The CLIP Region of Invariant Chain Plays a Critical Role in Regulating Major Histocompatibility Complex Class II Folding, Transport, and Peptide Occupancy

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Summary

Invariant chain (Ii) contributes in a number of distinct ways to the proper functioning of major histocompatibility complex (MHC) class II molecules. These include promoting effective association and folding of newly synthesized MHC class II α and β subunits, increasing transit of assembled heterodimers out of the endoplasmic reticulum (ER), inhibiting class II peptide binding, and facilitating class II movement to or accumulation in endosomes/lysosomes. Although the cytoplasmic tail of Ii makes a key contribution to the endocytic localization of class II, the relationship between the structure of Ii and its other diverse functions remains unknown. We show here that two thirds of the lumenal segment of Ii can be eliminated without affecting its contributions to the secretory pathway events of class II folding, ER to Golgi transport, or inhibition of peptide binding. These same experiments reveal that a short (25 residue) contiguous internal segment of Ii (the CLIP region), frequently found associated with purified MHC class II molecules, is critical for all three functions. Together with other recent findings, these results raise the possibility that the contributions of Ii to the early postsynthetic behavior of class II may depend on its interaction with the class II binding site. This would be consistent with the intracellular behavior of unoccupied MHC class I and class II molecules as incompletely folded proteins and imply a related structural basis for the similar contributions of Ii to class II and of short peptides to class I assembly and transport.

Upon synthesis and translocation into the endoplasmic reticulum (ER)[1], the component chains of class I and class II MHC molecules need to acquire a transport-competent conformation for effective post-ER transport. To achieve proper folding and a stable heavy chain–β_2 microglobulin interaction suitable for secretory pathway transit, MHC class I molecules require binding site occupancy with short peptides primarily generated in the cytosol and imported into the ER via TAP (1–3). In contrast, MHC class II molecules assemble as a stoichiometric complex with trimers of the type II integral membrane glycoprotein invariant chain (Ii) (4). In the secretory pathway, this association with Ii has effects on the properties of class II analogous to those resulting from peptide interaction with class I (3). Thus, interaction with intact Ii contributes to efficient, stable association of the class II α and β subunits in the ER (5, 6), promotes the transport of class II α/β heterodimers from the ER through the Golgi complex (5, 7–10), and inhibits the binding of other ligands (peptides) to the class II molecules (11–13). Whether these multiple effects of Ii require the entire molecule or are mediated by one or more structurally independent subregions of the protein is presently unknown. A particularly intriguing question is whether the effects of Ii on class II assembly, transport, and peptide binding represent discrete activities of this protein, or, as is true for short peptides binding to class I, whether they represent the consequences of a single underlying molecular event.

To investigate how Ii structure relates to its functions in the secretory pathway, and to examine the relationships between Ii-dependent class II folding, ER to Golgi transport, and inhibition of peptide binding, we have studied the behavior of class II molecules synthesized in the presence of mutant Ii proteins with truncations in the lumenal portion of the molecule. This strategy has allowed us to identify a short contiguous segment of Ii that is necessary for and coordinately influences all three of these functions in the absence of the majority of the lumenal segment of this protein, revealing a modular structure-function organization of the Ii molecule. The identity of this critical region of Ii with invariant chain–derived peptides (CLIP) frequently eluted from purified class II molecules (14–18), when considered with other recent data on the structural consequences of peptide binding by class II molecules (18a), suggests that a common mechanism of binding site occupancy may underlie the functional

[1] *Abbreviations used in this paper:* endo H, endoglycosidase H; ER, endoplasmic reticulum; Ii, invariant chain.

J. Exp. Med. © The Rockefeller University Press • 0022-1007/94/09/1107/07 $2.00
Volume 180 September 1994 1107–1113

similarities in the contributions of peptides to class I and Ii to class II assembly and export.

Materials and Methods

Plasmid Constructions. cDNA expression vectors containing inserts coding for various wild-type mouse class II α and β chains have been previously described (19, 20). A cDNA expression construct containing a recombinant Aβ chain with the β1 strands of Aβ^k and the β1 helix and remaining portion of A^b (Aβ^{kbb}) was provided by Dr. Ned Braunstein (Columbia P & S, New York). In the assays reported in this study, this construct yields the same results as wild-type Aβ^b when either β chain is coexpressed with Aα^b.

