian cells or from transfected insect cells are bound by peptides or other low molecular weight species that must dissociate prior to the binding of labeled peptide. Many peptides bind to form a short-lived intermediate that then slowly converts to the long-lived terminal complex.[51] Because of the complex binding kinetics, affinities or IC_{50} values estimated from direct binding and binding inhibition

Table 1. Peptide Sequences and Their Dissociation Half-Lives

peptide	$t_{1/2}$ (h)	peptide	$t_{1/2}$ (h)
fOVA, **6**	18	f331azaAla, **26**	2.5
f326Gly, **7**	6.6	f331azaGly, **27**	0.5
f326azaAla, **8**	5.7	f332Gly, **28**	19
f326azaGly, **9**	4.9	f332azaAla, **29**	10
f327Ala, **10**	30	f332azaGly, **30**	54
f327Gly, **11**	1.2	f333Ala, **31**	6.9
f327azaAla, **12**	3.9	f333Gly, **32**	1.9
f327azaGly, **13**	1.3	f333azaAla, **33**	1.7
f328Ala, **14**	5.2	f333azaGly, **34**	1.4
f328Gly, **15**	1.4	f334Ala, **35**	27
f328azaAla, **16**	2.8	f334Gly, **36**	1.0
f328azaGly, **17**	2.4	f334azaAla, **37**	0.4
f329Gly, **18**	12	f334azaGly, **38**	0.6
f329azaAla, **19**	1.3	f335Ala, **39**	120
f329azaGly, **20**	1.2	f335Gly, **40**	160
f330Gly, **21**	2.6	f335azaAla, **41**	42
f330azaAla, **22**	3.9	f335azaGly, **42**	140
f330azaGly, **23**	6.4	f333azaGlu, **43**	1.1
f331Ala, **24**	6.0	f333azaGly335Gly, **44**	4.6
f331Gly, **25**	1.5		

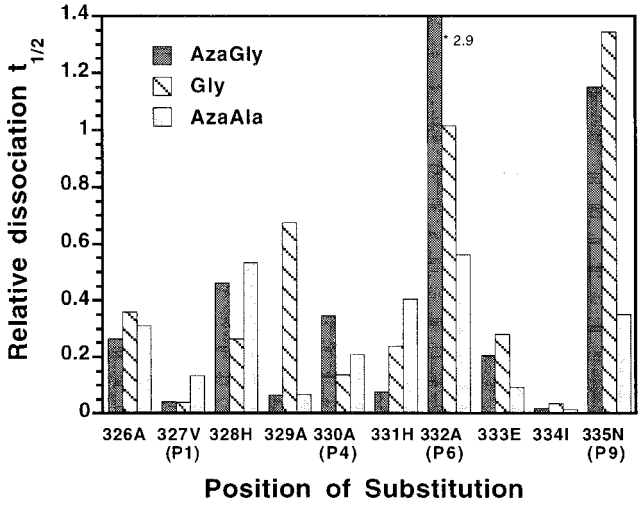


Figure 2. Effect of aza-amino acid substitutions on binding stability. Dissociation of fluorescein-labeled peptides was measured as described for Figure 1, and the dissociation half-times ($t_{1/2}$) were calculated from exponential functions fit to the data. The $t_{1/2}$ values for each azaGly, Gly, and azaAla substitution were normalized to the $t_{1/2}$ value for the peptide with Ala substituted at that position.

