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\textit{J Immunol} 2000; 165:2059-2067; doi: 10.4049/jimmunol.165.4.2059

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The MHC Class II Molecule I-A^d7 Exists in Alternate Conformations That Are Peptide Dependent

Lynne S. Arneson, Mary Peterson, and Andrea J. Sant

Insulin-dependent diabetes mellitus is an autoimmune disease that is genetically linked to the HLA class II molecule DQ in humans and to MHC I-A^d7 in nonobese diabetic mice. The I-A^d7 β-chain is unique and contains multiple polymorphisms, at least one of which is shared with DQ alleles linked to insulin-dependent diabetes mellitus. This polymorphism occurs at position 57 in the β-chain, in which aspartic acid is mutated to a serine, a change that results in the loss of an interchain salt bridge between αArg^76 and βAsp^57 at the periphery of the peptide binding groove. Using mAbs we have identified alternative conformations of I-A^d7 class II molecules. By using an invariant chain construct with various peptides engineered into the class II-associated invariant chain peptide (CLIP) region we have found that formation of these conformations is dependent on the peptide occupying the binding groove. Blocking studies with these Abs indicate that these conformations are present at the cell surface and are capable of interactions with TCRs that result in T cell activation. The Journal of Immunology, 2000, 165: 2059–2067.

The DQ allele associated with diabetes in humans shares a structural variation with the I-A^d7 class II molecule expressed in NOD mice. In both these molecules, but not in any other class II molecule, position 57 of the β-chain is mutated from an aspartic acid to a serine or other noncharged amino acid. In NOD mice, position 56 of the β-chain is also mutated from a proline, expressed in most other alleles, to a histidine. Crystal structures of I-A^d and I-A^d have indicated that Asp^57 of the β-chain forms an interchain salt bridge with Arg^76 in the class II α-chain at the carboxyl end of the peptide binding groove of the class II molecule. The interchain salt bridge between Asp^57 and Arg^76 probably stabilizes the interaction between the α- and β-chains and may stabilize the integrity of the peptide binding pocket of the class II molecule. The crystal structure of I-A^d7 indicates that the loss of the salt bridge results in a substantially wider peptide-binding groove around B57, which may account for differences in peptide preferences.

Because MHC class II molecules function to present peptide to CD4^+ T cells, a change in class II structure can affect systemic immunity by altering peptide binding, resulting in a change in either the repertoire of peptides bound or the affinity with which the peptide associates. Alternatively, changing the class II structure can alter the TCR-class II interaction. Recent evidence suggests that much of I-A^d7 class II expressed in APCs may not be associated with peptide (19). In vitro assays indicated that peptide binding to I-A^d7 is undetectable and that even immunogenic peptides rapidly dissociate from I-A^d7. Although another study reported success in eluting peptides from I-A^d7 and found that some peptides were able to stably associate with the I-A^d7 class II molecule, the authors conceded that much of the I-A^d7 class II appeared to be empty based on low yields of eluted peptides and on peptide binding studies (20). Large amounts of empty I-A^d7 would result in decreased cellular concentrations of I-A^d7 associated with specific peptide ligands. In the thymus, this decreased concentration of peptide:class II complexes would result in inefficient negative selection, thus allowing increased numbers of autoreactive T cells to emerge into the periphery (for review, see Refs. 21–23). Recent studies suggest that the peptide binding specificity of the class II I-A^d7 molecule is influenced dramatically by the presence of the absence of Asp^57 in the β-chain (18, 24). Thus, it is possible that changing the amino acid at β57 may affect the selection of
autoantigenic T cell epitopes. Finally, a variant class II structure induced by the unique sequence of I-A^\alpha7 could affect class II interaction with the TCR. This altered structure could decrease the affinity of the class II-TCR interaction and thus could decrease the efficiency of negative selection, allowing autoreactive T cells to escape to the periphery.

Previously our group investigated the association of NOD MHC class II molecules with invariant chain and DM, both known cofactors of class II molecules. Invariant chain participates in a number of events in class II biosynthesis; most notably it facilitates MHC class II folding in the endoplasmic reticulum and localizes to endosomes. In endosomes, invariant chain is proteolysed, leaving a short fragment, called the class II-associated invariant chain peptide (CLIP), in the peptide binding pocket of the MHC class II molecule (25, 26). An MHC-encoded heterodimer term DM associates with class II molecules in endosomes and facilitates the exchange of CLIP for antigenic peptide in the class II binding pocket (27, 28). To test whether class II molecules that had loaded with Ag in the presence of DM possessed any unique conformational features, cells coexpressing NOD class II, invariant chain, and DM were tested for reactivity with a panel of monoclonal anti-class II alloantibodies. These experiments revealed that a class II-specific mAb, 40M, preferentially stains I-A\(^\text{g7}\) molecules bio- synthetically in the presence of invariant chain and DM (29). Additional experiments demonstrated that 40M does not recognize I-A^\alpha7 when CLIP is associated. Thus, DM expression apparently enhances the reactivity of I-A^\alpha7 with 40M by facilitating the release of CLIP. These results indicate that 40M recognizes an epitope on I-A^\alpha7 that is disrupted by CLIP association. Another epitope on I-A^\alpha7 that is disrupted by CLIP association.