DNA fragments containing Ii coding sequences, 5′ translation control elements and EcoRI, BamHI and HindIII cloning sites were generated by PCR amplification (30 cycles: 94°C 1 min, 55°C 1 min, 72°C, 2 min) using the cDNA expression construct pcEXV-3mIi31 (19) as template and the following oligonucleotide primers: mIi1-82: upstream, 5′ CCGAATTCAAGCTTACTAGAGGCTAGAGCCATG 3′: downstream, 5′ GGAATTCCTATTATTTCGGAAGCTTCATGCG 3′: mIi1-107: upstream, 5′ CCGAATTCAAGCTTACTAGAGGCTAGAGCCATG 3′; downstream, 5′ GGAATTCCTATTACCCAAGGAGCATGTTATC 3′; mIi19-107: upstream, 5′ CCGAATTCAAGCTTCGTCATGAACCGCCCTAGAGAGCCAGAA 3′; downstream, 5′ GGAATTCCTATTACCCAAGGAGCATGTTATC 3′; mIi19-215: upstream, 5′CCGAATTCAAGCTTCGTCATGAACCGCCCTAGAGAGCCAGAA 3′; downstream, 5′ GGAATTCTTATCACAGGGTGACTTGACC 3′. The amplified fragments were digested with EcoRI and inserted into the EcoRI cloning site in the cDNA expression vector pcEXV-3. Clones with the insert in the proper transcriptional orientation were selected for use.

Transient Expression. For transient expression in COS cells, a modified DEAE-dextran procedure (21) was used as previously described (7). Briefly, COS 7.2 cells plated at 10^6 per 25 cm^2 tissue culture flask were washed twice with DMEM/10 mM Hepes. Cells were then incubated in 3 ml DMEM/10 mM Hepes containing 400 μg/ml DEAE dextran, 100 μM chloroquine, and DNA (1 μg of each plasmid encoding Aα and Aβ, plus 4 μg of plasmid encoding mIi31, mIi1-82, mIi1-107, mIi19-215, or mIi19-107). After 4 h at 37°C in 10% CO_2, the cells were treated with 10% DMSO in PBS for 2 min at room temperature, then incubated overnight in DMEM/10% FCS. 48 h after transfection, cells were used for immunoprecipitation, surface staining, or peptide binding.

Metabolic Radiolabeling, Immunoprecipitation, and NaDod/SO$_4$/Polyacrylamide Gel Analysis. Transfected COS cells were incubated for 1 h in leucine-free RPMI 1640 medium. [^{3}H]leucine (500 μCi/ml) was then added and the cells incubated for 20 min at 37°C. Some samples were lysed immediately using NP40 lysis buffer (13) (pulse). Other labeled cell samples were incubated for 4 h at 37°C in RPMI 1640 medium containing an excess of cold leucine (chase), then lysed in NP40 buffer. The precleared lysates were immunoprecipitated using mAbs previously bound to protein A–Sepharose beads or protein G–Sepharose beads, as previously described (13). The eluted samples were analyzed by SDS-PAGE in reducing conditions.

For endoglycosidase H (endo H) treatment, before elution, samples were incubated in 0.05 M sodium citrate buffer, pH 5.5, in the presence or absence of 15 mU of endo H (Boehringer Mannhein Corp., Indianapolis, IN) at 37°C for 16 h.