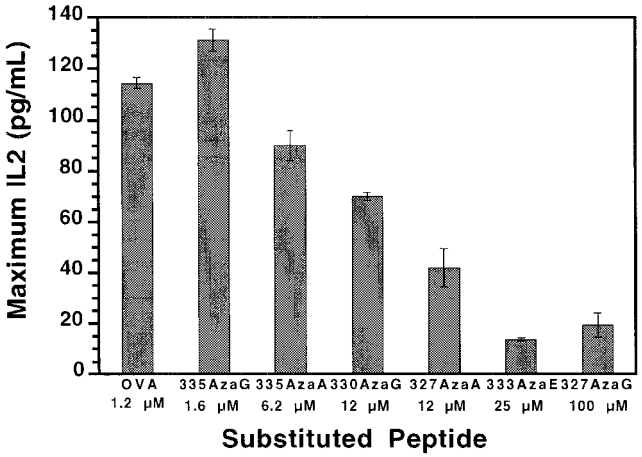


Figure 3. Effect of aza-amino acid substitution on activation of the 3DO54.8 T-cell hybridoma. Shown are the maximum concentrations of IL2 secreted in response to each peptide tested. The concentration of peptide required to achieve maximum IL2 secretion is listed below each peptide label. The error bars represent the standard error of the mean ($n = 3$).

assays do not correlate with binding thermodynamics.[51] However, if a peptide-receptive state of the protein is preformed by partial dissociation of a very low-affinity peptide, association rates can be estimated. The association rate constants measured in this manner vary less that 4-fold even among peptides with highly variable dissociation rate constants.[55] Thus, the more readily determined dissociation rate constants are the most reliable measure of relative binding affinities. Since binding of peptides to MHC II proteins is a chaperone-mediated process while cell surface expression correlates with kinetic stability of a complex, the dissociation rates are also of more physiological relevance.

The dissociation of native and aza-substituted peptides from the I-A^d MHC II protein was measured according to established techniques.[56] In brief, detergent-solubilized protein was incubated at 37 °C and pH 7.4 with fluorescein-labeled peptide. Excess free peptide was removed quickly with a small size exclusion column at 4 °C, and the labeled peptide complex was then incubated at 37 °C and pH 7.4. Aliquots of the solution were periodically removed and assayed on an analytical size exclusion column with a fluorescence detector. The amount of fluorescence associated with the protein peak was taken as a measure of the amount of peptide bound. The dissociation data were fit to an exponential function, and the derived rate constants are expressed as dissociation $t_{1/2}$ values (Table 1). All of the substituted OVA(325−339) peptides formed readily detectable complexes with the I-A^d protein. The majority of aza-substituted peptides formed complexes that were less stable than the corresponding peptide with an alanine substitution at the same residue (Figure 2). On average, the dissociation $t_{1/2}$ decreased by ∼50% for azaGly and ∼70% for azaAla substitution. By comparison, the Gly substitution at the same site also decreased the $t_{1/2}$ by an average of 60%, an amount within expectations. Given that the K_d for the OVA(325−339) peptide is ∼100 pM, the observed decreases in stability correspond to energetic changes of about 0.5−1 kcal mol^{-1}.[57]

The aza-substituted peptides were also assayed for their ability to activate the 3DO-54.8 T cell hybridoma cell line,[58] which secretes IL2 when activated by the OVA(325−339) peptide bound to I-A^d on the surface of antigen presenting cells. The 3DO-54.8 T cell responses to OVA peptides with natural Ala and Gly substitutions have been reported previously.[59] Peptides with aza-amino acids substituted at MHC anchor residues (peptides **12**, **13**, **22**, **23**, **29**, **30**, **41**, **42**) and with the 333azaGlu TCR contact substitution (peptide **43**) were assayed. The native OVA peptide induces maximal T-cell stimulation at a concentration of approximately 1 μM peptide, and several of the aza-substituted peptides induce a similar level of activation at concentrations ranging from 10 to 50 μM peptide (Figure 3). Peptide **43** in which the known TCR contact Glu333 is replaced with azaGlu produces maximal IL2 release of ∼20% of the maximum induced by the native peptide, and thus, **43** is a partial agonist. The aza-substituted peptides **22**, **29**, and **30** induced little or no stimulation at 100 μM (data not shown). A 2-fold excess of the doubly substituted 333azaGly, 335Gly peptide reduced OVA binding by 50%, and a ∼50-fold excess inhibited maximal OVA stimulation of 3DO-54.8 T cells by 50% (data not shown). These results confirm that the azapeptides compete for the same binding site.