Materials and Methods

Reagents and cell lines

NOD and CBA/J mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained in a pathogen-free barrier facility. B cell hybridomas secreting the mAb 10.2-16 (30), which binds both I-A^\alpha7 and I-A^\delta, and 16-1-11N (31), which binds H-2K^\alpha class I molecules, were obtained from American Type Culture Collection (Manassas, VA). Cells secreting 40M, which binds both I-A^\delta7 and I-A^\alpha (32); the anti-invariant chain Ab In-1 (33), and K24-199, which recognizes the I-A^\alpha \alpha chain (34), were provided by Dr. Jan Miller (University of Chicago, Chicago, IL). 39E supernatant, which recognizes I-A^\delta \beta-chain, was originally described by Pierres et al. (32). 20-8-4S recognizes H-2 K^\delta (35), and cells secreting the Ab were obtained from American Type Culture Collection. The anti-\beta-chain antiserum was made by collecting serum from rabbits originally immunized with a peptide corresponding to the cytoplasmic region of the I-A^\alpha \beta-chain conjugated to keyhole limpet hemocyanin in CFA, followed by subsequent immunizations in IFA.

Generation and expression of invariant chain constructs

Invariant chain cDNA constructs in which the CLIP region was either mutated at single amino acids or replaced with heterologous peptides were generated by ligation of annealed oligonucleotides into a mutated invariant chain construct designed to contain a unique restriction site on each side of the CLIP region (Fig. 1). The mutated invariant chain cDNA was constructed using PCR-mediated site-directed mutagenesis. The plasmid containing the new restriction sites was digested with KpnI and XhoI, gel isolated, and treated with calf alkaline phosphatase, then ligated to oligonucleotides (Life Technologies, Gaithersburg, MD) pretreated with polynucleotide kinase in the presence of 1 M ATP at 37°C for 1 h, and heated to 65°C to inactivate the kinase. The 5' and 3' oligonucleotides were mixed, placed in a beaker of 65°C water, and allowed to cool to room temperature to permit annealing. After ligation, plasmid clones were sequenced to verify the insertion of the modified CLIP segment. The constructs were then transiently transfected in Ltk^- cells stably expressing I-A^\alpha7. Briefly, Ltk^- cells expressing I-A^\alpha7 were incubated with 5 M cDNA in DMEM/10 M HEPES/25.8 mM glucose/0.25 mg/ml DEAE-dextran at 37°C for 3 h. The DNA solution was aspirated and replaced with DMEM and 10% DMSO (Sigma, St. Louis, MO) at room temperature for 1 min. The DMEM/DMSO was replaced with complete medium, and the cells were incubated for 72 h before harvesting from culture and biochemical analysis.

Metabolic labeling

Spleen cells were isolated from either NOD or CBA/J mice and labeled in leucine-free medium for 1 h at 37°C. Cells were then transferred to plates containing leucine-free medium plus 0.25 mM of [1^\text{H}]leucine (New England Nuclear, Boston, MA)/ml of medium and incubated at 37°C for 2 h, after which they were recovered by centrifugation and incubated in complete medium at 37°C for 5 h. Detergent lysates of the labeled cells were prepared by lysis using ice on 20 min in 0.5% Nonidet P-40, 150 mM NaCl, 50 mM Tris (pH 7.6), 5 mM EDTA, and 5 mM iodoacetamide plus protease inhibitors. The presence of iodoacetamide prevents disulfide bond formation between invariant chain molecules by air oxidation following cell lysis. Class II molecules were isolated from the postnuclear supernatant by immunoprecipitation using anti-class II Abs prebound to protein A-Sepharose (Pharmacia, Piscataway, NJ). Samples were dissociated in SDS-sample buffer containing 2% 2-ME, then electrophoresed on SDS-10% PAGE, and the gel was processed for autoradiography using Fluoro-Hance (Research Products International, Natick, MA) for 30 min. The treated gel was dried and exposed to film at ~70°C.

Western blot analysis

Cells were lysed in a buffer containing 150 mM NaCl, 50 mM Tris (pH 7.6), 5 mM EDTA, 5 mM iodoacetamide, protease inhibitors, and 10% glycerol (pH 6.8), and boiled for 2 min. Samples were electrophoresed on a SDS-10% PAGE and transferred to nitrocellulose for Western blot analysis as described previously (29). Postnuclear supernatants were incubated with mAbs prebound to protein A-Sepharose (Pharmacia). Immunoprecipitated proteins were eluted at room temperature with 2% SDS, 0.0625 M Tris (pH 6.8), and 10% glycerol, then boiled for 2 min. Samples were electrophoresed on a SDS-10% PAGE and transferred to nitrocellulose for Western blot analysis as described previously (29). The nitrocellulose membrane was blocked with 5% dry milk in TBST (10 mM Tris (pH 7.6), 0.15 M NaCl, and 0.1% Tween 20), then probed with the anti-invariant chain Ab In-1 in 5% milk/TBST overnight. Bound In-1 was detected with HRP-conjugated anti-rat Ab in 5% milk/TBST and developed with chemiluminescence using LumiGLO (Kirkegaard & Perry, Gaithersburg, MD).