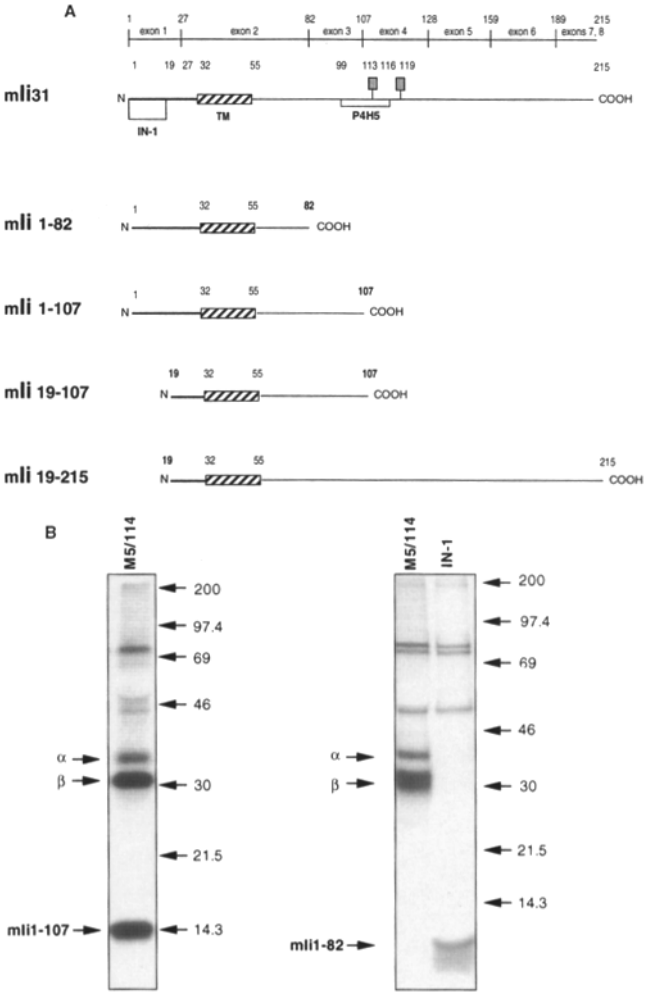

Figure 1. (*A*) Schematic representation of wild-type mouse Ii31 and truncated forms of Ii used in this study. The exon organization of the Ii gene is indicated. The *striped box* represents the Ii transmembrane region. *Dotted boxes* indicate Ii N-linked glycosylation sites. The putative binding sites of P4H5 and IN-1 mAb are indicated. Bold numbering indicates the residues at the termini of the mutant proteins. (*B*) Association of Ii mutants with MHC class II molecules. COS cells expressing either Aα^bAβ^{kbb} plus mIi1-107 or Aα^bAβ^{kbb} plus mIi1-82 were pulse-labeled, lysed in the presence of NP40, and immunoprecipitated with an anti-MHC class II mAb, M5/114, or an anti-Ii mAb, IN-1. The samples were eluted without heating and analyzed by SDS gel electrophoresis. *Arrows* on the left of the figure indicate the migration of α, β, mIi1-107, and mIi1-82. The positions of molecular weight (M_r) markers, expressed as $10^{-3} \times M_r$, are indicated on the right. Similar results were obtained for Aα^bAβ^b, and in experiments in which only mIi1-107 was tested, for EαEβ^d, and Aα^dAβ^d.

Peptide Binding. 48 h after transfection, COS cells were exposed to 100 μM biotinylated Eα (52-68) peptide and incubated for 4 h at 37°C. The cells were then washed, stained with PE-streptavidin for 20 min on ice, and washed twice. The stained cells were analyzed with a FACScan® using LYSYS II software (Becton Dickinson & Co., Mountain View, CA). Dead cells were eliminated from the analysis by staining with propidium iodide and appropriate gating.

Surface Staining. 48 h after transfection, COS cells were detached using versene, washed twice in PBS containing 5% FCS and 0.02%

