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Chemistry

The invariant chain forms complexes with class II major histocompatibility complex molecules and antigenic peptides *"in vivo"*

(antigen presentation)

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ABSTRACT **The binding of a chicken ovalbumin peptide (residues 323–339), Ova-(323–339), to I-A^d molecules was investigated *in vitro* and *in vivo*. By using antigenic peptides labeled either with a hapten or with fluorescein, complexes formed *in vitro* between I-A^d and antigenic peptides were detected by Western blot analysis with an antibody recognizing the hapten 7-nitrobenzo-2-oxa-1,3-diazole and by scanning gels for fluorescence emitted by fluoresceinated peptide. Both techniques reveal that Ova-(323–339) binds not only to I-A^d α/β heterodimers and separated α and β chains but also to complexes of higher molecular mass. Additional analysis shows that one of these additional complexes contains I-A^d heterodimers, antigenic peptides, and also invariant chain. To explore the physiological role of these complexes, cells were incubated with haptenated peptide and the I-A^d–peptide complexes formed *in vivo* were purified by affinity chromatography using hapten-specific antibody. The complexes formed migrate with a significantly higher apparent molecular mass than the α/β heterodimers. A band at 180 kDa contained the α/β heterodimer, the antigenic peptide, and the invariant chain. These results show that *in vivo* high molecular mass complexes formed by the I-A^d heterodimer and the invariant chain bind antigenic peptides.**

During the last decade, accumulated data have shown that a key role in immune recognition is played by a trimolecular complex formed by the T-cell receptor, major histocompatibility complex (MHC) molecules, and immunogenic peptides. Whereas B cells recognize antigens in their native form through their specific receptors, immunoglobulins, T cells do not (1–3). T cells recognize immunogenic peptides associated with syngeneic restriction MHC molecules. These peptides arise from the proteolytic degradation of protein antigens inside acidic compartments of antigen-presenting cells (4–7). A molecular model for this recognition has been delineated from experiments using antigenic peptides specifically bound to isolated MHC molecules to induce T-cell responses (8–11). This immune recognition requires the selective binding of peptides to MHC molecules. Differences among the various alleles of class II MHC molecules in their ability to bind antigenic peptides account for the molecular basis of MHC gene control of the immune response (12, 13).

Beside the major role of MHC molecules in recognition of antigenic peptides, several reports suggest the involvement of an additional protein: the invariant (Ii) chain. The highly polymorphic class II MHC molecules consist of two noncovalently linked glycoproteins, the α and β chains of apparent molecular masses 34 and 29 kDa. Inside the cells, α/β heterodimers are found to be associated with a third nonpolymorphic chain with various apparent molecular masses among which the prominent ones are of 31 and 41 kDa (14, 15). The Ii chain is encoded by a single-copy gene unlinked to the MHC (16) but exists in multiple forms resulting from a number of events ranging from alternate initiation of translation to post-translational modifications (17–21). Despite extensive studies, the function of the Ii chain has remained unclear. It has been suggested that Ii chain is involved in the assembly and transport of class II MHC molecules (16, 22, 23) and in the antigen processing and presentation (24, 25). A widely accepted view is that the Ii chain binds to the antigen binding site, protecting it from binding endogenous peptides until encountering antigenic peptides in acidic compartments where Ii is released from class II MHC (26–28). No evidence has been given previously for the formation of a tetrameric complex involving α, β, and Ii chains and antigenic peptide.

In the present study, we investigated the biochemical characteristics of the complexes containing class II MHC molecules that bind antigenic peptides *in vitro* and *in vivo*. Experiments described in this paper demonstrate that the chicken ovalbumin peptide (residues 323–339) [Ova-(323–339)] binds to various molecular complexes, all involving I-A^d molecules but of higher apparent molecular mass than the α/β heterodimers. By using Western blot analysis with monoclonal antibody (mAb) ANO9, that recognizes the hapten 7-nitrobenzo-2-oxa-1,3-diazole (NBD) (29), we were able to detect *in vitro* specific binding of NBD-conjugated Ova-(323–339)-Tyr [NBD-Ova-(323–339)-Tyr] not only to I-A^d heterodimers but also to complexes of higher molecular mass. Fluorescence scanning of proteins separated on SDS/PAGE under nonreducing conditions confirmed these results and revealed binding of fluorescein isothiocyanate-conjugated Ova-(323–339) [F-Ova-(323–339)] to I-A^d conformers with apparent molecular masses of 55 and 64 kDa, as described (30, 31). Two additional molecular species of 105 and 180 kDa appear even more effective in binding the antigenic peptide. Further characterization by Western blot analysis of these molecular complexes indicates that the 180-kDa complex is composed of I-A^d heterodimers, antigenic peptide, and also an Ii chain. Furthermore, when mAb ANO9 was used to purify I-A^d–peptide complexes formed *in vivo*, it was found that the high molecular mass complexes, rather than the α/β heterodimers alone, bind antigenic peptides *in vivo*. Thus these data demonstrate that the Ii chain is directly involved *in vivo* in a multimolecular complex with the class II MHC molecules and antigenic peptides.