Surface biotinylation

Spleen cells were washed in cold PBS three times before biotinylatation to remove competing proteins. NHS-SS-biotin (Pierce, Rockford, IL) was dissolved in DMSO at 300 mg/ml, then diluted to 1.5 mg/ml in cold PBS immediately before use. Washed splenic cells were suspended in NHS-SS-biotin/PBS at a concentration of 2 \times 10^7 cells/ml and incubated on ice for 30 min. The biotinylation reaction was stopped by washing cells three times with 50 M glycine in cold PBS. The cells were then washed twice with excess amounts of iodoacetamide in cold PBS (5 mg/ml to prevent biotinylation of internal material following cell lysis and solubilized, and the lysate was subjected to immunoprecipitation as described above.

Ag presentation assays

The OVA-specific and HEL-specific, I-A^\alpha7-restricted T cell hybridomas were generated by fusing Ag-specific T cells to the TCR-negative variant of the T cell lymphoma BW5147. The T cells used in the fusions were obtained by in vivo priming with 50 M of 1 mg/ml OVA or HEL in CFA (Sigma), followed by subsequent restimulations in vitro with Ag and syngeneic splenocytes at 10 days. Fusion was performed 3 days after a secondary Ag restimulation, and fused cells were cloned by limiting dilution. Ag-specific hybridomas were identified by coculture with Ag and nodal splenocytes as APCs. T cell hybridomas are specific to OVA (30, 5, HEL) and 97.4) or HEL (90.5, S17.10, and S70.4). S70.4 has a different fine specificity than 90.5 because it does not react with the HEL peptide 9-29.

Production of I-A^\alpha7

The OVA-specific and HEL-specific, I-A^\alpha7-restricted T cell hybridomas were generated by fusing Ag-specific T cells to the TCR-negative variant of the T cell lymphoma BW5147. The T cells used in the fusions were obtained by in vivo priming with 50 M of 1 mg/ml OVA or HEL in CFA (Sigma), followed by subsequent restimulations in vitro with Ag and syngeneic splenocytes at 10 days. Fusion was performed 3 days after a secondary Ag restimulation, and fused cells were cloned by limiting dilution. Ag-specific hybridomas were identified by coculture with Ag and nodal splenocytes as APCs. T cell hybridomas are specific to OVA (10.10, 30, 5, and HEL (90.5, S17.10, and S70.4). S70.4 has a different fine specificity than 90.5 because it does not react with the HEL peptide 9-29.

Cell Ag presentation assays were performed as previously described (29). Briefly, 25,000 T cells and 250,000 NOD spleen cell APCs were cocultured in 96-well dishes in the presence of submaximal doses of appropriate Ag. Ab supernatant, either 10.2-16 or 40M, was added to 25% of the final
Variability in Ab recognition of I-A<sup>87</sup> is peptide dependent

We have previously shown that the presence of the CLIP peptide in the peptide binding groove of I-A<sup>87</sup> disrupts recognition by the mAb 40M (29). Assaying whether the presence of other peptides in the groove can also affect 40M reactivity with I-A<sup>87</sup> is hindered by the fact that I-A<sup>87</sup> binds peptides poorly (19). Thus, attempts to selectively accumulate known soluble synthetic peptides on I-A<sup>87</sup> would probably fail. To circumvent this problem, we used the natural chaperone of class II, invariant chain, to hold a heterologous peptide in the groove of I-A<sup>87</sup> (36). As discussed above, the CLIP region of invariant chain associates with the peptide binding groove of the class II molecule. The regions of invariant chain flanking CLIP also make contact with the associated class II molecule, thus serving to tether CLIP within the binding pocket (37–39). We capitalized on this natural association to engineer different peptides into the class II binding pocket (36). Invariant chain constructs were derived in which the CLIP region was either mutated at single amino acids or completely replaced with other heterologous peptides (Fig. 1). These constructs were then transiently transfected into L cell fibroblasts expressing I-A<sup>87</sup>. To determine whether the presence of these peptides in the binding groove of I-A<sup>87</sup> affects 40M recognition, detergent lysates were prepared from cells expressing the alternate invariant chain constructs described in Fig. 1, and class II molecules were assayed for reactivity with mAbs. Class II molecules were immunoprecipitated from the lysates with either 10.2-16 or 40M. Class II molecules associated with CLIP or the replacement peptide were identified from the lysates with either 10.2-16 or 40M as indicated. The precipitate was analyzed by SDS-PAGE and blotted with an Ab that recognizes the cytoplasmic tail of invariant chain, and the Western blot was developed with enhanced chemiluminescence. The position of invariant chain is indicated. Both experiments shown include the CLIP construct to allow comparison of the relative amounts of invariant chain isolated with 10.2-16 and 40M. This experiment was performed five times.

