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# One of Two Unstructured Domains of Ii Becomes Ordered in Complexes with MHC Class II Molecules

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## Summary

**We studied the role of the invariant chain (Ii) protein's structure in its ability to form complexes with major histocompatibility complex class II molecules. Multidimensional nuclear magnetic resonance experiments demonstrated that Ii contains two unstructured, flexible domains: a 39 residue sequence that contains a region (CLIP) critical for Ii/class II complex formation and becomes rapidly ordered when Ii/class II complexes are assembled, and a 30 residue sequence that contains the insertion point for a protease inhibitor domain included in an alternative splice form of Ii. Mobility of these domains guarantees accessibility to CLIP and the inhibitor insert, and ordering of the CLIP-containing domain may provide protection against proteolysis and contribute, along with Ii's compact 118–192 domain, to allotype-independent class II binding.**

## Introduction

Antigen presentation by specialized cells in the immune system depends on the interplay of major histocompatibility complex (MHC) class II molecules, which bind and present antigenic peptides, the two chaperone molecules invariant chain and HLA-DM, which regulate transport and peptide-loading of class II, and proteases, which degrade endocytosed proteins. Membrane-anchored Ii trimers form nonameric complexes with newly synthesized MHC $\alpha\beta$ heterodimers in the endoplasmic reticulum, mediated primarily by interactions between luminal portions of the molecules (Roche et al., 1991; Romagnoli and Germain, 1994; Newcomb et al., 1996). Directed by a targeting sequence at the cytoplasmic N terminus of Ii, $(\alpha\beta\text{Ii})_3$ assemblies then migrate to *trans*-Golgi compartments. There Ii is proteolytically degraded, generating complexes of class II $\alpha\beta$ with CLIP, a peptide drawn from residues in Ii (81–104) which drastically reduce binding to class II when deleted (Bakke and Dobberstein, 1990; Lotteau et al., 1990; Avva and Cresswell, 1994; Bijlmakers et al., 1994; Freisewinkel et al., 1993).

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CLIP is subsequently replaced by antigenic peptides in an exchange catalyzed by the class II-like molecule HLA-DM (Denzin and Cresswell, 1995; Sherman et al., 1995; Sloan et al., 1995 ).

MHC class II molecules require up to several days to form stable complexes with peptides in vitro (Sadegh-Nasseri et al., 1994). By contrast, the process of peptide loading onto class II molecules in professional antigen-presenting cells takes only about 1–3 hr because of the participation of Ii and HLA-DM (Cresswell, 1985; Neefjes et al., 1990). Although the molecular mechanisms by which this rate enhancement is achieved are poorly understood, a necessary step is clearly the fast formation of Ii/class II complexes observed in the endoplasmic reticulum (Teyton et al., 1990). Recent evidence that tissue-specific and developmentally timed modulation of protease activity can affect positive selection of $CD4^+$ T cells specifically by altering the pattern of Ii degradation (Nakagawa et al., 1998; Pierre and Mellman, 1998) suggests that the kinetics of Ii cleavage, as well as the kinetics of class II binding, is critical to Ii's chaperonin functions. Structural features of Ii, and in particular large segments of the protein that are apparently unstructured in the intact molecule (Jasanoff et al., 1995; Park et al., 1995), may account for the schedule of Ii proteolysis and for its rapid and promiscuous binding to MHC class II variants in vivo.

## Results and Discussion

### Ii Has Two Mobile Domains

We used heteronuclear nuclear magnetic resonance (NMR) methods to delimit Ii's mobile segments. In spectra of a 54 kDa histidine-tagged luminal domain of Ii (Ii h72–216 [Figure 1A]), only rapidly reorienting residues give rise to sharp NMR signals. Data from double- and triple-resonance experiments allowed us to assign the sharp resonances (Figure 1B) and establish that they correspond to two continuous mobile segments of Ii h72–216: the 39 N-terminal residues 72–110, and the 30 C-terminal residues 187–216. In these two segments all residues were uniquely assigned, with the following exceptions: prolines (which did not contribute to the $^{15}N$-separated spectra we used), K90, M91, R92, M104 [due to peak overlaps in HNCA and HN(CO)CA spectra], K81 and K83 (which are both flanked by prolines and can be assigned but not distinguished from each other), and M216, the C-terminal residue (tentatively assigned only because of the apparent absence of a succeeding residue). The longest unassigned segment is three residues (90–92) and it is highly unlikely for physical reasons that any of the unassigned residues could constitute rigid interruptions to the otherwise continuous mobile regions.