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Abbreviations: MHC, major histocompatibility complex; Ii chain, invariant chain; Ova-(323–339), synthetic peptide representing amino acids 323–339 of chicken ovalbumin; F-Ova-(323–339), Ova-(323–339) labeled at its N terminus with fluorescein isothiocyanate; NBD, 7-nitrobenzo-2-oxa-1,3-diazole; NBD-Ova-(323–339)-Tyr, Ova-(323–339) with a tyrosine at position 340 and labeled at its N terminus with NBD; mAb, monoclonal antibody.

MATERIALS AND METHODS

Antibodies. mAbs MKD6 (I-A^d-specific) (32), 14.4.4S (I-E^d-specific) (33), ANO9 (dinitrophenyl-specific) (29) were purified from culture supernatant on a protein A-Sepharose column (Pharmacia). mAb In1 (Ii-specific) (34) was purified on a protein G-Sepharose column. Bound mAb was eluted at acidic pH and coupled to cyanogen bromide-activated Sepharose at a final concentration of 2 mg of mAb per ml of swollen gel.

Peptides. The ovalbumin peptide derivative, Ova-(323–339)-Tyr-340, was synthesized by the solid-phase technique on a Milligen model 9050 peptide synthesizer, using the pentafluorophenyl esters of side-chain- and N^{α}-fluorenylmethoxycarbonyl-protected amino acids. After removal of side-chain protecting groups with trifluoroacetic acid and carbocation scavenger, the crude peptide was isolated, dried, and redissolved in 0.1% trifluoroacetic acid. After purification by reverse-phase high performance liquid chromatography, the purified lyophilized peptide was dissolved in 0.1 M $NaHCO_3$ and labeled on the N-terminal amino group by reaction for 24 hr with an excess of 4-(*N*-hydroxysuccinimidyl)-7-nitrobenzo-2-oxa-1,3-diazole. The later reagent, which was prepared by reaction of NBD chloride with *N*-hydroxysuccinimide, quantitatively reacted with only the amino group of the peptide. The NBD-peptide solution was acidified with 1% trifluoroacetic acid and isolated by reverse-phase high performance liquid chromatography. F-Ova-(323–339) was purchased from Peninsula Laboratories.

Purification of I-A^d and I-E^d. A20.1.11 cells (35) were harvested and washed three times in RPMI medium without fetal calf serum. The cells (5×10^8 cells per ml) were lysed by suspension for 20 min at 4°C in 0.5% Nonidet P-40/10 mM Tris/150 mM NaCl/1 mM phenylmethylsulfonyl fluoride/0.02% merthiolate, pH 8.6. The lysate was then centrifuged at $2000 \times g$ for 20 min. The cellular extract was immediately applied to a lentil lectin-Sepharose column (Pharmacia) equilibrated in lysis buffer. Glycoproteins were eluted in 10% (wt/vol) α-methyl mannoside and directly applied to either MKD6 or 14.4.4S mAb immunoadsorbents. After detergent exchange with 30 mM *n*-octyl glucooside in Dulbecco's modified phosphate-buffered saline (DPBS; pH 7.4), elution of the specifically bound material was done in 50 mM $NaHCO_3$/0.5 M NaCl/1 mM phenylmethylsulfonyl fluoride/0.02% merthiolate/30 mM *n*-octyl glucoside, pH 10.65. Collected fractions were immediately neutralized with 1.5 M Tris (pH 8.8) and dialyzed in 30 mM *n*-octyl glucoside DPBS, pH 7.4.