Discussion

The reported success, and failure, of some azapeptides as peptide mimetics is probably strongly influenced by the inherent structural bias constraining the available conformational space of azapeptides with respect to the requirements of the ligand−receptor system studied. With a probable rotational energy barrier of >10 kcal mol^{-1} for the C−N "ψ" angle in azapeptides,[28,60,61] rotation about this bond is at least partly restricted and constrained within the range of 180 ± 30°, as reported in crystal structures.[25−27] The energetics of rotation about the N−N bond that forms the "ϕ" angle is uncertain; a dialkyl-diacyl hydrazine has a reported

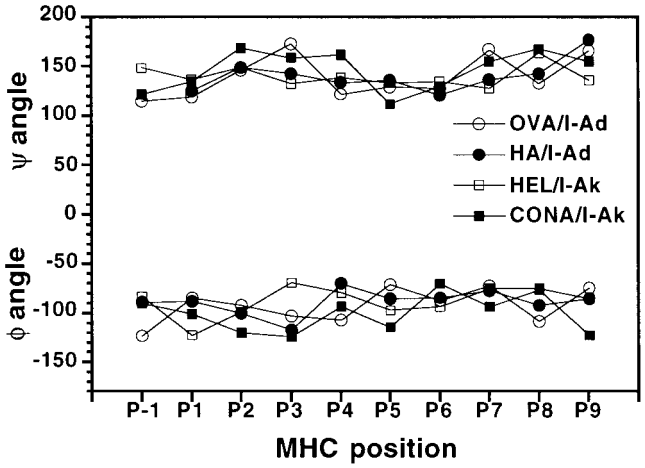


Figure 4. Conservation of peptide ϕ and ψ angles in peptide−MHC II complexes. Shown are the ϕ and ψ angles from four different peptides: OVA and HA bound to I-A^d, and HEL and ConA bound to I-A^k. Dihedral angles were obtained from the crystal structures. PDB registry numbers are 1IAO, 2IAD, 1IAK, and 1D9K, respectively.

dihedral energy barrier of 23 kcal/mol,[62] but given the variation of this angle in crystal structures, the less substituted azapeptides may well enjoy greater rotational freedom. A measured rotational energy barrier in monoalkyl-diacyl hydrazines has not been reported, although calculated and experimental data support the preference for a twisted orientation close to ±90°.[29] Thus, Han and Janda observed no binding of an azaleuenkephalin analogue to an antibody[44] while Bold et. al. synthesized HIV protease inhibitors composed of inverse aza-dipeptide isosteres with subnanonmolar affinities.[63] We have shown that aza-substituted peptides meet the requirements for the MHC class II binding *and* TCR interaction. All of the aza-substituted peptides formed measurably stable complexes with I-A^d, and 7 out the 10 that were assayed produced measurable T-cell activation.

The reported peptide ϕ/ψ angles for the OVA and an influenza hemaglutinin peptide bound to I-A^d are shown in Figure 4. Also shown are the highly similar ϕ/ψ angles for two peptides bound to the homologous MHC II protein I-A^k. For I-A^d the average ϕ and ψ angles are −94° and 135°, respectively, for the peptide residues found in the binding groove. The disposition of the "ϕ" angle in azapeptides thus appears to be an excellent match with known MHC II ligands. The observed "ψ" angle appears, on average, some 15° less than the lower limit of the range of values for this dihedral and so might be expected to be a possible reason for the generally decreased stability of aza-substituted peptides relative to that of alanine. That binding is not eliminated suggests that the energetic preference for this "ψ" angle is low enough such that it can be accommodated at any location in the binding groove. In support of this, if an unrefined nonamer azaAla peptide model sequence is constructed by setting ϕ and ψ angles to −90° and 180°, respectively, the distance spanning the C_α atoms from C1 to C9 is calculated to be 25.4 Å.[64] This distance is the same as the distance connecting the P1 and P9 C_α atoms in the OVA peptide bound to I-A^d, and the structure also propagates a qualitatively similar polyproline II helical structure. Given that the peptide backbone conformation is highly conserved in all reported MHC II protein complexes, azaamino acid substitutions are likely to be well tolerated in all MHC II alleles.

