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DO11.10 and OT-II T Cells Recognize a C-Terminal Ovalbumin 323–339 Epitope[1]

Jennifer M. Robertson,* Peter E. Jensen,† and Brian D. Evavold[2]*

The $OVA_{323–339}$ epitope recognized by DO11.10 (H-2^d) and OT-II (H-2^b) T cells was investigated using amino- and carboxy-terminal truncations to locate the approximate ends of the epitopes and single amino acid substitutions of $OVA_{323–339}$ to identify critical TCR contact residues of the $OVA_{323–339}$ peptide. DO11.10 and OT-II T cells are both specific for a C-terminal epitope whose core encompasses amino acids 329–337. Amino acid 333 was identified as the primary TCR contact residue for both cells, and amino acid 331 was found to be an important secondary TCR contact residue; however, the importance of other secondary TCR contact residues and peptide flanking residues differ between the cells. Additional $OVA_{323–339}$-specific clones were generated that recognized epitopes found in the N-terminal end or in the center of the peptide. These findings indicate that $OVA_{323–339}$ can be presented by I-A^d in at least three binding registers. This study highlights some of the complexities of peptide Ags such as $OVA_{323–339}$, which contain a nested set of overlapping T cell epitopes and MHC binding registers. ***The Journal of Immunology*****, 2000, 164: 4706–4712.**

A T cell is activated when its TCR interacts with a specific ligand comprised of an antigenic peptide bound to an MHC molecule on the surface of an APC (1, 2). This interaction leads to a defined series of events that results in effector functions such as cytokine production, cytolysis, and proliferation (3). A typical $CD4^+$ T cell epitope contains a core 9 aa with a primary TCR contact residue located at position 5 and MHC anchor residues at positions 1, 4, 6, and 9. The location and number of secondary TCR contact residues vary between T cells and are usually found at positions 2, 3, 7, and/or 8 (4–8). In addition, some T cells are dependent on peptide flanking residues located outside of the core 9-aa epitope (9, 10). Many MHC molecules have a preferred peptide-binding motif, which often includes large charged or polar amino acids that are required to stabilize the peptide-MHC interaction (4–8, 11, 12). Because of this it is assumed that a given antigenic peptide is always presented by a particular MHC molecule in the same binding register, which in turn causes all of the ligand-specific T cells to recognize the same epitope. For example, the primary TCR contact residue (position 5) of all $hemoglobin_{64–76}$:I-E^k-specific T cells is amino acid 72 (5, 11).

Despite the widespread use of DO11.10 TCR transgenic mice, a detailed analysis of the T cell epitope has not been reported (13, 14). Grey and coworkers (15) characterized an N-terminal epitope of $OVA_{323–339}$ recognized by many T cells and identified residues 327–333 as critical for peptide binding to I-A^d by comparing the affinity of $OVA_{323–339}$ to a series of truncated peptides and single amino acid substitutions. These early findings are supported by the recently published crystal structure of $OVA_{323–339}$ bound to I-A^d, which reveals the positioning of residues 324–332 within the binding groove of the MHC molecule (4). However, Shimonkevitz et al. (13) showed that DO11.10 T cells are much less sensitive to $OVA_{323–336}$ compared with $OVA_{323–339}$, despite the observation that these peptides have equal affinities for I-A^d (15). Similarly, it has recently been demonstrated that tetrameric complexes of $OVA_{328–338}$ bound to I-A^d can be used to detect DO11.10 T cells, suggesting that DO11.10 recognizes a carboxy-terminal region of the peptide (16). Collectively, these studies indicate that multiple epitopes within $OVA_{323–339}$ can be presented by I-A^d, a possibility supported by the lack of strong MHC anchor residues observed in the crystallized $OVA_{323–339}$:I-A^d molecule (4, 14–16).

In this study the T cell epitopes recognized by the $OVA_{323–339}$-specific DO11.10 (H-2^d) and OT-II (H-2^b) TCR transgenic mice were mapped using a series of $OVA_{323–339}$ analogue peptides. Amino- and carboxy-terminal truncations were used to find the approximate ends of the T cell epitopes, and single amino acid substitutions were used to identify critical TCR contact residues. Our analysis demonstrates that DO11.10 and OT-II T cells recognize the same 9-aa core epitope (329–337) located in the C-terminal end of the OVA peptide; however, the importance of secondary TCR contact residues and peptide flanking residues differs between the cells. Because the epitope recognized by DO11.10 and OT-II was not the epitope seen in the $OVA_{323–339}$:I-A^d crystal structure (4), additional OVA-specific clones were generated and analyzed. These clones were specific for other areas of the peptide, indicating that $OVA_{323–339}$ contains multiple T cell epitopes.

Materials and Methods

Mice

DO11.10 TCR transgenic mice (H-2^d) were generated by Murphy et al. (14), and the DO11.10 recombination activating gene-deficient ($RAG^{-/-}$)[3] mice were a gift from Terrence Barret (17). OT-II TCR transgenic mice (H-2^b), originally generated by Barnden et al. (18), were obtained from Dr. Judith A. Kapp (Emory University). BALB/CAnNCr (H-2^d) and C57BL/6NCr (H-2^b) mice were purchased from the National Cancer Institute (Frederick, MD). All mice were housed and maintained in the Emory University Department of Animal Resources facility.