Purification of I-A^d–NBD-Ova-(323–339)-Tyr Complexes. A20.1.11 cells (5×10^7 cells per ml) were incubated for 2 hr at 37°C in a 5% CO_2/95% air atmosphere in the presence of 2 μM NBD–Ova-(323–339)-Tyr. The incubation was ended by transferring cells at 4°C and washing three times in DPBS (pH 7.4). Cells were then lysed and their proteins were extracted and purified as described above using the ANO9 mAb immunoadsorbent.

Peptide Incubation and Detection. NBD-Ova-(323–339)-Tyr (25 μM) was incubated overnight at 20°C with either I-A^d or I-E^d (2.5 μM) in 30 mM *n*-octyl glucoside/DPBS, pH 7.4. Free peptide was dialyzed on a Centricon-30 (Amicon). NBD-Ova-(323–339)-Tyr peptides bound to class II MHC were resuspended in a final volume of 2 ml and a 10-μl fraction was separated on SDS/PAGE. F-Ova-(323–339) (100 μM) was incubated with I-A^d (1 μM) for 2 hr at 37°C and directly separated on SDS/PAGE. We used 12, 10, and 7% acrylamide gels (36). Samples were diluted in sample buffer without 2-mercaptoethanol and without boiling. F-Ova-(323–339) was detected by scanning gels on a fluorescence microscope as described (30, 31). Proteins were then visualized by silver staining (37). Alternatively, proteins separated on gels were transferred onto nitrocellulose membranes at 25 V overnight in 20 mM Tris/150 mM glycine; pH 8.

Immunolabeling was done in DPBS/1% bovine serum albumin, pH 7.4, in the presence of specific purified mAb at 10 μg/ml. A second antibody, specific for immunoglobulins from rat or mouse and coupled to alkaline phosphatase, was added at 10 μg/ml and its presence was revealed by adding nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

RESULTS

Because the binding of antigenic peptides to MHC molecules modifies the peptide conformation and environment, it is not generally possible to target the molecular complexes of peptide and MHC using antibodies specific for peptides. By using a mAb specific for NBD, an haptenic structure, we were able to detect binding of NBD-Ova-(323–339)-Tyr to the I-A^d molecule. We choose to use a mAb specific for the hapten dinitrophenyl ($K_a = 1.6 \times 10^7\ M^{-1}$) (29) but displaying a strong cross-reactivity with the hapten NBD ($K_a = 1.8 \times 10^6\ M^{-1}$, data not shown). In this experiment we used detergent-solubilized I-A^d or I-E^d (2.5 μM) incubated with NBD-Ova-(323–339)-Tyr (25 μM) overnight at 20°C. After removal of the free peptide by extensive dialysis, we visualized complexes with mAb ANO9 specific for NBD. Results obtained (Fig. 1, lane a) showed that the complexes formed migrate on nonreducing SDS/polyacrylamide gels at ≈67 kDa and >94 kDa. These bands were labeled in parallel with antibodies specific for I-A^d (Fig. 1, lane c) and I-E^d (Fig. 1, lane d), respectively, mAb MKD6 and mAb 14.4.4S. These results confirm that NBD-Ova-(323–339)-Tyr binds preferentially (30, 54) to I-A^d (Fig. 1, lane a) and to a lesser extent to I-E^d (Fig. 1, lane b) heterodimers, reflecting the I-A^d restriction of this antigen. Furthermore, using these nonreducing gels, we confirm the binding of antigenic peptides to the α/β heterodimers but reveal also peptide binding to various complexes of higher apparent molecular mass, involving I-A^d molecules.

To better define these I-A^d–Ova-(323–339) complexes, we used an alternative technique (30, 31). Fig. 2 shows the fluorescence scanning of proteins separated on nonreducing gels. I-A^d (1 μM) was incubated for 2 hr at 37°C with F-Ova-(323–339) (100 μM) and then separated by SDS/PAGE. F-Ova-(323–339) binds to the conformations "compact and floppy," which migrate at 55 and 64 kDa, respectively. Furthermore, we found peptide bound to separate α and β chains (30, 31). We did not detect peptide bound to separate chains with Western blot analysis using mAb ANO9. This is probably due to the lower affinity or faster off-rate of the peptide, which might have dissociated during transfer to nitrocellulose or staining. Beside these previously described