On average, substitution by an azaamino acid residue at any peptide position within the MHC II binding groove produces a complex that is approximately 2-fold less stable than for a corresponding alanine substitution, but with large variations within this average. This is interesting in light of the observation that disruption of any one backbone hydrogen bond at the peptide N-terminus in an I-A^d complex causes a 20- to 200-fold decrease in stability, while a similar disruption at the peptide C-terminus causes only a 5- to 20-fold decrease.[12] The lack of an obvious correlation between the site, or type, of azapeptide substitution and binding stability suggests that the perturbation is not simply a local effect. This is borne out by a more detailed examination of the OVA crystal structure, which reveals that the ψ angle at the P9 position is 166°, well within the azapeptide "ψ" range of 180° ± 30°. Together with the generally favorable ϕ angles, this is strongly suggestive of a particularly good site for azapeptide substitution, and azapeptide substitutions at P9 (peptides **41** and **42**) do indeed improve stability of the complexes with I-A^d. However, no such relationship holds at positions P3 and P7, where the ψ angles are an equally favorable 173° and 167°, respectively (e.g., aza mutations at P3, peptides **43** and **43**, were among the most disruptive). Furthermore, at the P6 position, azapeptide substitution produced highly stable complexes, yet at 128° the I-A^d ψ angle appears highly unfavorable. We conclude that the perturbation extends beyond the site of substitution, and because each individual position might be differentially sensitive, the overall effect is not easily predicted.

The primary goal of this research is to discover ligands capable of exerting subtle effects on the TCR in order to achieve T-cell antagonism, or partial agonism, by design. This has hitherto only been achieved on a trial and error basis with the more limited set of available L-amino acids. A method for routinely achieving such a modulation of TCR signaling would be a tool of great value, especially in the context of the heteroclitic nature of the in vivo T-cell response. The results reported here suggest that azapeptide substitutions may be such a tool. Although only 10 aza-substituted peptides were studied for T-cell activation, several ligands with altered T-cell reactivity were identified. The peptides **41** and **42** are clearly full agonists, while peptides **22**, **29**, and **30** are null ligands. Peptides **12** and **23** are weak agonists, while peptides **13** and **43** display the activity of partial agonists. The latter is perhaps the most interesting of the ligands surveyed in that Glu333 is a TCR contact residue that cannot be mutated to other natural amino acid residues and still activate the 3DO-54.8 T cells.[59] The partial agonism observed with 333azaGlu peptide **43** indicates that a conservative aza substitution at a TCR contact site potentially causes only modest structural perturbations that are nevertheless sufficient to alter T-cell signaling.

It is tempting to ascribe the majority of the observed changes in T-cell potencies as being simply due to reduced or increased MHC binding capabilities and hence the effective ligand concentration at the interface

between the T cell and the antigen presenting cell. At sites where encoded amino acid substitutions have little effect on TCR recognition this may indeed be the case, but, as with the effects upon MHC binding, this simple view is contradicted by our results. For example, the ligand concentration required to elicit maximal response to peptides **12** and **42** differs by approximately 1 log unit, but the difference in the stability of their MHC complexes is an order of magnitude larger still. Also, both of the sites modified in **12** and **42** are located distal to the TCR-sensitive central portion of the peptide. Similarly peptide **30**, one of the most stable complexes observed, was inactive, and yet the same mutation at a different position, peptide **23**, yielded a full agonist ligand that only weakly binds to I-A^d (Gly mutants here share similar effects at the MHC and TCR[59]). These two positions are proximal to the most TCR-sensitive sites, but they differ in one respect: the 332 position is flanked by two important T-cell contacts (331His and 333Glu), while position 330 is only flanked by one (331His). From these results we argue that a backbone conformational perturbation due to aza-amino acid substitution has a relatively large local effect at the site of substitution that is also partly propagated to more distant sites of the peptide.