Alternative conformations of I-A<sup>87</sup> are recognized by 10.2-16 and 40M

In the previous experiment the alternate conformations of I-A<sup>87</sup> were present in the biosynthetic pathway of the cell in the presence of associated intact invariant chain. We next sought to determine...
whether the alternative conformations of the class II molecule could be induced by naturally occurring peptides. To determine whether naturally occurring peptide:class II complexes can be distinguished by their reactivity with 10.2-16 vs 40M, newly synthesized proteins expressed by NOD spleen cells were radiolabeled with [3H]leucine. The cells were then chased in complete medium to allow the labeled protein to traffic through the secretory system and into endosomes, where endosomally acquired peptide would be loaded onto the class II molecules. To identify class II molecules exclusively reactive with 10.2-16 or 40M, a sequential immunoprecipitation strategy was employed. Following preparation of a detergent lysate from the radiolabeled spleen cells, class II molecules reactive with 10.2-16 were exhaustively removed from one-third of the lysate by successive immunoprecipitations, while molecules reactive with 40M were depleted from one third, and the last third of the lysate was treated with a control Ab. Class II molecules remaining in the lysate following depletion were assayed for Ab reactivity by immunoprecipitation with either 10.2-16 or 40M, then MHC class I molecules were immunoprecipitated to demonstrate that equivalent amounts of cell lysate were used for each immunoprecipitation following immunodepletion (Fig. 3C). Spleen cells from CBA mice expressing a different, but serologically related, class II allele (I-Ak) were also radiolabeled, chased, immunodepleted, and precipitated to determine whether specific monoclonal reactivity, and thus adoption of specific alternative conformations, is limited to the I-Ak class II allele. The results of this experiment are shown in Fig. 3. Following immunodepletion of NOD spleen lysate with 10.2-16, very little 10.2-16-precipitable I-Ag7 persisted in the lysate, showing that quantitative depletion was obtained. Examination of the 40M immunoprecipitation revealed that there was a significant amount of 40M-reactive class II molecules remaining in this lysate (Fig. 3A, compare lanes 3 and 4). Similarly, 40M immunodepletion was complete (Fig. 3A, lane 6), yet there was a substantial amount of I-Ag7 precipitated with 10.2-16 (Fig. 3A, compare lanes 7 and 8). From these data we can identify three distinct sets of class II-peptide complexes in NOD APCs. The major set of complexes, comprising ~80% of all class II molecules, is reactive with both 10.2-16 and 40M. The remaining class II molecules are in two equally represented sets, one reactive with only 10.2-16 and one reactive with only 40M. By contrast, there are no I-Ak molecules that are only precipitable by 40M (Fig. 3B, lanes 3 and 4), although 10.2-16 precipitates some I-Ak proteins following 40M immunodepletion (Fig. 3B, compare lanes 7 and 8). These data indicate that I-Ag7, but not I-Ak, is present in at least three peptide-dependent alternate conformations that can be distinguished by 10.2-16 and 40M reactivity. These alternate conformations of I-Ag7 are present in splenic APCs and can be induced by naturally occurring peptide in association with mature class II molecules.

Careful analysis of the autoradiographs in Fig. 3 suggest that the major population of class II-Ag7 molecules that are 10.2-16+/40M- appears to be associated with invariant chain (Fig. 3A, lane 7). To determine whether the 10.2-16+/40M- class II molecules were exclusively comprised of class II-invariant chain complexes containing CLIP within the peptide binding groove, successive immunoprecipitation was again employed, with one modification. Added to the protocol used in the previous experiment was an invariant chain depletion step to remove any class II-invariant chain complexes. Spleenic cells from NOD mice were radiolabeled and then chased to allow the labeled class II molecules to mature. Class II molecules reactive with 40M were exhaustively removed from the detergent lysate by immunoprecipitation. Following this immunodepletion step, half the lysate was tested for reactivity with 10.2-16, while the other half of the lysate was first immunode-
then precipitated with either 10.2-16 or 40M to assess the remaining levels of class II α- and β-chain precipitable by 10.2-16. If, following 40M depletion, 10.2-16 only precipitates class II associated with invariant chain, then the 10.2-16 precipitation following the anti-invariant chain depletion should contain no class II molecules. As seen previously, 10.2-16 precipitates class II α- and β-chains as well as some invariant chain following quantitative immunodepletion with 40M. Following immunodepletion of invariant chain, 10.2-16 still precipitates class II α- and β-chains, but no associated invariant chain molecules (Fig. 4). Thus, these data suggest that the subset of class II-peptide complexes precipitated only by 10.2-16 contains other peptides besides CLIP in the class II binding groove.

The alternate conformations of I-A\textsuperscript{d7} are present on the cell surface

To determine whether the class II molecules in the alternate conformations are present at the cell surface, and thus are able to interact with T cells, surface proteins expressed on NOD spleen cells were biotinylated. Sequential immunoprecipitation was again used to isolate class II molecules reactive with only 10.2-16 or 40M. These molecules were then detected by streptavidin in a Western blot. The results (Fig. 5) indicate that all three conformations, 10.2-16/40M\textsuperscript{1}, 10.2-16/40M\textsuperscript{2}, and 10.2-16/40M\textsuperscript{3}, are present at the cell surface and thus potentially able to interact with TCRs.