In principle, it was possible that parts of the 39 residue N-terminal segment or the 30 residue C-terminal segment could form small compact domains connected to the rest of the protein by flexible links; such minidomains would still be rapidly reorienting compared with the

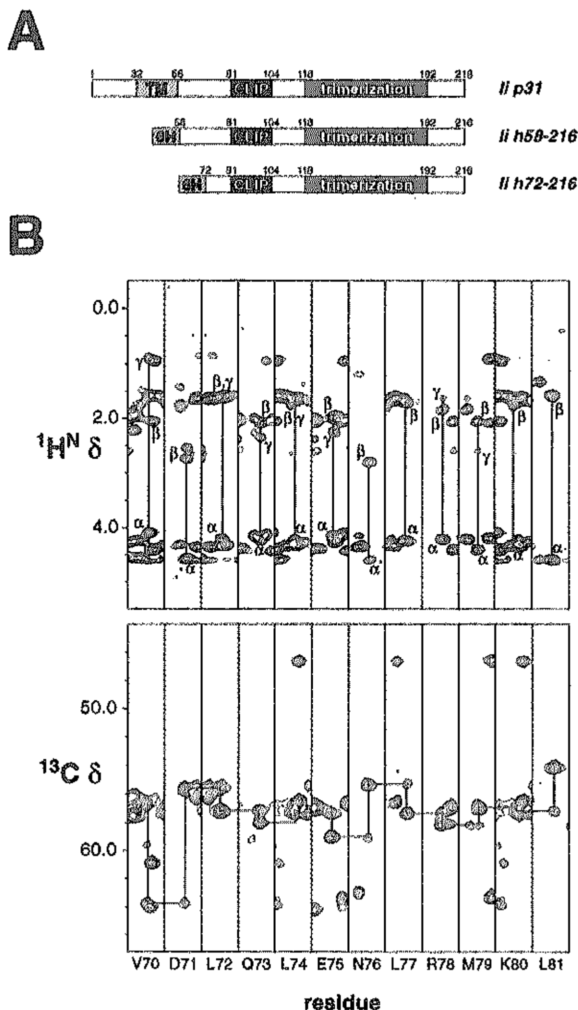


Figure 1. Primary Structure of Ii Molecules and NMR Sequential Assignments

(A) Primary structure of Ii molecules. Ii p31 is the major form of Ii in vivo. The Ii cytoplasmic domain (1–32) includes a cellular localization signal, and the luminal domain (56–216) contains the CLIP segment (81–104) and a trypsin-resistant trimerization domain (118–192). Ii h58-218 includes an N-terminal hexahistidine tag sequence (MRGSHHHHHHGSVD) followed by residues 58–216 of p31 Ii. Ii h72–216 includes the same histidine tag sequence followed by residues 72–216 of p31 Ii.

(B) Strips showing the NMR resonances of 12 sequential residues in $^{15}$N-TOCSY-HSQC and HNCA spectra. Specific identification of disordered residues in Ii h72–216 was accomplished using $^{15}$N-TOCSY-HSQC, $^{15}$N-NOESY-HSQC, HNCA, and HN(CO)CA experiments on uniformly $^{15}$N-labeled or $^{15}$N- and $^{13}$C-labeled protein. Sequential residues could be correlated through $H^N(i)$-N(i)-$C^\alpha(i-1)$ peaks in the HNCA and HN(CO)CA, and in some cases through weak (i, i+1) NOEs as well. All spin systems in the TOCSY spectra matched patterns of resonances found in randomly coiled peptides (Wüthrich, 1986), confirming that Ii residues contributing to the NMR spectra are disordered and simplifying assignment of each observed spin system or set of resonances to a specific residue type.

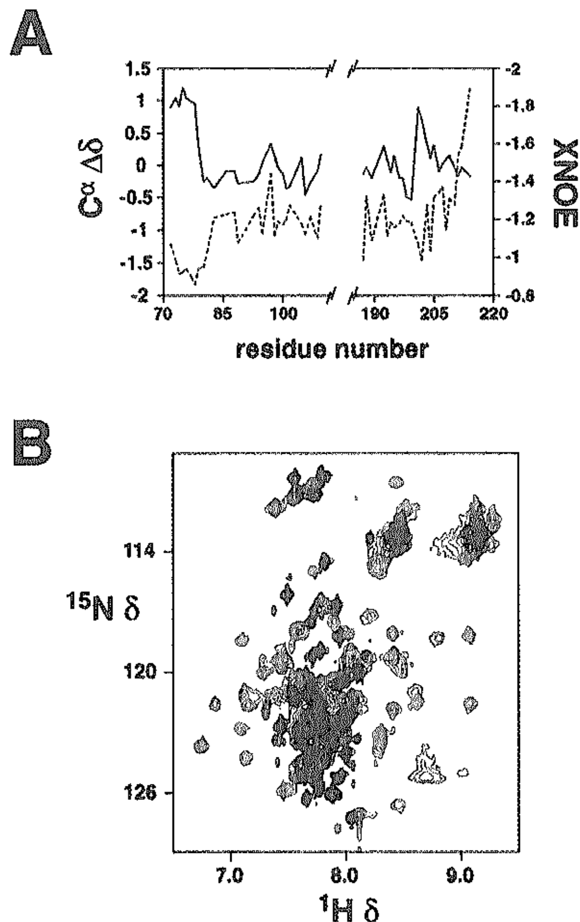


Figure 2. Chemical Shift Data and $^{15}$N-HSQC Spectra

(A) $C^\alpha$ secondary chemical shifts (solid line, left vertical axis) and $^1$H-$^{15}$N heteronuclear NOE enhancements [XNOE = $(I_{sat} - I_0) / I_0$] (dashed line, right vertical axis) for assigned mobile residues (horizontal axis) in Ii h72–216. Secondary chemical shifts are small throughout the 72–110 and 187–216 ranges. Δδ of roughly +1 ppm for residues 72–78 may indicate a slight helical propensity in this region but may be an artifact of their proximity to the hexahistidine tag in Ii h72–216. These are also the residues with the smallest (least negative) XNOE enhancements. Calculation of $(I_{sat} - I_0)/I_0$ is complicated by overlap of peaks in $^1$H-$^{15}$N two-dimensional NMR spectra. The C-terminal residue M216 has the largest enhancement of −2.7 (not shown).