As noted above, mutations of MHC II proteins have shown that some of the backbone hydrogen bonds are of much greater relative importance than others for MHC binding, and it would not be surprising if this is also the case for T-cell stimulation. Indeed, it might be expected that peptide backbone atoms that are solvent-exposed will frequently be used as hydrogen-bond binding contacts by the TCR, as are more distant residues in the MHC II protein that contact the complementarity determining regions of the TCR. Thus, we cannot rule out the possibility that the changes in T-cell responses observed with the aza-substituted peptides are not only due to indirect perturbations of TCR contacts through the peptide backbone. Regardless, it is evident that substitution of some peptide residues with azaamino acids can produce partial agonist activity. It is worth emphasizing the slightly unusual criteria of an idealized T-cell partial agonist. Simple avidity does not provide a good mechanistic model of activation through the TCR. Rather, it appears to be a kinetic discrimination based on the triggering of many TCRs by the few peptide−MHC complexes that bind sufficiently long to signal.[65,66] Complexes that dissociate only slightly more rapidly initiate incomplete signals that apparently lead to partial agonism and/or antagonism. The kinetic window that discriminates between agonist and partial agonist is narrow, about a factor of 5−10.[13,14] Consequently the goal is not to design a ligand of higher TCR affinity but to design a slightly inferior one. The ligand must still bind the MHC II protein, although it can afford to do so with lower affinity than its cognate ligand. Thus, the design of T-cell partial agonists is fundamentally distinct from attempts to abrogate TCR signaling by high-affinity MHC blockade. Because the aza-substituted peptides studied here retain MHC II protein binding while causing subtle alterations in T-cell activation, they represent a potential synthetic platform for the design of altered peptide ligands that modulate T-cell activity. In vivo, these could be valuable as synthetic vaccines and as potential therapeutics for the treatment of autoimmune diseases.

Conclusions

We have shown that substitution of an aza-amino acid into an antigenic peptide is permissible for both binding to an MHC II protein and the activation of T cells by the azapeptide−MHC II complex. Since each position along the length of an antigenic peptide contributes differentially to either MHC II binding or T-cell activation, it was anticipated that there would be a correlation between the substitution site, known structure, and the effect on either binding or activation. The lack of any such correlation led to the conclusion that the backbone conformational constraint of an aza-peptide induces a local perturbation of the peptide structure that is also propagated to more distant positions along the length of the peptide. The propagation of conformational disturbance to more distant sites would suggest that an aza-peptide substitution at a position not directly involved in TCR recognition would nevertheless affect T-cell activation. Indeed, it was found that some of the peptides with azaAla and azaGly substitutions at MHC II binding sites were also partial agonists for the 3DO54.8 T-cell hybridoma. We suggest that the strategic substitution of one or more azaAla or azaGly units into an antigenic peptide may be sufficient to generate an APL with therapeutic potential for the treatment of autoimmune diseases or vaccine development.

Experimental Section

Chemical Abbreviations. Fmoc, (9-fluorenylmethyloxycarbonyl)-; HOBt, 1-hydroxybenzotriazole; HOAt, 1-hydroxy-7-azabenzotriazole; HBTU, *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HATU, *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; NMP, *N*-methyl-2-pyrrolidone; TFA, trifluoroacetic acid; DMF, dimethylformamide; EDIA, ethyldiisopropylamine; FmocAA-OH, *N*-(9-fluorenylmethyloxycarbonyl)amino acid; MeCN, acetonitrile; ES-MS, electrospray mass spectrometry.