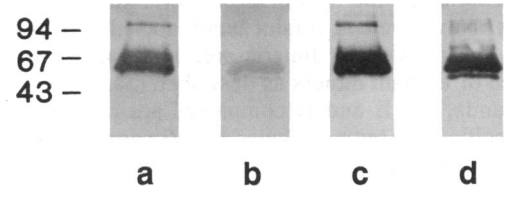

FIG. 1. Western blot analysis of NBD-Ova-(323–339)-Tyr recognition by I-A^d and I-E^d. Class II MHC molecules were purified. Proteins were separated by SDS/PAGE (12% gels) under nonreducing conditions. Lanes: a and c, I-A^d–peptide complexes revealed by mAb ANO9 (recognizing NBD) and by mAb MKD6 (I-A^d specific), respectively; b and d, I-E^d-peptide complexes revealed by mAb ANO9 or mAb 14.4.4S (I-E^d specific), respectively. Apparent molecular mass markers in kDa are indicated by arrows on left side of the figure. We used hen egg ovalbumin (43 kDa), bovine serum albumin (67 kDa), and phosphorylase b (94 kDa) (Pharmacia).

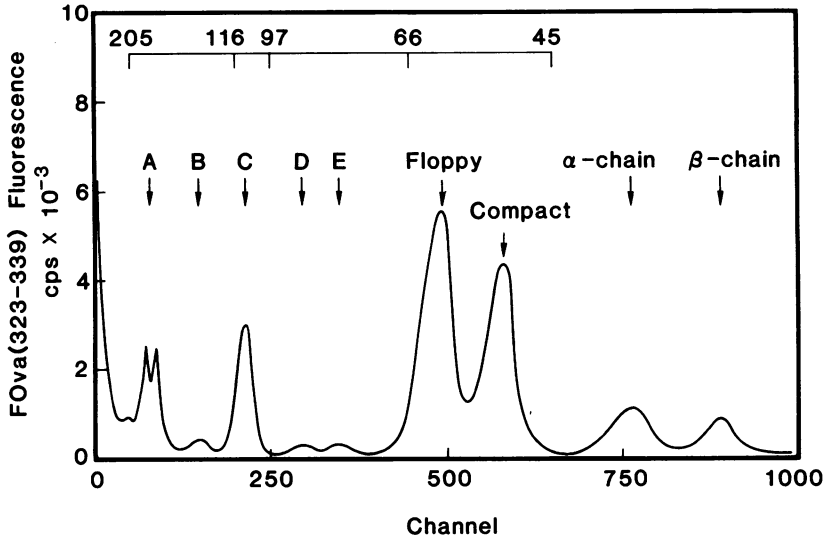

FIG. 2. Scan for fluorescent peptides bound to I-A^d. Purified I-A^d was incubated with F-Ova-(323–339). Proteins were separated by SDS/PAGE on a 7% nonreducing gel. F-Ova-(323–339) bound not only to the α/β heterodimeric conformations floppy and compact and to the separated α and β chains but also to complexes of apparent molecular masses of 180 kDa (referred to as peak A) and 105 kDa (peak C). Positions determined for complexes identified by other techniques (see Fig. 3) are identified as peak B (130 kDa), peak D (87 kDa), and peak E (79 kDa). Peptide binding to these complexes was weak. Positions of the molecular mass standard proteins are indicated. We used myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), and hen egg ovalbumin (45 kDa) (Sigma), which were boiled and reduced.

peptide–I-A^d complexes, we found that antigenic peptides bind to two molecular species, named bands A and C in Fig. 2, with apparent molecular masses of approximately 180 and 105 kDa. These bands are only faintly stained by silver when compared to α/β heterodimers and thus apparently bind peptides even more effectively. Finally, several bands from I-A^d preparations did not bind significant amounts of the peptide, namely, bands B (131 kDa), D (87 kDa), and E (79 kDa). These proteins might be either immature or complexes denatured during purification procedure. The relative amount of complexes migrating at apparent molecular masses greater than the α/β heterodimers varies considerably in different preparations of I-A^d. These complexes were found to disassemble upon storage to yield the "floppy" and "compact" conformations and the α and β chains (M.V. and K.J.M., unpublished results).