(B) $^{15}$N-HSQC spectra of nondeuterated Ii h72–216 (black) and Ii 118–192 (red) at 500 MHz superimposed on the spectrum of mostly deuterated Ii h72–216 taken on a 750 MHz Varian Unity INOVA spectrometer (blue). Almost all well-resolved peaks in the spectrum of Ii 118–192 correspond to small broad peaks that appear in the Ii h72–216 spectrum at high field, strongly suggesting that the compact trimeric 120–180 domain retains its structure in the context of the longer Ii luminal domain molecules. $^{15}$N-HSQC spectra were also obtained for deuterated and undeuterated Ii h72–216 at 750 MHz in the absence of $t_1$ and $t_2$ decoupling (data not shown); the distribution of line widths among each group of four crosspeaks per residue in these spectra was not as would be expected if dipole-dipole coupling and chemical shift anisotropy provide the only significant relaxation mechanisms, and TROSY-type experiments (Pervushin et al., 1997) are therefore unlikely to provide further information about the structured domain of Ii h72–216.

compact trimerization domain and could contribute sharp lines to spectra of Ii h72–216. Two lines of evidence argue against this possibility (Figure 2A): (1) the $C^\alpha$ atoms of residues in the putative flexible segments show no pronounced secondary chemical shifts, as would amino acid sequences with strong tendency to form secondary structures, and (2) the $^{15}$N-$^1$H heteronuclear NOE enhancements are severe—more negative

than −1.0 for over 90% of those measured—for residues in the mobile segments, and thus show no evidence of motionally restricted subdomains. The C terminus of Ii h72–216 is identical to the C terminus of Ii in vivo, but flexibility of the N-terminal region of the protein could be an artifact of introducing a terminus at residue 72. To address this possibility, we looked at spectra of a longer construct, Ii h58–216, which includes all residues N-terminal to the Ii transmembrane region, together with an N-terminal histidine tag (Figure 1A). Although we did not perform an independent assignment of peaks in the Ii h58–216 spectra, we found that $^{15}$N-HSQC spectra of Ii h72–216 and Ii h58–216 could be superimposed, with the only major differences being apparent perturbations to the chemical shifts of residues 72–74 adjacent to the added residues 58–71 and the presence of extra peaks (at least 10), presumably due to further mobile residues in the 58–71 segment.

### Structure of Ii

Assignment of residues 72–110 and 187–216 to mobile segments of Ii h72–216 complements our recently presented structure of the trimeric Ii 118–192 domain (Jasanoff et al., 1998) to produce a virtually complete picture of the conformation of Ii. Residues 181–192 at the C terminus of Ii 118–192 are disordered in the isolated domain and overlap the C-terminal 187–216 disordered segment we identified in Ii h72–216. Amino acids between P111 and K117 are the only residues in the Ii luminal domain not accounted for either in assignment of the flexible segments in Ii h72–216 or in the structure of Ii 118–192. As a qualitative assurance that the structure of the 118–192 domain is the same in the longer protein as it is in its isolated form, we looked at the $^{15}$N-HSQC spectrum of ~70% deuterated Ii h72–216 at 750 MHz (Figure 2B). The sharp peaks of the flexible residues were visible as well as broad, faint peaks due to the compact domain. Most peaks in the Ii 118–192 spectrum corresponded closely with the broad peaks in Ii h72–216 HSQC, strongly suggesting that the 118–192 trimerization domain structure is preserved in the larger protein.

How does the structure of Ii and in particular its mobile domains facilitate interactions with MHC class II molecules and Ii function in antigen processing? The significance of the 30 residue disordered C-terminal segment may lie in its ability to incorporate a 64 residue domain that is inserted by differential splicing in the minor p41 form of Ii but not in the major p31 form (from which the Ii h72–216 sequence is drawn) (Koch et al., 1987). Ii p41 accounts for widely varying fractions of total Ii in vivo, depending on tissue type (Kampgen et al., 1991), and its extra domain, a potent cathepsin L inhibitor (Bevec et al., 1996), is the most highly conserved sequence in Ii. Incorporation of the p41 inhibitory domain into the middle of the C-terminal loop (after residue 192) presumably disrupts only minimally the rest of the Ii protein. A clear consequence of the presence of the N-terminal disordered segment spanning Ii residues 72–110 and containing the CLIP region is that in the context of this segment, Ii could potentially offer the CLIP residues P87-A101 as an antigenic peptide surrogate for binding in the MHC class II peptide-binding groove. For many