*Department of Microbiology and Immunology, Emory University; and †Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, GA 30322

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[2] Address correspondence and reprint requests to Dr. Brian D. Evavold, Department of Microbiology and Immunology, Emory University, 1510 Clifton Road, Atlanta, GA 30322. E-mail address: evavold@microbio.emory.edu

[3] Abbreviations used in this paper: RAG, recombination activating gene; CLIP, class II-associated invariant chain peptide; SWM, sperm whale myoglobin; HA, hemagglutinin.

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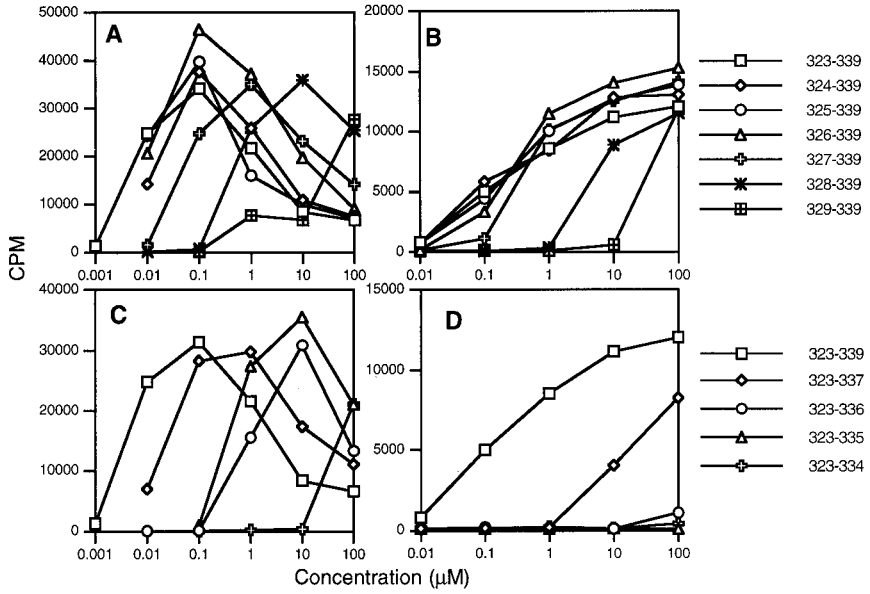

FIGURE 1. DO11.10 and OT-II T cells recognize a C-terminal epitope of $OVA_{323–339}$. The dose-response curve of DO11.10 $RAG^{-/-}$ T cells was measured in response to N-terminal (*A*) and C-terminal (*C*) truncations of $OVA_{323–339}$. Removal of amino acids 327 and 328 from the N terminus or 337 and 336 from the C terminus of the peptide causes a substantial decrease in DO11.10 proliferation, indicating that these amino acids are located on the edges of the DO11.10 T cell epitope. The proliferation of OT-II T cells was similarly measured in response to N-terminal (*B*) and C-terminal (*D*) truncations of $OVA_{323–339}$. The response of OT-II was affected by the removal of amino acids 328 or 337 and 338, indicating that these amino acids mark the edges of the OT-II epitope.

Cells and reagents

Transgenic T cell lines were obtained by stimulating spleen cells from DO11.10 $RAG^{-/-}$ or OT-II mice with either 0.1 μM or 1 μM chicken OVA peptide 323–339 (ISQAVHAAHAEINEAGR), respectively. Other T cells were generated by s.c. priming BALB/c mice with 10 μg OVA protein (Sigma, St. Louis, MO) in CFA (Difco, Detroit, Michigan). Draining lymph nodes were removed after 9 days, and T cells were cloned by limiting dilution upon stimulation with 0.3 μM $OVA_{323–339}$ peptide and gamma-irradiated syngeneic splenocytes (2000 rad). T cell lines and clones (2×10^5/well) were restimulated every 2 wk in a 24-well plate with appropriate peptide and 5×10^6 gamma-irradiated splenocytes (2000 rad) from either BALB/c or C57BL/6 mice along with 50 U of IL-2 obtained from the culture supernatants of IL-2-secreting P815 cells (19). Cell culture media consisted of RPMI 1640 supplemented with 2 mM L-glutamine, 0.01 M HEPES buffer, 100 μg/ml Gentamycin (Mediatech, Herndon, VA), 10% FBS (Atlanta Biologicals, Norcross, GA), and 2×10^{-5} M 2-ME (Sigma). All peptides were synthesized using florenyl methoxycarbonyl chemistry on a Symphony/Multiplex Peptide Synthesizer and were analyzed by HPLC (Rainin Instruments, Woburn, MA).

Proliferation assay

T cells (3×10^4/well) were incubated with the indicated peptide and 5×10^5 irradiated syngeneic spleen cells in duplicate in a 96-well plate. Proliferating cells were labeled after 48 h with 0.4 μCi/well of [^{3}H]thymidine, and after another 18 h, the assays were harvested and analyzed on a Matrix 96 Direct Beta Counter (Packard, Meriden, CT). Data shown represent one of at least three similar experiments performed.