Reagents. Peptide synthesis grade NMP, HBTU, and HOBt were purchased form Advanced Chemtech. Rink amide MBHA resin and all amino acid derivatives were purchased from NovaBiochem. Phosgene (20% solution) in toluene was purchased from Fluka. 5(6)-Carboxyfluorescein-*N*-hydroxysuccinimidyl ester (mixed isomers) was purchased from Molecular Probes. All other solvents and reagents were purchased from Sigma-Aldrich.

Peptide Synthesis and Labeling. OVA(325−339) peptide (and all variants) were synthesized as the C-terminal amide on Rink amide MBHA resin by standard Fmoc-HBTU/*tert*-butyl protection chemistry on an Applied Biosystems 433A automated peptide synthesizer (100 μmol scale). After deprotection and NMP washes, the N-terminus was fluorescein-labeled with 5(6)-carboxyfluorescein-*N*-hydroxysuccinimidyl ester plus 1% triethylamine in DMF and the mixture was stirred for 12 h. (All compounds containing fluorescein were shielded from light, especially so after purification and during MHC binding assays.) The resin was then washed three times with DMF and twice with methanol and dried for 24 h under high vacuum. The peptide was cleaved from the resin by stirring with 95% trifluoroacetic acid plus 5% water for 3 h, filtered to remove resin, precipitated with diethyl ether, dried, dissolved in water, and purified by RP-HPLC on a C18 Alltech Prosphere column (300 Å, 5 μm, 250 mm × 10 mm) with an acetonitrile/water gradient and 0.1% TFA buffer, flow rate of 5 mL/min, detection at 220 nm. After lyophilization the peptide was characterized by ES-MS. Purity was assessed by analytical RP-HPLC (acetonitrile/water and methanol/water gradients

with 0.1% TFA buffer). Peptides were dissolved in water and the concentration measured by absorbance of the fluorophore at 495 nm in 0.1 M sodium carbonate buffer.

***N*-(9-Fluorenylmethyloxycarbonyl)-*N′*-methylhydrazine, "Fmoc-azaAlanine", 1.** This was synthesized according to literature methods (Scheme 1A).[46]

3-(1-Dimethylethoxy-1-oxopropyl)hydrazine Hydrochloride, "AzaGlutamate", 2 (Scheme 1B). Hydrazine hydrate (8 mL, 0.16mol), 2-propanol (20 mL), and *tert*-butyl acrylate (2.29 mL, 0.016 mol) were mixed and allowed to stand at room temperature for 10 min. Toluene (50 mL) was added, the mixture shaken, and the lower layer discarded. The toluene layer was then washed five times with 10 mL of saturated sodium chloride solution and extracted twice with dilute hydrochloric acid. The acidic aqueous extracts were washed with toluene, adjusted to pH 3−4 with sodium bicarbonate, frozen, and lyophilized. The white solid was digested with 50 mL of ethanol, the mixture was filtered and evaporated, and the residue was dissolved in 6 mL of acetonitrile and refiltered through a small amount of silica gel. The residue was again evaporated and redissolved in 7 mL of acetontrile, and 7 volumes of diethyl ether was added. After the mixture was stirred for12 h the white crystals were filtered, washed with ether, and dried to give 1.2 g (39%) of product (as the monohydrochloride). ES-MS: *m/z* 161.1 $(M + H^+)^{1+}$. ^{1}H NMR (500 MHz, D_2O): δ 1.23 (s, 9H), 2.49 (t, 2H), 3.12 (t, 2H). ^{13}C NMR (D_2O): δ 28.0, 31.8, 46.9, 84.2, 172.4. Elemental analysis ($C_7H_{17}ClN_2O_2$) found: C 42.8%, H 9.0%, N 14.3%, Cl 18.8%. Expected: C 42.8%, H 8.7%, N 14.2%, Cl 18.0%