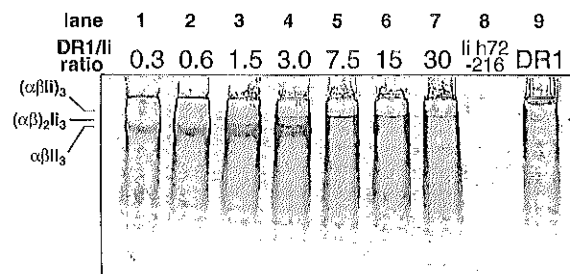


Figure 3. Western Blot of a Nondenaturing Polyacrylamide Gel Showing Complex Formation between Ii h72–216 and HLA-DR1

The anti-DR1 polyclonal antibody CHAMP was used to blot proteins transferred to polyvinylidene chloride. Numbers above lanes 1–7 denote the ratios (wt/wt) of DR1 to Ii h72–216. The DR1 concentration was kept fixed at 270 μg/mL and the Ii concentrations were 920, 460, 180, 92, 37, 28, and 9.3 μg/mL in lanes 1–7, respectively. Lane 8 contains 920 μg/mL Ii h72–216 only and lane 9 contains 270 μg/mL DR1 only. At low ratios of DR1 αβ to Ii, only one DR1-containing band is visible. At intermediate concentrations two new bands appear and the lowest-mobility band predominates at high DR1/Ii ratios. The highest- to lowest-mobility bands therefore correspond respectively to $\alpha\beta Ii_3$, $(\alpha\beta)_2Ii_3$, and $(\alpha\beta Ii)_3$. The molecular weights of Ii h72–216 and DR1 αβ are 18 and 54 kDa, respectively. The high ratios of DR1 to Ii h72–216 required to form complexes indicate that over 50% of the DR1 did not participate in Ii binding. Size-exclusion chromatography indicates that that fraction is aggregated in preparations of peptide-free DR1.

allotypes of class II, the CLIP sequence is required for tight Ii/class II complex formation, and recent experiments in which the CLIP region was modified or replaced suggest that it does in fact bind to the class II peptide-binding groove in $(\alpha\beta Ii)_3$ nonamers (Stumptner and Benaroch, 1997).

### The N-Terminal Ii Loop Becomes Ordered in $(\alpha\beta Ii)_3$ Complexes

We formed complexes between recombinant Ii and peptide-free (empty) HLA-DR1 class II molecules in vitro by mixing the two proteins and incubating them at room temperature for 4 hr. The efficiency of complex formation could be increased roughly 5-fold by the addition of 5% isopropanol to mixtures of Ii and HLA-DR1. We confirmed by NMR that isopropanol had little effect on the structure of Ii h72–216, so the effect of the alcohol is probably either to solubilize a greater fraction of empty DR1 molecules, which tend strongly to aggregate, or to influence a conformational equilibrium of empty DR1 to favor Ii binding; alcohols and medium-length fatty acids have been observed previously to stimulate peptide binding to MHC (Avva and Cresswell, 1994). Figure 3 shows the Western blot of a nondenaturing gel of a titration of empty class II with Ii h72–216, using a polyclonal antibody against HLA-DR1. Empty DR1 molecules run as a diffuse smudge on the gel (lane 9) and lane 8, containing Ii h72–216 alone, does not blot. Three DR1-containing bands are observed to varying extents as the Ii h72–216 concentration is decreased from lanes 1 to 7. The uppermost band must be the nonameric complex and the lower two bands contain one and two DR1 αβ molecules per Ii trimer. The fact that $\alpha\beta Ii_3$, $(\alpha\beta)_2Ii_3$, and $(\alpha\beta Ii)_3$ form progressively as the DR1 to Ii ratio increases indicates that binding of each class II molecule