Results

DO11.10 and OT-II T cells recognize a C-terminal epitope

DO11.10 (H-2^d) and OT-II (H-2^b) $CD4^+$ T cells were originally generated by priming either a BALB/c or a C57BL/6 mouse, respectively, with OVA protein (18, 20). Responses of these T cells are $OVA_{323–339}$-specific even though the MHC backgrounds of the primed mice are different (13, 18). In fact, DO11.10 and OT-II T cells can respond to $OVA_{323–339}$ presented by I-A^d and I-A^b (Refs. 20 and 21 and our unpublished data). Despite the widespread use of these T cell systems, the fine specificities of the epitopes recognized by the cells are unknown. A panel of N- and C-terminal truncations of the OVA peptide 323–339 was used to find the approximate ends of the DO11.10 and OT-II T cell epitopes (Fig. 1 and summarized in Table I). The stepwise removal of amino acids from the N-terminal end of the OVA peptide has little or no effect on the DO11.10 response until amino acids 327 and 328 are deleted. These truncations cause a substantial decrease in DO11.10 proliferation, indicating that these amino acids are located on the

Table I. *Summary of T cell reactivity to $OVA_{323–339}$ truncations*[a]

	T Cell Clone				
Peptide	DO11.10	OT-II	A6	A7	A1
323–339	0.004	0.2	0.06	0.033	0.0027
324–339	0.011	0.12	**>100**	0.09	0.003
325–339	0.004	0.2	>100	**5.2**	0.018
326–339	0.004	0.21	>100	>100	**0.12**
327–339	0.04	0.33	>100	>100	6
328–339	**0.4**	4.8	>100	>100	50
329–339	25	**30**	>100	>100	>100
323–337	0.025	**30**	0.06	0.04	0.003
323–336	**1**	>100	0.115	0.1	0.003
323–335	0.35	>100	0.15	0.06	**0.15**
323–334	45	>100	0.2	0.16	2.5

[a] The concentration (μM) of peptide required to reach the half-maximal proliferation induced by $OVA_{323–339}$ is shown. The values in bold indicate the edges of the T cell epitopes as determined by the requirement for a 100-fold increase in peptide to reach the half-max.

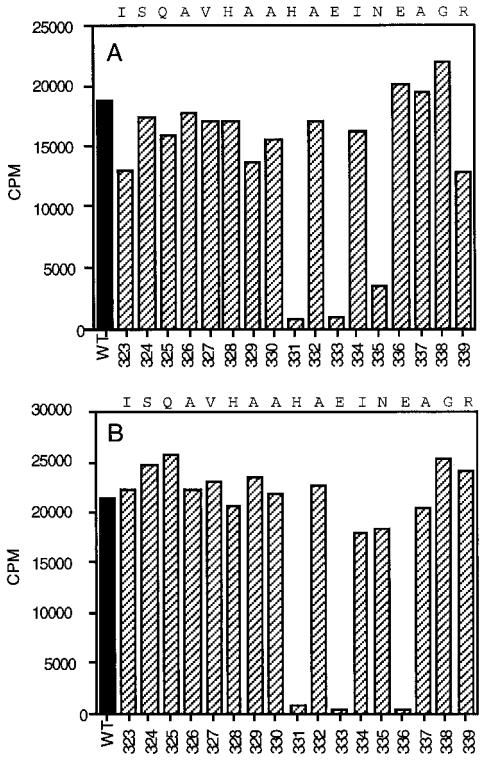

FIGURE 2. Identification of critical T cell contact residues for DO11.10 and OT-II. *A*, Proliferation of DO11.10 $RAG^{-/-}$ T cells was measured at a full range of concentrations in response to a series of alanine- (or serine- if the original amino acid was an alanine) substituted peptides. Proliferation at 1 μM peptide is shown revealing that amino acids 331, 333, and 335 are important to the DO11.10 response. *B*, OT-II proliferation was similarly measured, and the maximum T cell response obtained for each peptide is shown. Proliferation was affected by the substitution of residues at 331, 333, and 336. The filled bars represent proliferation in response to $OVA_{323–339}$.

N-terminal edge of the DO11.10 T cell epitope. The response of OT-II is similarly affected by the removal of amino acid 328. Truncations from the C-terminal side of $OVA_{323–339}$ reveal that amino acids 337 and 336 are important to DO11.10, whereas the removal of 338 and 339 have minimal effect on T cell proliferation. OT-II is dependent on residues 337 and 338, indicating that these amino acids mark the C-terminal edge of the OT-II epitope. Because T cells can differ in their dependency on residues flanking the core 9 aa (9, 10), truncated peptides cannot be used to determine the precise boundaries of the epitopes, yet our data indicate that DO11.10 and OT-II T cells recognize a C-terminal region of the $OVA_{323–339}$ peptide (Fig. 1 and Table I).

Critical T cell contact residues for DO11.10 and OT-II

To further map the OVA epitope recognized by DO11.10 and OT-II, the residues important to the T cell response were identified by measuring proliferation of the cells to peptides with an alanine (or serine if the original amino acid was an alanine) substituted at each position of $OVA_{323–339}$. Proliferation of DO11.10 to these "alanine scan" peptides on $I\text{-}A^d$ depended on the conservation of amino acids 331, 333, and 335 (Fig. 2*A*). The epitope recognized by OT-II on $I\text{-}A^b$ was similarly mapped and residues 331, 333, and 336 were identified as being important to T cell recognition (Fig. 2*B*). These results confirmed the data obtained using truncated peptides, locating the core epitope for DO11.10 and OT-II in the C terminus of $OVA_{323–339}$.