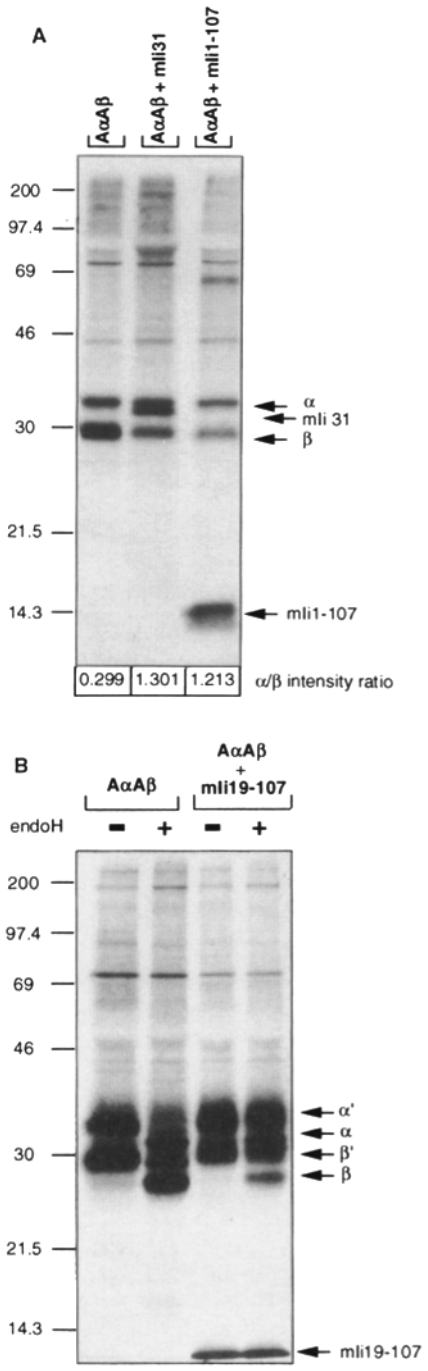

Figure 2. (*A*) Protection of class II dimers from aggregation with free class II β chains by stably associated wild-type and truncated Ii. COS cells expressing either $A\alpha^bA\beta^{kbb}$ (lane *1*), $A\alpha^bA\beta^{kbb}$ plus mIi31 (lane *2*), or $A\alpha^bA\beta^{kbb}$ plus mIi1-107 (lane *3*) were pulse-labeled, lysed in the presence of NP-40, and immunoprecipitated with an anti-MHC class II mAb, Y-3P. *Arrows* on the right of the figure indicate the migration of α, mIi31, β, and mIi1-107. The positions of molecular weight (M_r) markers, expressed as $10^{-3} \times M_r$, are indicated on the left. The numbers below each lane give the α/β band intensity ratio as determined by densitometric analysis. (*B*) Enhancement of MHC class II ER egress and Golgi transit by the mIi19-107 truncated form of Ii. Metabolically labeled MHC class II molecules were immunoprecipitated with the mAb Y-3P from lysates of COS cells after pulse-labeling and a 4-h chase period. (Lanes *1* and *2*), $A\alpha^bA\beta^{kbb}$ alone, (lanes *3* and *4*) $A\alpha^bA\beta^{kbb}$ plus mIi19-107. Immunoprecipitates were either mock treated (lanes *1* and *3*) or treated with endo H before gel analysis (lanes *2* and *4*). The eluted boiled samples were analyzed by SDS-PAGE under reducing conditions. *Arrows* on the right indicate the migration of immature α (α), immature β (β), mature α (α') and mature β (β'). *Arrows* on the left point to the positions of the molecular weight markers. The displayed figure used $A\alpha^bA\beta^{kbb}$; similar data were obtained using $A\alpha^bA\beta^b$, and enhanced surface expression, previously shown to correlate with Ii-dependent secretory pathway trafficking (7), was also observed using $A\alpha^dA\beta^d$ and $A\alpha^dA\beta^k$.

NaN_3, and used for indirect immunofluorescence. Cells were incubated on ice either with antibodies for 20 min or with biotinylated TSST-1 for 2 h and washed twice. Culture supernatants containing Y-3P and M5/114 mAb were used to stain the cells, followed by fluorescein isothiocyanate–conjugated goat anti–mouse or anti–rat immunoglobulin. Cells incubated with biotinylated TSST-1 were stained with PE-streptavidin. The stained cells were analyzed with a FACScan® using LYSYS II software (Becton Dickinson & Co.). Dead cells were eliminated from the analysis by staining and propidium iodide and appropriate gating. Fluorescence units were calculated as the product of the mean fluorescence of all positive cells times the percentage of positive cells (7).

Results and Discussion

Because the ER-related functions of wild-type Ii involve stable association with class II, we first established the minimum structure necessary for such interaction. cDNA expression constructs were prepared encoding COOH-terminal (lumenal) truncations of Ii with or without deletion of endosomal localization signals in residues 2–19 of the cytoplasmic tail (20, 22–24) (Fig. 1 *A*). Ii mutants were expressed in COS cells together with mouse class II Aα and Aβ chains and proteins from lysates of pulse-labeled cells were immunoprecipitated using mAbs specific for AαAβ or the cytoplasmic tail of Ii (IN-1 [25]). mIi1-107 was the shortest form of Ii coprecipitating with class II (Fig. 1 *B*). mIi1-82 was not detectably associated with MHC class II molecules in NP-40 lysates, although it could be readily precipitated by IN-1 (Fig. 1 *B*). Small amounts of class II were inconsistently coprecipitated with mIi1-82 from digitonin lysates (data not shown). Thus, the exon 3–encoded region (26) between residues 83 and 107 of Ii makes a critical contribution to association with MHC class II, and sequences beyond residue 107 are unnecessary for stable class II–Ii interaction in the ER. These data on the contribution of this region to class II–Ii association complement those recently obtained using an internal deletion approach (27).

The mAb Y-3P (28) only reacts with assembled class II $\alpha\beta$ dimers, and AαAβ dimers precipitated by Y-3P from lysates of [^{3}H]leucine-labeled spleen cells show better labeling of the Aα than Aβ chain (5, 13). Yet in Y-3P immunoprecipitates from COS cells expressing class II without Ii, a greater Aβ than Aα signal was observed (Fig. 2 *A*). Recent experiments indicate that this represents coprecipitation of unassembled, aggregated, disulphide-linked β chains with the small number of true α/β heterodimers that form in cells lacking Ii (6). Strikingly, coexpression of either mIi31 or mIi1-107, but not mIi1-82, eliminated coprecipitation of these excess

β proteins. These results confirm that Ii promotes effective formation of class II heterodimers that resist interaction with incompletely folded proteins or their associated chaperones and demonstrate that the Ii segment 1-107 is both necessary and sufficient for this function.