Conformational states identified by mAbs are functionally relevant

To determine whether T cells are able to interact with the alternate conformations of I-A\textsuperscript{d7}, the abilities of 10.2-16 and 40M to block T cell recognition of class II-peptide complexes on APCs were assessed. A set of T cells specific for either HEL or OVA in the context of NOD class II were generated, and dose-response curves to Ag were determined (data not shown). These T cells were then tested for their relative ability to be blocked by 10.2-16 vs 40M at a suboptimal dose of Ag (Fig. 6). One set of T cells restricted to OVA (10.10, 30.5, and 97.4) in the context of I-A\textsuperscript{d7} was more efficiently blocked by 10.2-16 than 40M. Another set of T cells restricted to HEL (S70.4, 90.5, and S17.10) in the context of I-A\textsuperscript{d7} was also generated. We know the fine specificity of these T cells tested for their relative ability to be blocked by 10.2-16 vs 40M at a suboptimal dose of Ag (Fig. 6). One set of T cells restricted to OVA (10.10, 30.5, and 97.4) in the context of I-A\textsuperscript{d7} was more efficiently blocked by 10.2-16 than 40M. Another set of T cells restricted to HEL (S70.4, 90.5, and S17.10) in the context of I-A\textsuperscript{d7} was also generated. We know the fine specificity of these T cells

FIGURE 4. I-A\textsuperscript{d7} class II molecules reactive exclusively with 10.2-16 are not comprised entirely of class II-invariant chain complexes. Spleen cells from NOD mice were radiolabeled with [\textsuperscript{3}H]leucine for 2 h, then incubated in unlabeled complete medium for 5 h. Following lysis, the lysate was immunodepleted by four successive rounds of 40M (immunodepletions 1 and 4 are shown). The lysate was then divided. Half the lysate was divided again and immunoprecipitated with either 10.2-16 or 40M. The remaining lysate was first immunodepleted by four successive rounds of a combination of anti-invariant chain Abs (P4H5 and In-1), then divided and immunoprecipitated with either 10.2-16 or 40M. The α- and β-chains are indicated, as is the p31 invariant chain. This experiment was performed twice.

FIGURE 5. The alternative conformations of I-A\textsuperscript{d7} are present on the cell surface. Surface molecules on NOD spleen cells were biotinylated on ice, then lysed. The lysate was divided in half and immunodepleted by four successive rounds of either 10.2-16 or 40M. Following immunodepletion, the lysate was again divided in half, and the class II molecules were immunoprecipitated by either 10.2-16 or 40M. The α- and β-chains are indicated. This experiment was performed twice.

FIGURE 6. Alternative conformational states of I-A\textsuperscript{d7} are recognized by Ag-specific T cells. T cell hybridomas specific to OVA (10.10, 30.5, and 97.4) or HEL (90.5, S17.10, or S70.4) were generated as described in Materials and Methods. S70.4 has a different fine specificity than 90.5, because it does not react with the HEL peptide 9–29. Briefly, 25,000 T cells and 25,000 NOD spleen cells were incubated in the presence of suboptimal doses of Ag at a suboptimal dose of Ag (Fig. 6). One set of T cells restricted to OVA (10.10, 30.5, and 97.4) in the context of I-A\textsuperscript{d7} was more efficiently blocked by 10.2-16 than 40M. Another set of T cells restricted to HEL (S70.4, 90.5, and S17.10) in the context of I-A\textsuperscript{d7} was also generated. We know the fine specificity of these T cells
to be different, because 90.5 and S17.10, but not S70.4, recognize HEL peptide 9–29 (data not shown). The mAb blocking studies revealed that the T cells 90.5 and S17.10 were both more efficiently blocked by 40M than by 10.2-16, consistent with the data shown in Fig. 2. The T cell S70.4, by contrast, is more efficiently blocked by 10.2-16 than by 40M. Together these data indicate that I-A<sup>q7</sup> associated with antigenic peptides form different conformations that are differentially recognized by 10.2-16 and 40M. Further, these class II-peptide conformations are capable of interacting with T cells, resulting in T cell activation, indicating that they are present on the cell surface and may be functionally relevant in vivo.

Conformational changes in I-A<sup>q7</sup> are not confined to a local region of the molecule

The data shown above suggest that the I-A<sup>q7</sup> class II molecule can adopt multiple conformations that are dependent upon the peptide present in the class II peptide binding groove. However, the increased flexibility of the I-A<sup>q7</sup> molecule may be limited to a localized region of the class II molecule. To determine whether the preceding data reflect a global flexibility of the I-A<sup>q7</sup> molecule or merely increased flexibility in a localized region, we screened additional anti-class II mAbs for relative reactivity with I-A<sup>q7</sup> bound to different peptides. In this experiment we used an antiserum directed against the cytosolic tail of the β-chain of MHC class II molecules as a measure of total I-A<sup>q7</sup> precipitable from lysates. In addition to 10.2-16 and 40M, reactivity of I-A<sup>q7</sup>-peptide complexes with K24-199, directed against the I-A<sup>q7</sup> α-chain, and 39E, directed against the β-chain, was tested. Cells stably expressing I-A<sup>q7</sup> were transiently transfected with the constructs shown in Fig. 1, and the cells were harvested and lysed 3 days post-transfection. After dividing the lysate into equal portions for precipitation, 12.5% of each portion was analyzed by Western blot for invariant chain levels to verify that equal amounts of lysate were used for each immunoprecipitation, and that the relative levels of invariant expression for each transfection were equivalent. Compared with the yield of class II-invariant chain complexes isolated with the anti-β tail antiserum, I-A<sup>q7</sup> associated with CLIP was recognized well by 10.2-16, to an intermediate level by K24-199 and 40M, and not at all by 39E (Fig. 7). I-A<sup>q7</sup> associated with invariant chain in which the CLIP peptide has been exchanged for the HEL peptide was precipitated by 10.2-16, 40M, and 39E in amounts similar to those precipitated by anti-β tail antiserum, but was recognized poorly by K24-199 (Fig. 7). CLIP mutated at position 99 from Arg to Ala associated with I-A<sup>q7</sup> was recognized well by both 10.2-16 and 40M compared with anti-β tail antiserum, but was precipitated poorly by both K24-199 and 39E (Fig. 7). These data indicate that the peptide in the binding groove can affect recognition by Abs directed against both the α- and the β-chain of the class II molecule, suggesting that peptide-dependent alterations in conformation are not limited to local regions, but result in more global changes in the structure of the I-A<sup>q7</sup> class II molecule.