The definite location of the 9-aa epitope for DO11.10 and OT-II was placed by identifying the primary TCR contact residue for each T cell. The primary TCR contact residue is defined functionally as accepting very few amino acid substitutions compared with other secondary TCR contact residues (11, 22), and this position equates with the solvent exposed P5 residue identified in all peptide:MHC class II crystal structures (4–6, 8). The critical residues identified in Fig. 2 for T cell recognition were further analyzed

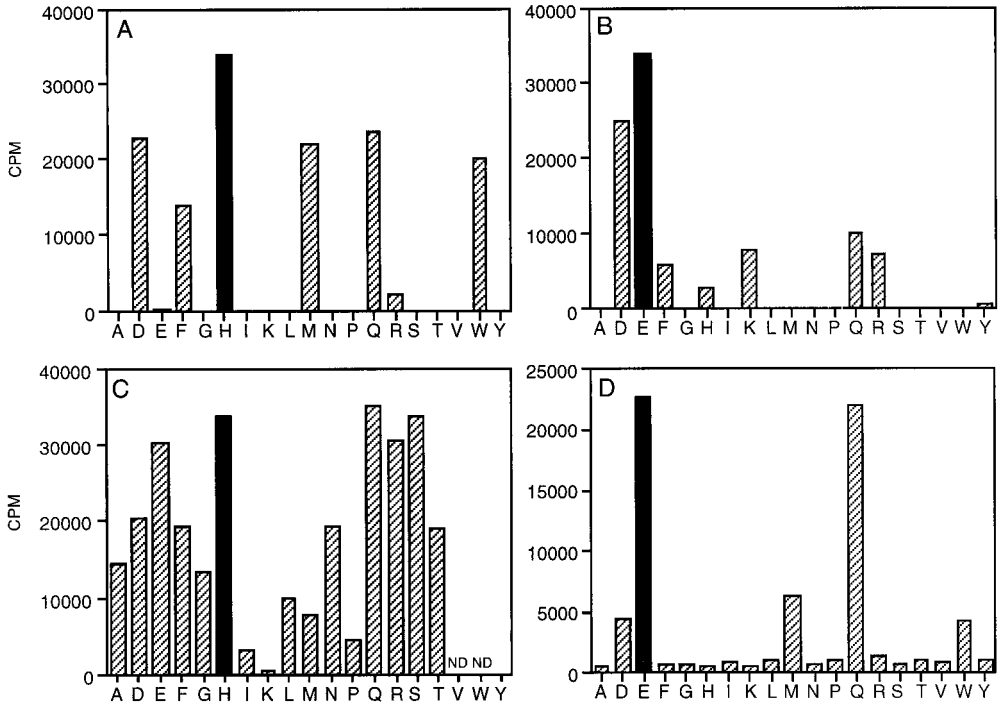

FIGURE 3. Residue 333 is the primary TCR contact residue for DO11.10 and OT-II T cells. Proliferation of DO11.10 $RAG^{-/-}$ (*A* and *B*) or OT-II (*C* and *D*) T cells was measured in response to a panel of peptides substituted with each amino acid (except cysteine) at residue 331 (*A* and *C*) or 333 (*B* and *D*). Amino acid 333 is the most critical amino acid for both T cells, indicating that this is the primary TCR contact residue for DO11.10 and OT-II. The filled bars indicate wild-type $OVA_{323–339}$ peptide. The peak proliferative response obtained with each peptide is shown.