***N*-3-(1-Dimethylethoxy-1-oxopropyl)-*N′*-(9-fluorenylmethyloxycarbonyl)hydrazine Hydrochloride 3 "Fmoc-azaGlutamate" (Scheme 1B).** To **2** (1.2 g, 6.1 mmol) in 20 mL of methanol was added *N*-(9-fluorenylmethyloxycarbonyloxy)succinimide (2.1 g, 6.2 mmol) in acetonitrile (20 mL) and triethylamine (0.87 mL, 1.2 mmol). Reaction was complete after 5 min by TLC. The reaction mixture was added to excess water, extracted twice with dichloromethane, dried with magnesium sulfate, and filtered, and the solvent was evaporated. The crude product so obtained contained approximately 30% of the desired regioisomer by HPLC. This was dissolved in toluene and 0.6 mL of benzaldehyde plus 2 drops of acetic acid, and the solution was heated to 70° for 15 min, by which time the undesired isomer had completely reacted to form the hydrazone, as indicated by HPLC analysis. The solution was washed with aqueous sodium bicarbonate, dried, and filtered, and the solvent was removed. The oil was subjected to column chromatography on silica gel, eluting with mixtures of ethyl acetate in hexane in 5% increments rising from 0% to 70% ethyl acetate. This achieved a baseline separation of the desired product. The relevant fractions were evaporated, dissolved in diethyl ether, and filtered through a small amount of silica gel. Addition of a solution of hydrogen chloride in ethanol gave the product hydrochloride as a white precipitate, which was filtered, washed with ether, and dried. Yield: 0.35 g (13%). TLC indicated that a substantial amount of product remained in the ethanol liquors, but this was not pursued. ES-MS: *m/z* 383.0 $(M + H^+)^{1+}$, 765.2.0 $(2M + H^+)^{1+}$. ^{1}H NMR (500 MHz, DMSO): δ 1.43 (s, 9H), 2.69 (t, 2H), 3.26 (t, 2H), 4.31 (t, 1H), 4.52 (d, 2H) 7.36 (t, 2H), 7.45 (t, 2H), 7.75 (d, 2H), 7.92 (d, 2H). ^{13}C NMR (DMSO): δ 28.9, 31.3, 46.3, 47.6, 68.2, 82.0, 121.2, 126.3, 128.2, 128.8, 141.8, 144.3, 156.0, 170.1.

General Procedure A: *N*-Alkyl-*N*-chlorocarbonyl-*N′*-(9-fluorenylmethyloxycarbonyl)hydrazine "Fmoc-azaamino Acid Chloride" Monomers, 4 and 5 (Scheme 1C). This was performed in essentially the same manner as the method described by Kessler et al.[46] Thus, the *N*-alkyl-*N′*-fmoc-hydrazine, or its hydrochloride (0.02 mmol), was dissolved in dry dioxane (∼10 mL) to which was added phosgene (0.04 mmol) as a 20% solution in toluene. (Caution! Phosgene is highly toxic and should only be used in a fume hood.) The mixture was then stirred for 2 h at room temperature. Excess phosgene was removed by evaporation of the solvent and by redissolving the residue in dry dioxane and evaporating. The residual oil was placed under high vacuum for 12 h and then used immediately.

General Procedure B: Coupling of Activated "Fmoc-azaamino" Acid Monomers (Scheme 1C). *N*-Alkyl-*N*-chlorocarbonyl-*N′*-fmoc-hydrazine (**4**, **5**, 0.1 mmol) was prepared from *N*-alkyl-*N′*-fmoc-hydrazine and phosgene as described in procedure A and dissolved in NMP to give a 0.1 M solution. This was added to 0.02 mequiv of resin-bound peptide preswollen in NMP. EDIA (17.4 μL, 0.1 mmol) was added, and the resin was stirred at room temperature for 18 h. Normal piperidine deprotection and NMP washes exposed the hydrazide moiety of the azapeptide at the N-terminus.