Discussion

The question of whether there is a unique structural characteristic of diabetogenic class II molecules is still not resolved. HLA-DQ class II genes in humans and the I-A<sup>q7</sup> class II gene in mice are genetically linked to diabetes susceptibility. The β<sub>57</sub>Asp→Ser polymorphism shared by these alleles is thought to be important in disease initiation or progression, but how this polymorphism affects class II and thus diabetes susceptibility is not clear. Studies presented here suggest that the murine class II molecule associated with diabetes susceptibility, I-A<sup>q7</sup>, exists in multiple alternative conformational states. We used an Ab (40M) whose epitope was previously found to be enhanced on I-A<sup>q7</sup> when coexpressed with invariant chain and DM (29) along with other anti-class II mAbs to identify alternate conformations of I-A<sup>q7</sup>. The formation of these conformations is peptide dependent, as shown by the change in relative reactivities of these Abs when different peptides are locked

**FIGURE 7.** Conformational changes in I-A<sup>q7</sup> are not confined to a localized region. L cells stably expressing I-A<sup>q7</sup> class II were transiently transfected with either vector alone or the invariant chain constructs shown in Fig. 1. After 3 days the cells were harvested and lysed, and the supernatant was divided into five equal portions. Aliquots of 50 μl were removed from each portion, and the class II molecules from the remaining supernatant were immunoprecipitated with anti-β tail antiserum, 10.2-16, 40M, K24-199, or 39E (A). The 50-μl aliquots were analyzed by SDS-PAGE and Western blot with In-1 for invariant chain expression to determine that equivalent amounts of cell lysate were used for each immunoprecipitation (B). This experiment was performed four times.
into the class II peptide binding groove. T cell blocking assays show that these Abs differentially block T cell clones, indicating that these conformations are present at the cell surface and are capable of interacting with TCRs resulting in T cell proliferation.

The I-A\textsuperscript{d} class II molecule is a unique hybrid molecule, composed of the I-A\textsuperscript{d} \(\alpha\)-chain and the unique I-A\textsuperscript{d} \(\beta\)-chain. The sequence at amino acids 12–14 in the \(\beta\)-chain is identical with I-A\textsuperscript{\(\alpha\)}\textsuperscript{d}, allowing the \(\beta\)-chain to dimerize with the I-A\textsuperscript{\(\alpha\)} \(\alpha\)-chain (41). This sequence is also shared with I-A\textsuperscript{\(\alpha\)} u, f, and q class II \(\beta\)-chains. The remainder of the I-A\textsuperscript{\(\alpha\)}\textsuperscript{d}\(\beta\)-chain most closely resembles I-A\textsuperscript{\(\alpha\)}, although polymorphisms at positions 8, 9, 26, and 63 are shared with I-A\textsuperscript{\(\alpha\)}. Amino acids at positions 65 and 67 are deleted in I-A\textsuperscript{\(\alpha\)}\textsuperscript{d}\(\beta\), as they are in k, u, and f, allowing the I-A\textsuperscript{\(\alpha\)}\textsuperscript{d}\(\beta\)-chain to be recognized by the mAb 10.2-16. Finally, I-A\textsuperscript{\(\alpha\)}\textsuperscript{d}\(\beta\)-chain contains polymorphisms at positions 56 and 57, resulting in the lack of potential to form an interchain salt bridge with the \(\alpha\)-chain. These multiple substitutions make it difficult to compare I-A\textsuperscript{\(\alpha\)}\textsuperscript{d} with any other class II molecule in terms of Ab reactivity and suggest that different conformational states could be due not just to the lack of the salt bridge, but possibly to the cumulative effect of multiple polymorphisms.