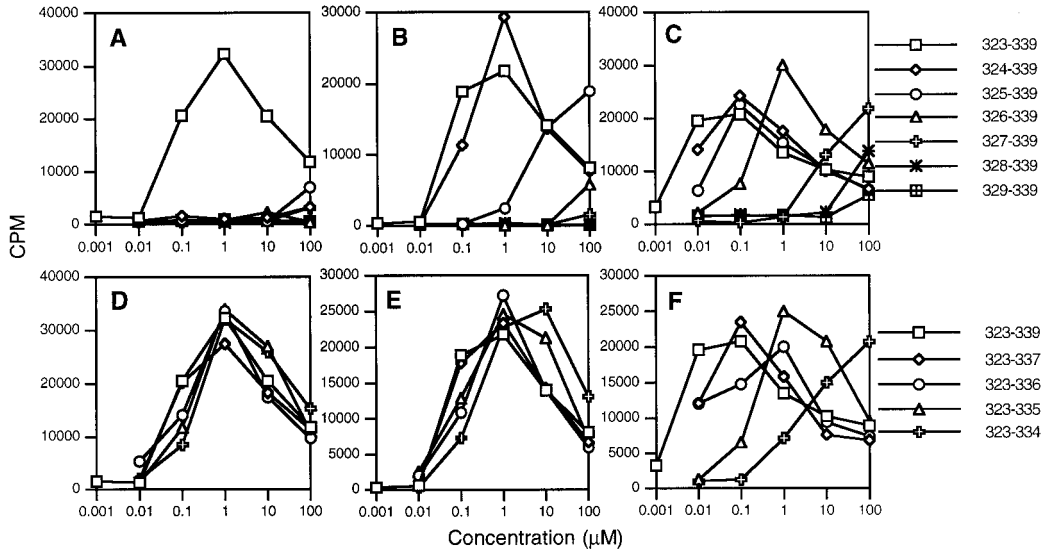

FIGURE 4. Identification of other T cell epitopes within $OVA_{323–339}$. Proliferation of additional $OVA_{323–339}$-reactive T cell clones was measured in response to N-terminal (*A*, *B*, and *C*) and C-terminal (*D*, *E*, and *F*) truncations. Clones A6 (*A* and *D*) and A7 (*B* and *E*) are both specific for an N-terminal epitope. Very few truncations are accepted by A6 and A7 from the N terminus; however, removal of at least five amino acids from the C terminus have no effect on proliferation. Clone A1 (*C* and *F*) recognizes an epitope located in the center of the peptide in that truncations from both sides are tolerated until amino acids 327 and 326 or 335 and 334 are removed.

using analogue peptides substituted at these residues with each amino acid. DO11.10 T cells were tolerant of only one very conservative substitution at 333, whereas five were accepted at amino acid 331 (Fig. 3, *A* and *B*) and the majority of substitutions were accepted at residue 335 (data not shown). The response of OT-II was similar to that of DO11.10 with only one conservative amino acid substitution allowed at amino acid 333, whereas ~10 substitutions were tolerated at residue 331 (Fig. 3, *C* and *D*) and four were allowed at 336 (data not shown). These results indicate that the primary TCR contact residue (P5) for both DO11.10 and OT-II is amino acid 333 with the core 9-aa epitope spanning peptide residues 329–337. The two T cells vary in their recognition of important secondary TCR contact residues (residue 335 vs 336; Fig. 2) and peptide flanking residues (Fig. 1 and Table I), which is expected because each uses a different TCR (18, 20). These results demonstrate that the OVA epitope 329–337 recognized by DO11.10 and OT-II T cells is presented by both $I\text{-}A^d$ and $I\text{-}A^b$ MHC molecules, which provides an explanation for why these cells are able to respond to OVA presented by either MHC molecule.

Additional T cell epitopes within $OVA_{323–339}$

DO11.10 recognition of a C-terminal epitope within $OVA_{323–339}$ agrees with data published by the labs of Kappler and Marrack and others studying DO11.10 T cells (13, 14, 16). However, reports of an N-terminal epitope suggest that $OVA_{323–339}$ can be presented by $I\text{-}A^d$ in multiple binding registers (4, 15). To investigate $OVA_{323–339}$ presentation by $I\text{-}A^d$, a panel of T cells specific for $OVA_{323–339}$ was generated by cloning T cells from an OVA protein-primed BALB/c mouse ($H\text{-}2^d$). The epitope recognized by each T cell was identified using truncations and substitutions of the wild-type $OVA_{323–339}$ peptide (Figs. 4 and 5). The response of these clones is vastly different from those of DO11.10 and OT-II, indicating that $OVA_{323–339}$ contains at least three different T cell epitopes. Two clones reacted similarly, with no reduction in proliferation in response to any of the C-terminal truncations. However, their response was greatly reduced upon removal of just one or two amino acids from the N-terminal side of the peptide (Fig. 4, *A*, *B*, *D*, and *E*). Amino acid 328 is crucial for these T cells, whereas other secondary T cell contact residues vary between clones, establishing that the core 9-aa epitope spans 324–332 (Fig. 5*A*). This is the binding register found in the crystallized

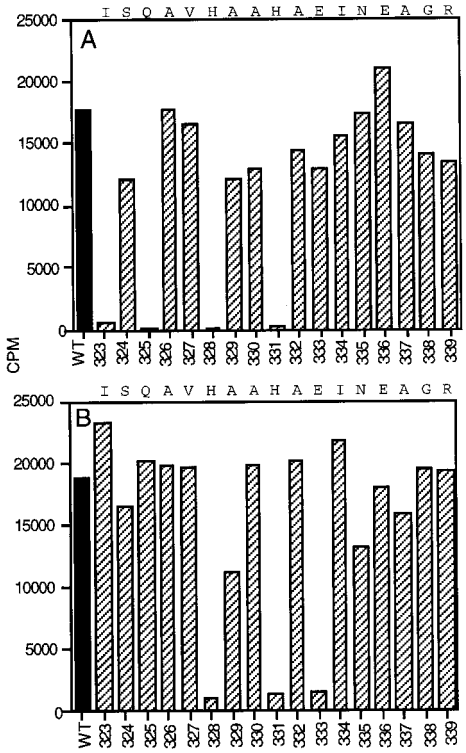

FIGURE 5. Critical TCR contact residues for alternate-register T cell clones. Proliferation of A6 (*A*) and A1 (*B*) T cells was measured in response to a series of alanine- (or serine- if the original amino acid was an alanine) substituted peptides. The maximum T cell response obtained for each peptide is shown. Proliferation of A6 is dependent on amino acids 323, 325, 328, and 331. Residues 328, 331, and 333 are important for recognition by A1. The filled bars represent proliferation in response to $OVA_{323–339}$.