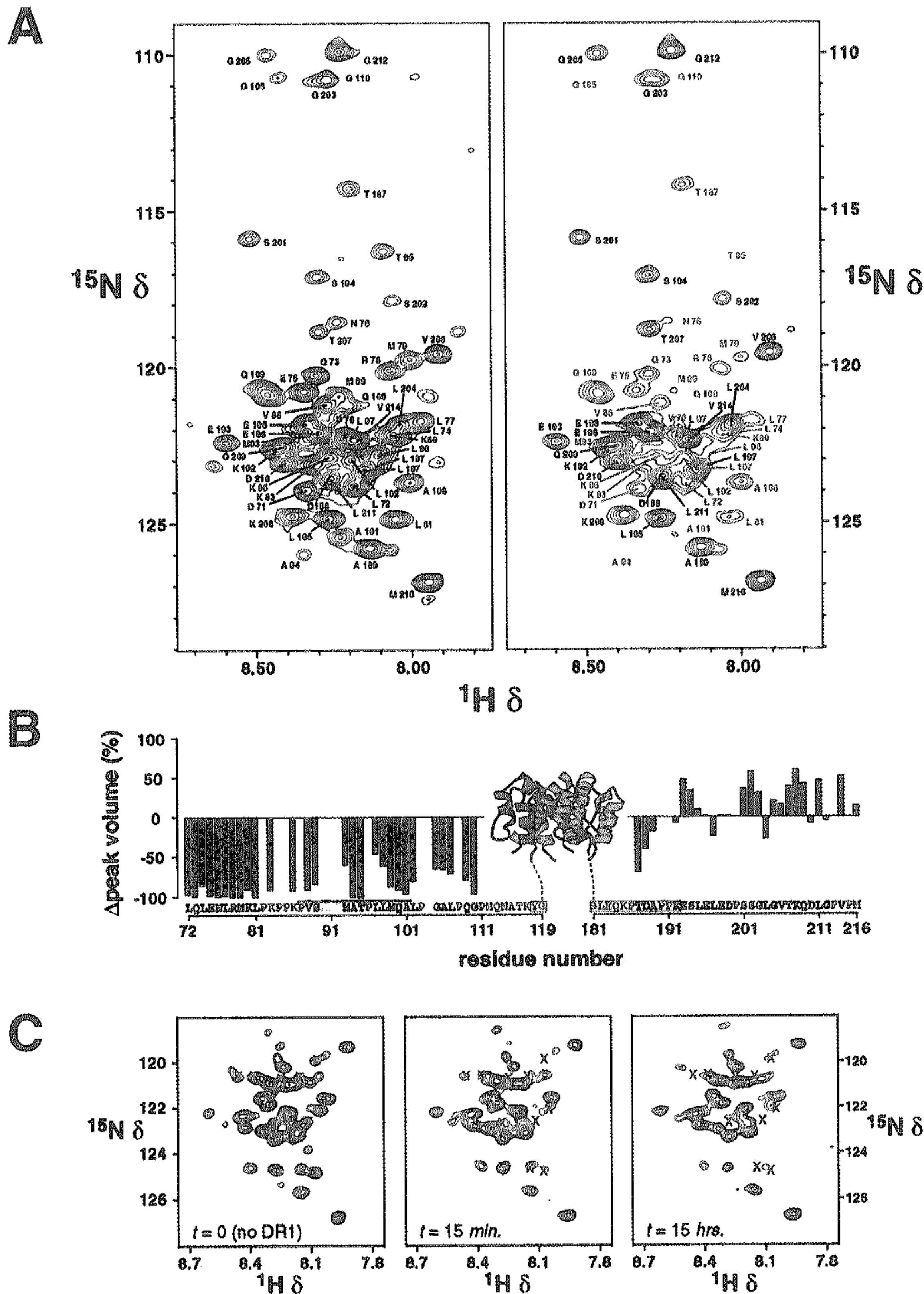


Figure 4. NMR of HLA-DR Binding to Ii

(A) HSQC spectra of 0.2 mM $^{15}$N-labeled Ii h72–216 in the absence (left) and presence (right) of 10:1 (wt/wt) excess unlabeled HLA-DR1. Residues whose $^{15}$N-TOCSY-HSQC signals diminish significantly on addition of DR1 are labeled in red in the spectrum of the complex. Spectra were recorded on a Bruker DMX-500 spectrometer under the same conditions as Figure 1B. A small amount of uncomplexed Ii (less than

to Ii is roughly independent and that nonameric $(\alpha\beta Ii)_3$ complex formation is not strongly cooperative in vitro.

The unfolded structure of Ii h72–216 changes dramatically upon complex formation with HLA-DR1. Figure 4A shows $^{15}$N-HSQC spectra of Ii h72–216 before (left panel) and after (right panel) addition of excess DR1. Each $H^N$-N peak (one per residue) is labeled with the residue number determined by the sequential assignment procedure. Many peaks are significantly diminished in the spectrum of the complex (red labels in Figure 4A), indicating that their corresponding backbone amide groups have become wholly or partially ordered in binding to DR1. Peak volumes in the $^{15}$N-TOCSY-HSQC spectrum were integrated above a threshold level and compared, and percent peak volume changes for each assigned residue are shown in Figure 4B. It is clear from the chart that amide peaks from the N-terminal mobile domain are all substantially diminished. By contrast, amide peaks in the C-terminal disordered region are only slightly reduced and, in some cases, enhanced. This most likely indicates that the entire N-terminal disordered region acquires significant structure upon binding of Ii to DR1. The fact that residues 187–216 do not also become ordered indicates that ordering of the Ii N terminus is not a nonspecific effect due to viscosity or binding of the structured trimerization domain to class II. To determine that the ordering is not an artifact of the Ii h72–216 recombinant molecule, we also studied DR1 complex formation with the longer Ii h58–216 (Figure 4C). A similar large-scale change in peak volumes was observed, consistent with results from Ii h72–216.