Because of the known association of the Ii chain with MHC class II molecules, we analyzed these apparent high molecular mass complexes with a mAb specific for the Ii chain, mAb In1. Results obtained are reported in Fig. 3. Western blot analysis reveals the presence of Ii chain in the high molecular mass complexes. Since floppy and compact conformations (Fig. 3, lane a) were labeled with I-A^d-specific mAb (Fig. 3, lane c) but not by Ii-specific mAb (Fig. 3, lane b), we confirmed the α/β heterodimeric structure of these proteins. Nevertheless, a faint band labeled with Ii-specific mAb is just above this dimeric area and may visualize the presence of Ii chain dimers as described (34, 38, 39). Above these bands, the E and D complexes are not labeled with I-A^d-specific mAb but are labeled with Ii-specific mAb and do not bind significantly peptide *in vitro* (Fig. 2). These populations might be α/Ii, β/Ii, or Ii/Ii dimers, released from I-A^d during purification and handling of these preparations. In the monomer regions, three bands are labeled with Ii-specific mAb and represent released Ii chains with apparent molecular masses 31, 33, and 41 kDa, which must have been originally associated with I-A^d as they were copurified on the MKD6 immunoadsorbent. The last set of molecular complexes (bands A, B, and C) is labeled with I-A^d-specific mAb but bands A and B are also labeled by Ii-specific mAb. Since band A has been shown to bind peptide *in vitro* (Fig. 2), we conclude that in band A the molecular complex responsible for binding peptide consists of α, β, and Ii chains.

To characterize complexes of MHC molecules responsible for binding antigenic peptides under physiological conditions, we incubated MHC-positive A20.1.11 cells with NBD-Ova-(323–339)-Tyr peptides. The complexes formed under such conditions were then isolated by detergent solubilization and purification on an immunoadsorbent constituted with mAb ANO9 that recognizes NBD hapten. The fractions obtained were analyzed under nonreducing conditions by SDS/PAGE. Eluates obtained are shown in Fig. 4. α/β heterodimers were detected only as a minor population. The two major bands obtained are complexes A and C, which are very effective in binding antigenic peptides *in vitro*. Several minor bands corresponding to B, D, and E complexes were also observed in I-A^d preparations. Because B, D, and E complexes contain Ii chain but do not bind peptide *in vitro*, they are probably released subunits, which suggest the copurification of Ii chain with anti-peptide immunoadsorbent. Thus these results confirm that Ii chain is found associated with I-A^d in molecular complexes responsible for binding peptide and indicate a major role *in vivo*.

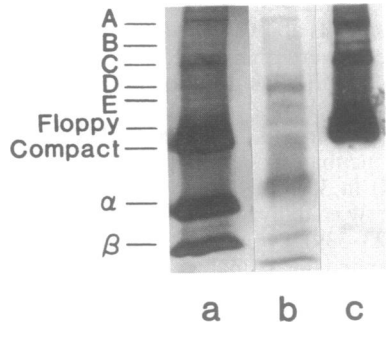

FIG. 3. Invariant chain is associated with I-A^d in the high molecular mass complexes. Lanes: a, silver staining of 7% polyacrylamide gel containing I-A^d purified proteins; b and c, Western blot with Ii-specific In1 mAb and with I-A^d-specific MKD6 mAb, respectively. Bands A–E, floppy and compact conformations, and separate chains described in Fig. 2 are indicated.

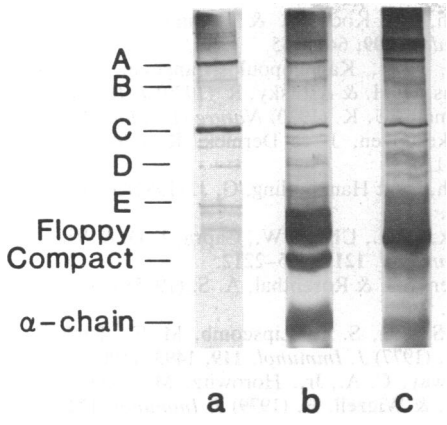

FIG. 4. Purification of high molecular mass complexes binding peptides *in vivo* with the mAb ANO9 recognizing the haptenated peptide. A20.1.11 cells were incubated with NBD-Ova-(323–339)-Tyr and their detergent-solubilized glycoproteins were sequentially purified. SDS/polyacrylamide (7%) gels of material eluted from mAb ANO9 recognizing haptenated peptide (lane a), MKD6 specific for I-A^d (lane b), and 14.4.4S specific for I-E^d (lane c) immunoadsorbents. Bands A–E, floppy and compact conformations and separate chains described in Fig. 2 are indicated.

A second set of experiments with a different invariant chain-specific mAb, P4H5 (55), gave confirming results. The detergent-solubilized fraction was first applied to an immunoadsorbent constituted with mAb P4H5 and the run-through then applied to the immunoadsorbent constituted with mAb ANO9. Gel electrophoresis of this eluate was essentially as in Fig. 4, lane a, except the band A was depleted (data not shown).