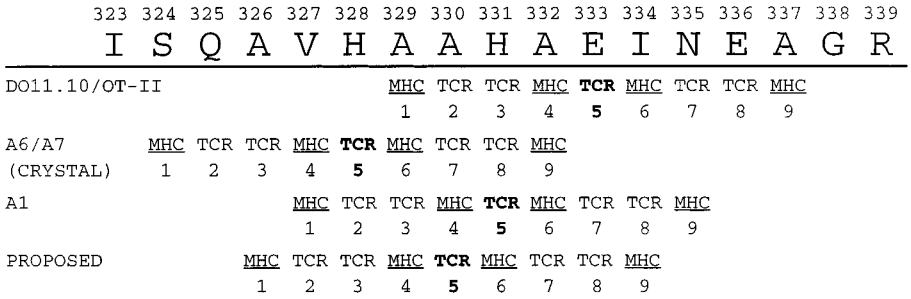

FIGURE 6. $OVA_{323–339}$ contains a nested set of $CD4^+$ T cell epitopes. The location of the core 9 aa for each $OVA_{323–339}$ epitope is shown with the positions of TCR and MHC contact residues noted.

$OVA_{323–339}$:$I\text{-}A^d$ complex (4). Analysis of the data from another T cell clone, A1, shows that this T cell recognizes a different epitope located in the center of $OVA_{323–339}$ (Figs. 4 and 5). The truncations and single amino acid substitutions reveal that this epitope spans from amino acid 327–335 with 331 as the primary TCR contact residue. Other important secondary TCR contact residues for this T cell clone are found at 328 and 333 (Figs. 4, *C* and *F*, and 5*B*).

MHC anchor swap peptides

All peptide:MHC class II crystal structures, including the $OVA_{324–332}$:$I\text{-}A^d$ structure, have shown MHC anchor residues located at positions 1, 4, 6, and 9 of the core 9-aa epitope (4–6, 8). The identification of residue 333 as the primary TCR contact residue for DO11.10 predicts that amino acids 329A, 332A, 334I, and 337A (positions 1, 4, 6, and 9) are the MHC anchor residues for the epitope recognized by DO11.10 (Fig. 6). MHC anchor residues are typically identified using peptide binding assays, which analyze the role of each amino acid residue in peptide affinity for the MHC (7, 15, 23, 24). However, we have shown that $OVA_{323–339}$ contains at least three distinct T cell epitopes, each of which should correspond to a unique MHC binding register (Fig. 6). Because of this, traditional peptide binding assays cannot be used to identify MHC anchor residues. The effect of altering a single MHC anchor residue for one epitope may have little or no effect on overall peptide affinity for $I\text{-}A^d$ because binding in other registers may compensate for any loss of binding of one epitope. Therefore, the influence of single amino acid changes on peptide binding would be inconclusive.

Because of these difficulties, we chose to use DO11.10 T cells as a readout for alterations in the affinity of their epitope. Whereas a change at a single MHC anchor residue normally has very little effect on the T cell response (7, 11, 24), the replacement of all four MHC anchor residues with those from peptides known to have high or low MHC affinities should alter T cell proliferation in a manner consistent with the expected affinity of the epitope. If the MHC anchor positions have not been correctly identified for DO11.10, the replacement of four amino acids would greatly affect proliferation by altering multiple TCR contact residues (Fig. 6). Substituted peptides were made in which amino acids 329A, 332A, 334I, and 337A (positions 1, 4, 6, and 9) were replaced with the corresponding anchor residues from the high-affinity sperm whale myoglobin (SWM) peptide 106–118 (329E, 332I, 334V, 337S) and the lower-affinity MHC class II-associated invariant chain peptide (CLIP) 85–99 (329 M, 332A, 334P, 337 M) (Fig. 7 and Refs. 24 and 25). As expected, proliferation of DO11.10 was unaffected by the anchor swap peptide containing high-affinity anchor residues from SWM, yet the weaker-affinity CLIP anchors decreased the dose-response curve 10- to 100-fold (Fig. 7). These data suggest that positions 1, 4, 6, and 9, corresponding to amino acids 329, 332, 334, and 337, are the MHC anchor residues for the epitope recognized by DO11.10.

Skewing epitope presentation

Our data suggest that at least three different epitopes are being presented simultaneously during a T cell response to $OVA_{323–339}$ (Fig. 6). To manipulate the ratio of ligands to favor presentation of one epitope over another, we designed altered peptides with a large biotinylated anchor residue to hinder binding in the small MHC binding pockets revealed by the $I\text{-}A^d$ crystal structure. For instance, a biotinylated lysine residue substituted at amino acid 337 (position 9 of the C-terminal register and position +5 for the N-terminal register; Fig. 6) decreases the proliferation of DO11.10 T cells by more than 10-fold (Fig. 8). In contrast, stimulation of DO11.10 with OVA 324K-biotin, which affects the N-terminal register (position 1) without changing the C-terminal epitope (position −5), yields a 10-fold increase in T cell sensitivity as well as a stronger peak proliferative response. These data demonstrate that an alteration outside of the core 9-aa epitope and its flanking residues can have significant affects on a T cell's response when multiple epitopes are presented.