The flexible domain that becomes ordered, Ii residues 72–110, includes the CLIP residues (87–101) found in the class II peptide-binding site of DR3/CLIP complexes. The domain also includes flanking residues found in LIP and SLIP, 21 kDa and 12 kDa early intermediates in Ii degradation that retain the membrane anchor and whose accumulation in complex with MHC class II molecules can be induced by treatment of antigen-presenting cells with leupeptin (Blum and Cresswell, 1988; Demotz et al., 1994; Ghosh et al., 1995). The ordering of these residues in Ii/class II complexes observed here suggests a reason for their relative stability against degradation, despite the fact that they are disordered in uncomplexed Ii. A sequential Ii proteolysis pathway in which membrane-anchored Ii fragments containing CLIP are the last to be degraded may be important to ensure Ii cytoplasmic domain–directed retention of class II molecules in endosomes until DM-catalyzed antigenic peptide loading has taken place. Ordering of the 72–110 domain may also be important in the kinetics of Ii/class II complex formation. We studied the kinetics of the disorder-to-order transition in Ii h72–216 and Ii h58–216 and found that with protein concentrations of 0.2 mM in both cases, mobile segments became ordered within the 5 min "dead time" of the experiment (shown for Ii h58–216 in Figure 4C). This is comparable to the rate of an initial peptide interaction with empty class II molecules but significantly faster than either their (probably first-order) acquisition of SDS-resistance in vitro or their formation of long-lived MHC/peptide complexes (Witt and McConnell, 1991; Sadegh-Nasseri et al., 1994). In binding to class II, Ii may have a kinetic advantage over peptides due to potentially cooperative binding of the compact trimerization domain, the CLIP region, and perhaps other elements of the 72–110 segment.

### Structure of $(\alpha\beta Ii)_3$

What is the structure of the Ii/MHC class II complex? Ordering of Ii residues 72–110 on $(\alpha\beta Ii)_3$ complex formation indicates that these residues either contact class II molecules directly or adopt an ordered conformation induced by Ii's binding to DR1. Residues 87–101 in Ii's CLIP region most probably bind in the class II peptide-binding groove as in CLIP/class II complexes. But in principle this places only mild conformational restraints on the geometry of the Ii/class II complex as a whole

15%) was detected by gel electrophoresis after the NMR experiment, despite the fact that the ratio of DR1 to Ii h72–216 was significantly higher than that required for full complex formation according to Figure 2. This indicates that a lower fraction of empty DR1 was available for binding Ii at the high concentration required for the NMR experiment (roughly 1 mM) than at the concentration used for the gel in Figure 2 (roughly 5 μM). Vestigial peaks at the chemical shift values of Ii h72–216 resonances, which significantly diminish on Ii/DR1 complex formation, are most likely due to the small amount of uncomplexed Ii.

(B) Percent change in the total volume of $^{15}$N-TOCSY-HSQC peaks for each disordered residue in Ii h72–216 on complex formation with HLA-DR1. Because visible peak intensity arises only from mobility of residues in Ii h72–216, decreases in the volume of the TOCSY peaks indicate partial ordering of the corresponding residues. NMR samples were the same as in (A). TOCSY-HSQC spectra were acquired, processed, and integrated identically for Ii h72–216 alone and Ii/DR1 complexes. Residues are color-coded as follows: black, assigned residues in double- and triple-resonance spectra of Ii h72–216; green, prolines (not observable in the $^{15}$N-separated NMR spectra we used); blue, ambiguously assigned residues K83, K86, and M216; yellow, unassigned residues in the flexible domains 72–110 and 187–216; and red, unassigned residues in (boxed) or flanking the Ii 118–192 trimerization domain (also red in Figure 1A). A horizontal bar marks Ii residues 87–101 observed in the crystal structure of HLA-DR3 complexed with CLIP peptide. Residues 120–180 form a well-ordered trimeric structure (ribbon diagram), which we recently determined from NMR studies of Ii 118–192. Residues 118–119 and 181–184 are disordered in this molecule but show some evidence of partial structure and could not be assigned in spectra of Ii h72–216. Residues 185–192 are completely disordered in Ii 118–192 and overlap the C-terminal 187–216 domain of Ii h72–216, which we show here to be disordered. Residues 114 and 120 are glycosylation sites, and residue 110 is a proteinase K cleavage site in αβIi complexes (Machamer and Cresswell, 1982; Newcomb et al., 1996), suggesting that the 111–120 region that is unassigned in Ii h72–216 is exposed both in free Ii and in αβIi complexes. Peak volumes and percent changes reported here may be affected by overlap of resonances in the TOCSY spectra. Increases in the peak volumes for some residues in the 187–216 sequence probably reflect uncertainty in the measurement as opposed to an "increase in mobility." Resonances corresponding to T187 in the $^{15}$N-TOCSY-HSQC spectrum decrease more significantly on Ii/DR1 complex formation than its resonance in the HSQC spectra of (A); its proximity to the ordered trimerization domain may be significant, particularly if (as is likely) the trimerization domain interacts directly with class II heterodimers.

(C) Portions of the $^{15}$N-HSQC spectra of $^{15}$N-labeled Ii h58–216 initiated before addition of 15:1 (wt/wt) excess unlabeled HLA-DR1 (left panel) 15 min after mixing (middle panel) and 15 hr after mixing (right panel). Peaks that diminish significantly after addition of DR1 are noted by red crosses. There are no notable differences between Ii h58–216 HSQC spectra (20 min acquisition time) initiated 15 min and 15 hr after mixing.