The formation of alternate conformations by I-A\textsuperscript{\(\alpha\)}\textsuperscript{d} suggests that this class II molecule is flexible, but the degree of flexibility in molecular terms is difficult to directly assess. The Abs used to identify the conformations recognize distinct regions in the class II molecule. The mAb K24-190 recognizes the I-A\textsuperscript{\(\alpha\)} \(\alpha\)-chain (34), which is the partner chain for the I-A\textsuperscript{\(\alpha\)}\textsuperscript{d} \(\beta\)-chain. The 10.2-16 epitope is located around aa 64 in the \(\beta\)-chain near the carboxyl end of the bound peptide (34), whereas recognition of class II by both 40M and 39E has previously been shown to be dependent on amino acid Arg\textsuperscript{99} in the middle of the groove (42). The region around \(\beta70\) contains a kink in the \(\alpha\) helix that may allow increased structural mobility and has been implicated in conformational shifts in both class I (43, 44) and class II molecules (45). Comparison of class II molecules with CLIP or other peptides in their binding grooves indicates that the largest structural differences attributable to peptide occur in this region around aa 70 in the \(\beta\)-chain (46, 47). Because I-A\textsuperscript{\(\alpha\)}\textsuperscript{d} lacks the potential to form a salt bridge at the carboxyl end of the peptide binding groove, more flexibility may be allowed at this end of the class II structure compared with other allotypic regions of class II. In fact, the recently solved crystal structure of I-A\textsuperscript{\(\alpha\)}\textsuperscript{d} indicates that the peptide binding groove around \(\beta73\) is substantially wider than that in other class II alleles (18). Previous studies have identified Ab epitopes that are influenced by bound peptide or by the absence of peptide, indicating that peptide can impart conformational features on both class I and class II MHC molecules (43–45, 48–50). These findings are consistent with MHC crystallization studies showing that structural features in the class II \(\alpha\) helices are influenced by the sequence of peptides in the class II peptide binding pocket (46, 47). From these studies the concept has emerged that MHC molecules have inherently flexible structures. Either the lack of a stabilizing salt bridge or the presence of other polymorphisms in the NOD class II molecule may result in increased flexibility of the molecule, possibly accentuating the impact of the peptide on the structure of the class II molecule.

Although the current studies demonstrate that the formation of the alternate conformations is dependent on the sequence of the peptide bound in the groove, we do not yet know the motif required to form each conformation. Previous reports have identified sequence motifs for peptides that interact well with I-A\textsuperscript{\(\alpha\)}\textsuperscript{d}. Using a competitive peptide binding assay, one group identified the I-A\textsuperscript{\(\alpha\)}\textsuperscript{d} binding motif to include a large hydrophobic amino acid at P6 and either an aromatic hydrophobic or basic amino acid at P9 (51). Using a similar approach, another group determined that peptides binding to I-A\textsuperscript{\(\alpha\)}\textsuperscript{d} are anchored at positions P4, P6, and P9. Leucine is preferred at P4, although Tyr, Ala, and Thr are accepted. P6 must be occupied by small residues, such as Ala or Thr, while Glu is preferred at P9, with any substitution resulting in decreased peptide binding (20). Similarly, utilization of a peptide elution sequencing strategy coupled with competitive binding assays by another group suggested that a primary feature of I-A\textsuperscript{\(\alpha\)}\textsuperscript{d} binding peptides is an acidic residue at P9 (40). This acidic residue is postulated to neutralize the unpaired basic amino acid at position 76 of the \(\alpha\)-chain. A recent study found that although CLIP does not bind well within the I-A\textsuperscript{\(\alpha\)}\textsuperscript{d} binding pocket, modification of Met\textsuperscript{99} at P9 to an acidic residue greatly increases binding affinity (52). The recently solved crystal structure of I-A\textsuperscript{\(\alpha\)}\textsuperscript{d} indicates that the peptide binding groove has an increased capacity for promiscuous peptide binding. Interestingly, the P9 pocket can accommodate two classes of amino acids in different orientations. Amino acids with small side chains pointing down into the pocket are accepted, but the positively charged environment favors acidic amino acids with their side chains oriented sideways (18). This difference in the orientation of the side chain would affect the interaction of the peptide backbone, which could, in turn, affect the conformation of the \(\alpha\) helices of the class II molecule through the hydrogen bonds and van der Waals interactions between the class II molecule and the peptide. These different I-A\textsuperscript{\(\alpha\)}\textsuperscript{d} binding motifs may, in fact, reflect the different subsets of peptides that lead to the alternate peptide-dependent conformations that I-A\textsuperscript{\(\alpha\)}\textsuperscript{d} can adopt. We are currently employing the invariant chain casserole strategy to evaluate this possibility.

One possible interpretation of the data presented is that rather than discriminating between different conformations of the I-A\textsuperscript{\(\alpha\)}\textsuperscript{d} class II molecule formed due to the peptide in the binding groove, the Abs may, in fact, be partly recognizing specific residues of the peptide itself. However, multiple mAbs directed against both the \(\alpha\)- and \(\beta\)-chains of the class II molecule differentially recognize the different peptide:class II complexes. The likelihood that all Abs tested, against both the \(\alpha\)-chain and the \(\beta\)-chain, interact with specific residues of the peptide in the peptide:class II complexes is very low. Also, arguing against this possibility is that although both 40M and 39E recognize epitopes in the same region of the class II molecule, specifically around amino acid 70 (42), they display different recognition patterns of I-A\textsuperscript{\(\alpha\)}\textsuperscript{d} complexed with different peptides. Specifically, although neither Ab recognizes I-A\textsuperscript{\(\alpha\)}\textsuperscript{d} associated with CLIP, recognition by 40M, but not 39E, is regained when Arg\textsuperscript{99} is mutated to Ala. Positions \(\beta61\) and \(\beta57\) have been modeled to interact with P9 of peptides associated with I-A\textsuperscript{\(\alpha\)}\textsuperscript{d} (20). Arg\textsuperscript{99} would probably be P10 in CLIP (52), suggesting that this amino acid would be far from \(\beta70\) and thus unlikely to be recognized by 40M and 39E. Collectively our data are more consistent with the possibility that mutation of the peptide in the binding groove at P10 results in a conformational change that is passed along the \(\alpha\) helix of the \(\beta\)-chain, which can be distinguished by Abs binding to distal sites in the class II molecule.