Discussion

Previous studies have shown that peptide residues near the N-terminal side of the $OVA_{323–339}$ peptide are important for binding to $I\text{-}A^d$ (15); however, these residues are not required for recognition by DO11.10. This discrepancy has led to our thorough investigation of the $OVA_{323–339}$ peptide and the $CD4^+$ T cell

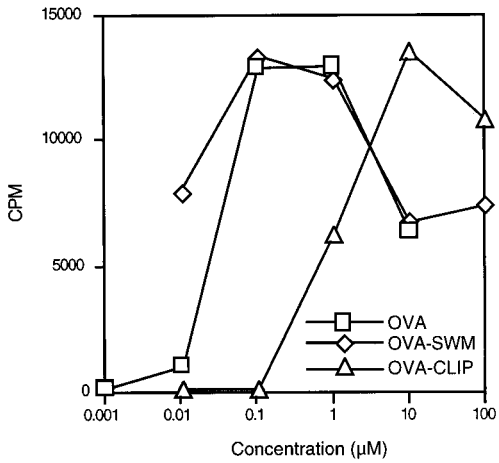

FIGURE 7. Identification of MHC anchor residues using "anchor swap" peptides. Proliferation of DO11.10 T cells was measured in response to $OVA_{323–339}$, OVA-SWM (329E, 332I, 334V, 337S), and OVA-CLIP (329 M, 332A, 334P, 337 M). These peptides were designed by swapping the anchor residues from the DO11.10 epitope of $OVA_{323–339}$ with the anchor residues from peptides with known binding affinities for $I\text{-}A^d$ MHC. Proliferation of DO11.10 is unaffected by OVA-SWM; however, OVA-CLIP causes a 10- to 100-fold decrease in the dose-response curve, which is consistent with the expected affinities of the altered C-terminal epitopes.

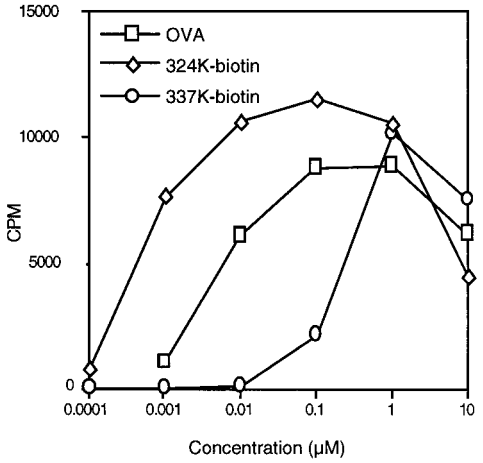

FIGURE 8. T cell proliferation can be skewed by altering the proportion of ligand presented. Peptides were designed with large residues at MHC anchor positions to inhibit binding of a specific $OVA_{323–339}$ epitope. Proliferation of DO11.10 T cells was measured in response to wild-type OVA, 324K-biotin, and 337K-biotin. Substitution of a biotinylated lysine residue amino acid 337 (position 9 of the C-terminal register and position +5 for the N-terminal register; see Table I) decreases the proliferation of DO11.10 T cells by more than 10-fold. In contrast, stimulation of DO11.10 with OVA 324K-biotin, which affects the N-terminal register (position 1) without changing the C-terminal epitope (position −5), yields a 10-fold increase in T cell sensitivity as well as a stronger peak proliferative response.

epitopes it contains. DO11.10, OT-II, and several other OVA-specific T cells were analyzed, and the epitope recognized by each was determined. We have used truncated peptides to locate the region of the peptide containing each epitope, peptides with single amino acid changes to identify the primary and secondary TCR contact residues, and "anchor swap" peptides to determine the position of MHC anchor residues. These studies revealed the existence of at least three distinct $CD4^+$ T cell epitopes within $OVA_{323–339}$ (Fig. 6). DO11.10 and OT-II T cells recognize the same C-terminal epitope spanning amino acids 329–337. Residue 333 is the primary TCR contact for both cells; however, the importance of secondary TCR contact residues and peptide flanking residues varies between the cells. T cells specific for the N-terminal epitope seen in the $OVA_{323–339}$:$I\text{-}A^d$ crystal structure as well as for an epitope located in the center of the peptide were also found.

Earlier analysis of two well-documented $OVA_{323–339}$-specific T cell hybridomas by Sette et al. (15) also used truncations and single amino acid substitutions to map the requirements for peptide interaction with $I\text{-}A^d$. These analyses can be reexamined to determine the epitope specificities of the studied T cell hybridomas. The 8DO-51.15 T cell hybridoma was dependent on the first five N-terminal amino acids and had important TCR contact residues at amino acids 325, 328, and 331, indicating that this T cell recognized the N-terminal $OVA_{323–339}$ epitope seen in the crystal structure and recognized by our clones A6 and A7 (Fig. 6). The 3DO54.8 T cell hybridoma did not require the first several N-terminal amino acids and had critical TCR contact residues at amino acids 328, 329, 331, and 333, consistent with recognition of the central $OVA_{323–339}$ epitope also seen by our clone A1 (Fig. 6).

Unlike $CD8^+$ T cell epitopes, all $CD4^+$ epitopes examined thus far have conformed to the same general structure, with major MHC anchor residues at positions 1, 4, 6, and 9, a primary TCR contact residue at position 5, and secondary TCR contact residues at 2, 3, 7, and/or 8 (4–8). In some cases, position 7 has been shown to interact with the MHC molecule (6, 8). Crystallized peptide:MHC class II complexes have revealed that this core 9-aa epitope is always located within the MHC binding groove (4–6, 8). The finding of three overlapping epitopes within $OVA_{323–339}$ indicates that there are also multiple binding registers. Early binding studies by Grey and coworkers (15) identified residue 332 as one of the most important $I\text{-}A^d$ binding residues, a finding which supports our argument of multiple binding registers because this amino acid is an MHC anchor residue for all three epitopes described here (Fig. 6).