DISCUSSION

By using an antibody directed against a haptenated antigenic peptide, we were able to isolate MHC–peptide complexes formed *in vivo*. To purify these populations from the majority of the MHC molecules, we used a mAb specific for the hapten attached to the N terminus of the peptide. [Antibodies against peptides do not usually bind to MHC–peptide complexes (40–42).] The possibility of using haptenated peptides was suggested by earlier work (43–45) that showed an anti-hapten mAb was able to block the specific recognition of hapten by T cells. mAb ANO9 is specific for the hapten dinitrophenyl (29) but cross-reacts with the hapten NBD with a lower affinity. Thus mAb ANO9 can be used for immunochromatography. By coupling NBD to Ova-(323–339)-Tyr, we combine the capacity to use a mAb specific for hapten to target antigens bound to MHC. The recognition of NBD-Ova-(323–339)-Tyr peptide by I-A^d molecules was assayed by Western blot with mAb ANO9. The results show the accessibility of the hapten at the N terminus of the peptide bound to class II MHC molecules. The results confirm the restriction of NBD-Ova-(323–339)-Tyr to I-A^d when compared to I-E^d, as reported (46). Previous experiments performed *in vitro* showed that detergent-solubilized class II MHC molecules bind peptides with a specific pattern that overlap the known allelic restriction (8–10). In those studies the specificity of the recognition of peptides by MHC was visualized by SDS/PAGE under reducing conditions and α and β chains were chemically cross-linked with peptides.

In contrast, nonreducing SDS/PAGE, Western blot, and gel scanning experiments show the binding of antigenic peptide not only to the expected α/β heterodimers but also to higher apparent molecular mass complexes. Besides the major area of α/β heterodimers where we detected the floppy and compact conformers of I-A^d (30, 31), we detected two bands of 105 and 180 kDa. These complexes were shown to contain I-A^d heterodimers but the 180-kDa complex also contained the Ii chain. Thus, our experiments *in vitro* demonstrate that a 180-kDa complex does exist, comprising I-A^d, peptide, and the Ii chain. Various hypotheses have been proposed for the function of invariant chain. A classical view is that the Ii chain may be involved in regulation and transport of MHC class II molecules (47–49). A current hypothesis is that Ii chain binds to the desetope of MHC class II molecules, Ii chains, and peptides thus competing for the binding site (26–28). More recently it has been shown that the Ii chain is necessary for presentation of native antigens but not for antigenic peptides (25). It was proposed that the Ii chain participates in the transport of internalized antigens to digestive compartments in which antigenic peptides are produced. In all these studies no evidence was reported for direct binding of the Ii chain to the well-characterized complex of class II MHC and peptide. Our results are not contradictory with the hypothesis that the Ii chain participates in transport of MHC but conflict with the hypothesis that peptide binding and Ii chain binding to class II MHC are mutually exclusive.

The physiological relevance of these multimolecular complexes was assessed by purifying the NBD-ovalbumin peptide-binding proteins with mAb ANO9 that recognizes NBD. This experiment showed that the two high molecular mass complexes of 180 and 105 kDa (Fig. 2, bands A and C), which have a high peptide binding capacity *in vitro*, are also those that bind the haptenated peptides *in vivo* predominantly. These results indicate their prominent role *in vivo* when compared to α/β heterodimers. Thus these high molecular mass complexes are not artifacts from the purification procedure, as has been suggested (7). The 180-kDa complex certainly consists of more than one α/β dimer and one Ii chain. The high molecular mass of complexes observed by nonreducing SDS/PAGE does not reveal their real molecular mass and composition, as it depends upon the shape of the micelle and the number of SDS molecules in such micelles. However, this observation raises the question whether this multimolecular structure might be composed only of dimers of Ii–α–β complexes or whether additional molecules are involved. It has been shown that the Ii chain, associated with class II MHC molecules, is the core protein of a proteoglycan with an apparent molecular mass of 40–70 kDa (50).

Studies on association kinetics indicate significant differences between *in vitro* and *in vivo* association of MHC and peptide (8, 10, 51). Our results raise the possibility that the Ii chain binding to class II MHC molecules modifies the affinity or reaction kinetics involving antigenic peptides (52, 53). Observations reported herein definitely argue that the Ii chain is physiologically important.

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