General Procedure C: Coupling of Amino Acid Residue to N-Terminal Azapeptide (Scheme 1C). Approximately 0.02 mequiv of azapeptide on Rink amide resin was preswollen with a minimal amount of NMP. To this was added a solution of the fmoc-amino acid (0.1 mmol) in 1 mL of NMP preactivated with 0.1 mmol of HATU, 0.2 mmol of EDIA, and 0.1 mmol of HOAt. The reaction was stirred for 18 h and the resin washed with NMP. The peptide was then further extended by normal automated peptide synthesis.

General Procedure D: Insertion of azaGlycine (Scheme 1D). Approximately 0.02 mequiv of peptide on Rink amide resin was stirred with disuccinimidyl carbonate (25.6 mg, 0.1 mmol) in NMP (1 mL) for 4 h at room temperature, and the resin was washed twice with NMP. The resin was then stirred with >20-fold excess hydrazine as a 5% solution in NMP for 12 h. The N-terminal hydrazide was acylated as in procedure C using HATU activation of the Fmoc-amino acid. The peptide was then further extended and labeled as before.

Purification of I-A^d Protein. Class II molecules were obtained and used as previously reported.[56] In brief, A20-1.11 antigen-presenting cells were lysed with NP40 detergent, and cell lysates were passed over a lentil lectin column. The lentil lectin column was eluted with 10% α-methylmannoside onto an antibody affinity column bearing the MKD6 antibody specific for I-A^d. The MHC protein was then eluted with a solution of 1 mM dodecyl maltoside (DM) in 0.5 M NaCl/0.1 M Na_2CO_3 (pH 11.3). Appropriate fractions were collected, neutralized, and dialyzed against 1 mM DM in PBS at pH 7.4. Protein concentration was measured by micro-BCA assay (Pierce) and diluted to ∼0.25 μM in the same buffer.

Peptide Dissociation Kinetics. Approximately 50 μL of the protein solution was incubated in 0.1 M sodium citrate/1 mM DM/10 mM sodium phosphate/150 mM NaCl (pH 5.3) at 37° for 3 h with a 50- to 1000-fold excess of the labeled peptide. Excess free peptide was removed at 4 °C with a Sephadex G50-SF column (∼1 mL) pretreated with 10 mg/mL BSA and then washed with 1 mM DM/10 mM sodium phosphate/150 mM NaCl (pH 7.4). Peptide/MHC complexes were then eluted with the same buffer at pH 7.4 (400−600 μL). The sample was then incubated at 37 °C, and samples were removed periodically and analyzed by high-performance size exclusion chromatography using a 7.5 mm × 30 cm TSK G3000SW$_{XL}$ column (Toso Haas). The amount of peptide bound to MHC protein was measured as the peak height of fluorescence as measured with an HPLC fluorescence detector. The series of decaying peak heights was fit to a first-order exponential function, and the apparent first-order dissociation rate constant was extracted. Rebinding of labeled peptide is not detectable at these concentrations. Inhibition of binding was measured by forming the labeled complex in the same manner in the presence of varying amounts of an unlabeled peptide. The solution was then injected directly onto the column, and the relative amounts of complex formed were measured as the observed peak height.

T-Cell Stimulation Assay. 3DO-54.8 T cells and A20-1.11 antigen-presenting cells were grown in RPMI 1640 media supplemented with 10% fetal calf serum (37 °C, 5% CO_2). In 96-well plates, 1×10^4 cells of each type were incubated for 48 h in 200 μL of media containing peptides serially diluted with media. Experiments were performed in triplicate. After 48 h, cell supernatants, 100 μL/well, were withdrawn and frozen at −80 °C. IL2 ELISA assays were carried out on 20

μL of the defrosted supernatants according to the manufacturer's instructions (Pharmingen "OptEIA" ELISA kit.).

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Supporting Information Available: Table listing peptide dissociation half-times, MS data, and HPLC retention times. This material is available free of charge via the Internet at http://pubs.acs.org.

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