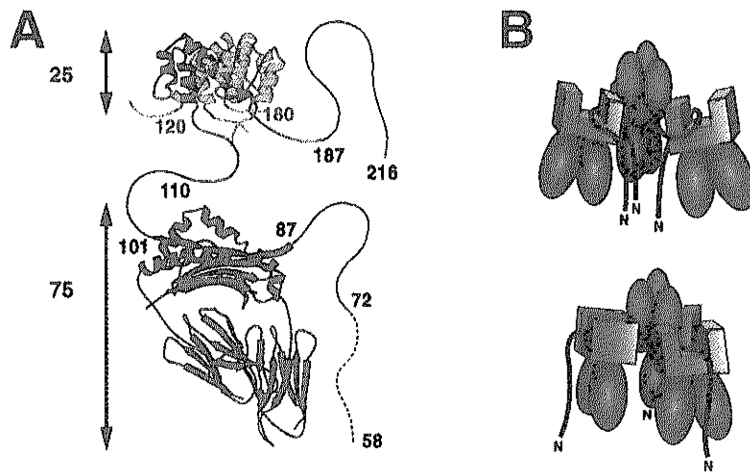


Figure 5. Geometry of Complex Formation between Ii and MHC Class II Molecules

(A) Ribbon diagram of an Ii homotrimer with the N- and C-terminal mobile domains of the red protomer rendered as extended polypeptide chains and drawn roughly to scale (3.5 Å per extended residue). The N-terminal mobile domain contains residues 72–110, of which residues 87–101 (gray ribbon) in the CLIP region probably bind in the peptide-binding groove of an MHC class II molecule (green ribbon structure) in $(\alpha\beta Ii)_3$ complexes. Residues 58–71 (dashed line) give rise to peaks in the HSQC spectra of the long Ii construct Ii h58–216 and are probably also flexible. Residues 111–119 and 181–186 (red line segments) do not give rise to assigned resonances in spectra of Ii h72–216 but do not form compact structure (residues 120–180, red ribbon) in the Ii trimerization domain. C-terminal residues 187–216 are assigned and mobile in Ii h72–216. The N terminus of Ii and C termini of class II $\alpha\beta$ are membrane proximal (bottom of figure). The diagram indicates that the mobile portions of Ii are long with respect to the heights of Ii (25 Å) and MHC class II molecules (75 Å) and that many Ii/class II interaction geometries are consistent with simultaneous interaction of MHC class II with Ii's CLIP region and compact domain.

(B) Schematic depiction of hypothetical nonameric $(\alpha\beta Ii)_3$ structures in which the N-terminal ordering domains (black strands) of Ii (compact domain in red) approach each other as they enter the plasma membrane (upper panel) or diverge and enclose three (green) class II molecules (lower panel). A complex with the geometry of the upper panel would be easier to assemble. The Ii N-terminal domains might interact directly or indirectly with the class II molecules in either complex geometry (no specific model shown). C-terminal flexible domains have been omitted for simplicity.

because of the length of the Ii N-terminal unfolded segment (Figure 5A). Assuming an extended chain length of about 3.5 Å per residue (Creighton, 1993), the 39 residue 72–110 segment could be over 130 Å long. Residues 72–86 can stretch 50 Å, and with the likely additional flexibility of residues 58–71, there can be over 100 Å of unfolded polypeptide between Ii's transmembrane region and the class II groove binding residues 87–101. These length considerations admit the possibility that in the $(\alpha\beta Ii)_3$ nonamer an Ii trimer "encloses" three class II molecules by passing its N-terminal segments over them and forming a ring of its three transmembrane regions around the class II transmembrane regions (Figure 5B, bottom). We consider it more likely that three class II molecules in $(\alpha\beta Ii)_3$ enclose the three Ii N-terminal segments (Figure 5B top); such a complex could be more easily assembled and is more easily reconciled with evidence that the Ii transmembrane trimerizes. Finally, it should be noted that because of the unfolded segment C-terminal to CLIP, occupancy of the class II binding site by the Ii 87–101 sequence is consistent with a wide variety of interaction geometries between class II and the compact Ii C-terminal trimerization domain, which is also known to form nonameric complexes with class II molecules (Newcomb et al., 1996).

Observation of a disorder-to-order transition of the entire 72–110 segment in Ii h72–216/DR1 complex formation provides the first direct structural evidence for involvement of non-groove binding CLIP residues (87–101) in MHC class II complex formation with Ii. Prior indications that Ii residues in 72–110 may interact with class II have included findings that (1) deletion of residues 81–87 abrogates class II binding activity of an Ii already missing CLIP groove-binding residues (Siebenkotten et al., 1998), (2) deletion of residues 103–117 abrogates class II binding of Ii 103–216 (C-terminal to CLIP) (Thayer et al., 1999), (3) a peptide corresponding to Ii residues 71–88 binds HLA-DR2 with micromolar affinity noncompetitively at the same time as groove-binding peptides (Vogt et al., 1995), and (4) a peptide containing Ii residues 81–89 promotes CLIP dissociation from DR3 complexes in *trans* (Kropshofer et al., 1995). In contrast to these earlier data, observation of the 72–110 disorder-to-order transition in the context of class II/Ii h72–216 complexes shows that all of the Ii residues thought to interact with class II can participate in interactions simultaneously in a virtually intact Ii molecule. The ability of Ii to present an extended and initially flexible interaction surface, as well as a high-affinity class II groove-binding sequence, could allow the protein to chelate a wide variety of class II molecules with high affinity and enhance Ii's ability to function as an allotype-independent class II chaperone.