The relative levels of each conformation were assessed in the current study. The metabolic labeling experiment followed by sequential immunodepletion indicates that \(\sim 80\%\) of the molecules are reactive with both 10.2-16 and 40M, whereas \(\sim 10\%\) of the molecules react with only 10.2-16 or 40M. However, by biotinylation, the conformation reactive with only 40M represents \(>25\%\) of the total class II molecules. This apparent discrepancy could be due to labeling differences, with the 10.2-16 /40M\textsuperscript{\(\alpha\)}\textsuperscript{d} conformation exposing additional biotinylation sites, while the biotin-available sites are masked on class II molecules reactive only with 10.2-16.
The disparity between biosynthetic labeling and surface biotinylation could also be accounted for by differing half-lives of the class II molecules expressing different conformations. Class II molecules that have unstable interactions with peptide are thought to have more rapid turnover rates within APCs. I-A\textsuperscript{b} has been shown to both bind peptide poorly and to have an atypically short half-life (19, 52, 53). Thus, the minor population of I-A\textsuperscript{b} that interacts only with 40M may persist at the cell surface, possibly due to its more stable interaction with peptide. These molecules would thus accumulate over time. We are currently examining these possibilities.

To be important in diabetogenesis, these alternate conformations must play a role in T cell selection and/or activation. The anti-class II mAb blocking data shown above suggest that the various conformations participate in T cell activation. If we consider what is known about the sites of interaction at the TCR:class II interface, we can speculate at least two events that could be affected by the alternative conformations of I-A\textsuperscript{b}. First, the alternative conformations could interact with the TCR with different affinities. The TCR docks onto the MHC class I:peptide complex in a diagonal fashion, with the complementarity-determining region loops interacting both with the MHC molecule and the peptide (54, 55). Comparison of a solved crystal structure of a class I:peptide complex with that of a class I:peptide:TCR complex indicates that the side chains of the class I molecule shift slightly when the complex engages the TCR (55). Thus, an altered conformation could result in differential engagement of the TCR, potentially affecting both T cell education in the thymus and stimulation in the periphery. The recently published crystal structure of the D10 TCR complexed with I-A\textsuperscript{b} and peptide (56) indicates that TCRs bind to class II molecules in a more orthogonal manner, with multiple TCR-MHC contacts occurring in the region around \(\beta 67-\beta 77\). In fact, \(\beta 70\), which is present in the epitope recognized by both 40M and 39E, forms three hydrogen bonds and eight van der Waals contacts with various residues of the TCR. Thus, an alternative conformation in this area of the class II-peptide complex could reasonably be expected to result in differential interaction with TCRs.

Additionally, different conformations of I-A\textsuperscript{b} could result in differential engagement of the CD4 molecule on the surface of the T cell. Mutagenesis studies suggest that the CD4 molecule interacts with two sites on the class II molecule, one on the \(\alpha 2\) domain (57) and one on the \(\beta 2\) domain (58, 59), each of which binds separate CD4 molecules. A mutation at either site impairs CD4 function in T cell activation, indicating that both sites must bind their receptor for full activation to occur (57). Thus, one of the I-A\textsuperscript{b} conformations may interact with the TCR in an altered manner, such that there is a change in the ability to recruit CD4, thus altering the ability to stimulate the T cell in the thymus or the periphery.

Although the conformations recognized exclusively by a single Ab represent a minor population of the total class II molecules, it is possible that this small population of class II molecules may play a disproportionately large role in T cell selection in the thymus, since, as discussed above, it is not known whether all conformational states of MHC molecules equally recruit TCRs and coreceptors. Although we do not yet know how the various I-A\textsuperscript{b} conformations may alter TCR:Class II interactions, there is evidence that the NOD class II molecule may have a general deficiency in effective interactions with the TCR. Data from Fathman’s group (60, 61) have suggested that the addition of another class II allele to NOD mice results in the majority of the developing T cells being educated on the new allele, a finding that suggests that I-A\textsuperscript{b} is a poor class II molecule for T cell education (60–62). It is possible that the inability to stably bind peptide may be solely responsible for the poor recruitment of the TCR by I-A\textsuperscript{b}.

Our studies raise the alternative possibility that deficiencies in the ability of I-A\textsuperscript{b} to engage TCRs may be due to an unusually high degree of structural flexibility. Future experiments are being designed to evaluate this intriguing possibility.

Acknowledgments

We thank Dr. Jim Miller for his helpful suggestions and reading of the manuscript. We are very grateful to Jun Cao for generating the invariant chain construct used in the experiments described in this manuscript. Flow cytometry was performed in the University of Chicago Cancer Center flow cytometry facility.

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