The crystal structures of $OVA_{323–339}$ and hemagglutinin $(HA)_{126–138}$ bound to $I\text{-}A^d$ published by Wilson and coworkers (4) reveals several unique characteristics that define peptide binding to $I\text{-}A^d$. For instance, peptide binding to $I\text{-}A^d$ does not require the insertion of large polar or charged peptide anchor residues into MHC pockets. The anchor residues for $HA_{126–138}$ and $OVA_{323–339}$ are mostly small- to medium-sized nonpolar amino acids like alanine and valine. Additionally, the majority of hydrogen bonds between $I\text{-}A^d$ and a bound peptide are to the peptide backbone, allowing binding to be virtually sequence-independent. These characteristics led the authors to predict that OVA may be able to bind to $I\text{-}A^d$ in multiple registers that all have a similar pattern of small anchor residues (4). We have shown that one of the two alternative registers suggested by Wilson and coworkers (4) is recognized by DO11.10 and OT-II T cells (Figs. 1–3 and 7). We did not identify any T cells specific for the second predicted register centered at amino acid 330, although the examination of additional clones may lead to their discovery. The existence of the central epitope recognized by the A1 clone was not predicted, most likely due to the asparagine located at position 9, which does not conform to the observed preference for alanine at this position. However, according to our data (Fig. 7), DO11.10 T cells are able to respond to OVA peptides with vastly different MHC anchor residues, suggesting that the $I\text{-}A^d$ binding motif is not simply restricted to small, nonpolar amino acids. In fact, the SWM peptide 106–118, which has a very high affinity for $I\text{-}A^d$ (Fig. 7), has a large negatively charged residue (glutamic acid) at position 1 (24).

Other molecules in the murine I-A and human HLA-DQ families are structurally similar to $I\text{-}A^d$, and therefore, the characteristics of the MHC binding pockets and peptide-MHC interactions should be comparable (4). The relative absence of dominant MHC anchors may allow for the presentation of a single peptide on multiple MHC molecules. For instance, $OVA_{323–339}$ is known to bind $I\text{-}A^b$, $I\text{-}A^u$, and $I\text{-}A^s$ as well as MHC molecules from other species (20, 21, 25–27). The bovine RNase peptide 90–105 has also been shown to be presented by multiple MHC class II alleles (28). We expect that further analysis of other I-A binding peptides will demonstrate similar promiscuity of MHC binding, and other nested sets of epitopes as described for $OVA_{323–339}$. The close proximity of these three T cell epitopes may explain the prevalence of T cells generated in an OVA protein-primed mouse, which are specific for $OVA_{323–339}$ (13). A single epitope within this peptide may not be a better ligand than an epitope found elsewhere in the protein, but the concentration of epitopes within this sequence makes $OVA_{323–339}$ a "hotspot" of T cell reactivity (13). Peptides with a nested set of epitopes like $OVA_{323–339}$ have advantages over single-epitope peptides because the potential pool of responding T cells is multiplied for each epitope.

Altering the effective concentration of a ligand when using a nested set of epitopes as observed with $OVA_{323–339}$ could be an effective mechanism for controlling the immune response. Several groups have demonstrated that the phenotype of a Th cell can be influenced by Ag dose (29, 30). We were able to change the effective dose of the C-terminal OVA epitope by two methods. First, the affinity of this OVA epitope was modulated by swapping its

MHC anchor residues with those from other peptides with a desired affinity for MHC such as SWM or CLIP (Fig. 7). DO11.10 proliferation to OVA-CLIP was decreased by 10- to 100-fold (Fig. 7). In the second method, a single anchor residue of the N-terminal register was altered, decreasing the affinity of this epitope and consequently increasing presentation of the C-terminal epitope as measured by the response of DO11.10 T cells (Fig. 8). Both of these techniques affect T cell proliferation in a manner consistent with the expected availability of the $OVA_{329–337}$:I-A^d ligand and could be used to favor the presentation of any given epitope over another.

One of the goals of research using altered peptide ligands is to develop in vivo therapies designed to control T cell populations in disorders such as autoimmunity or graft rejection (22). The presentation of multiple epitopes from peptides such as $OVA_{323–339}$ may make it difficult to manipulate the bulk T cell response in this manner because the TCR contact residues will vary depending on the epitope recognized. However, it is interesting that amino acid 331 is an important TCR contact residue for each of the analyzed T cell clones despite their recognition of different epitopes. Amino acid substitutions of the $OVA_{323–339}$ peptide at this residue may yield partial agonist and antagonist peptides able to control the responses of all $OVA_{323–339}$-reactive T cells (22). In contrast, substitutions at amino acid 335 or 336 would be expected to only affect T cells such as DO11.10 and OT-II, which recognize the C-terminal epitope.

Note added in proof: Since the submission of this manuscript, McFarland et al. (31) have biochemically confirmed the existence of the central I-A^d binding register recognized by clone A1 and the DO54.8 hybridoma.

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