## Experimental Procedures

### Protein Purification and Ii/HLA-DR1 Complex Formation

The soluble Ii luminal domains, Ii h58–216 and Ii h72–216 (Figure 1A), were produced from recombinant *Escherichia coli* (strain XA90) and purified by Ni-affinity (Qiagen) and anion exchange chromatography (Pharmacia). Isotopically labeled molecules were produced by growing recombinant bacteria on M9 minimal medium at 30°C, with 1.0 g/L $^{15}NH_4Cl$, 1.2 g/L $^{13}C_6$-glucose, or $D_2O$ (Cambridge Isotope Laboratories) as medium components, depending on the desired labeling pattern. MHC class II HLA-DR1 molecules were produced and secreted by recombinant S2 Schneider cells and immunoaffinity purified from the culture medium (Sloan et al., 1995).

Complexes were formed by incubating mixtures of Ii and DR1 in phosphate-buffered saline solution (pH 6.7) in the presence of 5% isopropanol and protease inhibitors aprotinin, leupeptin, and EDTA (Sigma) at standard concentrations. In the absence of the alcohol, complexes could also be formed, but with much lower yield. Products of the Ii/DR1 association were resolved by nondenaturing 10%

polyacrylamide gel electrophoresis and either stained with Coomassie blue or transferred to a polyvinylidene membrane and visualized with rabbit polyclonal anti-DR1 antibody (CHAMP) and alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (Boehringer Mannheim).

### NMR Spectroscopy

NMR experiments were performed with Ii concentrations of 0.2–1.0 mM at 27°C in phosphate-buffered saline solution (pH 6.7) with 5% perdeuterated isopropanol and standard concentrations of aprotinin, leupeptin, and EDTA. On a Bruker DMX-500 spectrometer, $^{15}$N-HSQC, $^{15}$N TOCSY-HSQC, and $^{15}$N NOESY-HSQC spectra were obtained with $^{15}$N-labeled Ii h72–216 and Ii h58–216 in the presence and absence of excess HLA-DR1 (10–15:1 wt/wt), and also with Ii h72–216 in the absence of the isopropanol; HNCA and HN(CO)CA experiments were performed with $^{15}$N,$^{13}$C-labeled Ii h72–216. $C^{\alpha}$ chemical shifts were referenced to tetramethylsilane with external 10% $^{13}C_6$-ethylbenzene in $CD_3Cl$.

The timecourses of 0.2 mM $^{15}$N-labeled Ii h72–216 and Ii h58–216 complex formation with unlabeled HLA-DR1 were monitored by measuring one-dimensional (128 transients/FID, 3 min acquisition time) and two-dimensional (two transients/FID and 128 $t_1$ increments, 20 min acquisition time) $^{15}$N-HSQC spectra at timepoints from 5 min to 15 hr following mixing. Heteronuclear $^{1}$H-$^{15}$N NOE (XNOE) measurements were made using approximately 70% deuterated Ii h72–216 and a sensitivity enhanced pulse-sequence (Farrow et al., 1994) on a Varian Unity Plus 500 spectrometer. $^{15}$N-HSQC spectra were obtained for deuterated and undeuterated Ii h72–216 on a Varian Unity INOVA 750 MHz spectrometer, with and without decoupling in both dimensions.

### Data Analysis

NMR data were processed and displayed with Felix (Molecular Simulations) and XEASY (Xia and Bartels, 1994). For analysis of XNOE data (Figure 2A), integrations were performed within XEASY and $(I_{sat} - I_0) / I_0$ was calculated for each residue, where $I_{sat}$ and $I_0$ are the peak intensities in spectra obtained with and without presaturation, respectively. Secondary chemical shifts reported in Figure 2A were calculated with $C^{\alpha}$ chemical shifts derived from the HNCA spectrum of Ii h72–216 and a set of random coil chemical shifts, which account for nearest-neighbor effects of proline in hexapeptides (Wishart et al., 1995). To give the changes in peak volumes upon Ii/DR1 complex formation (Figure 4B), $^{15}$N TOCSY-HSQC peaks were integrated identically for the spectra of Ii/DR1 and for Ii alone using the XEASY-associated program peakint. Because the Ii concentrations were the same in both the mixed and unmixed samples, the quotient $(I_{Ii/DR1} - I_{Ii})/I_{Ii}$ is reported for each measured residue without adjustment.

Figures were produced using Felix (Molecular Simulations), Molscript (Kraulis, 1991), and Adobe Photoshop and Illustrator (Adobe Systems).

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