The contributions of Ii lumenal subregions to class II heterodimer export from the ER and to inhibition of peptide binding were examined using constructs lacking the endosomal localization signals in residues 2–19 of the Ii cytoplasmic tail (22, 23, 29, 30). Class II molecules expressed in cells lacking Ii show retention in the ER (10, 20, 23, 24) and poor acquisition of endo H resistant N-linked glycans (5, 7–10). Coexpression of wild-type Ii augments the fraction of class II dimers acquiring endo H resistant carbohydrates. In agreement with our failure to detect their stable association with class II, mIi1-82, or mIi19-82 had no measurable effect on class II glycan maturation and mIi1-82 could not be detected outside the ER by immunofluorescence microscopy, whether or not class II was also present (data not shown). In contrast, mIi19-107 was highly effective in promoting the ER egress and medial Golgi transit of Y-3P–reactive class II dimers, based on their acquisition of endo H–resistant glycans (Fig. 2 *B*). As would be expected from these biochemical data, coexpression of mIi19-107 also increased cell surface expression of class II (data not shown).

Intact Ii inhibits stable peptide–MHC class II interaction (11, 12), and in vitro studies with soluble synthetic peptides have led to the suggestion that a segment of Ii between residues 83–107 (CLIP) is involved in this function (16, 18). To examine whether inhibition of peptide binding mapped to this region in membrane-anchored Ii proteins, COS cells were prepared that expressed on their surface class II alone (after intracellular dissociation from wild-type mIi31), class II stably assembled with mIi19-107, or class II stably assembled with mIi19-215. These cells were incubated with the biotinylated peptide Eα (52–68), then stained with PE-streptavidin. Fig. 3 shows that association of class II with mIi19-107, as with the longer mIi19-215 form, prevents formation of class II–Eα (52–68) complexes. Similar results were obtained using unconjugated peptide and an antibody (Y-Ae[31]) specific for the class II–Eα peptide complex, and class II–dependent peptide stimulation of T hybridoma responses was also prevented by mIi19-107 (data not shown). Thus, the same short 83–107 segment of Ii makes an essential contribution to both the class II assembly/transport promoting functions of Ii and to its ability to inhibit peptide binding.

mIi1-107 could carry out its functions by any of three general mechanisms: (*a*) It could form a cap across the superficial surface of the peptide binding site, preventing peptide entry while holding the two chains together; (*b*) It could bind outside the binding groove, inhibiting peptide interaction by an allosteric change in class II structure that also promotes α and β chain association; or (*c*) It could lie in the binding groove, blocking interaction with antigenic peptides while providing at least some of the stabilization of class II that is characteristic of conventional binding site occupancy. In an initial attempt to discriminate among these possibilities,

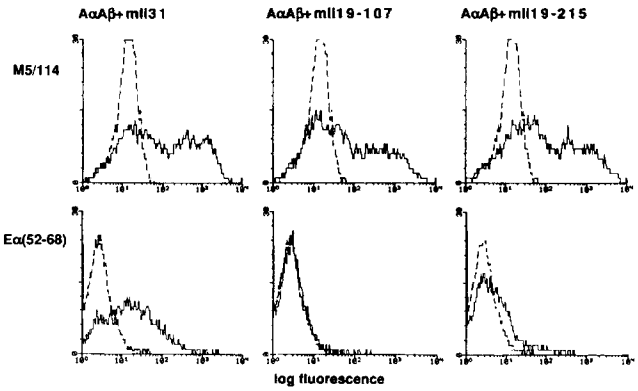

Figure 3. Inhibition of antigenic peptide binding by wild-type and truncated Ii. COS cells expressing either $A\alpha^bA\beta^{kbb}$ plus mIi31, $A\alpha^bA\beta^{kbb}$ plus mIi19-107, $A\alpha^bA\beta^{kbb}$ plus mIi19-215 (*solid line* in each panel) or mIi 31 alone (*dotted line* in each panel) were examined for peptide binding by surface class II using biotinylated Eα (52–68) and PE-streptavidin. The Eα (52–68) peptide binds well to $A\alpha^bA\beta^{kbb}$ and this complex reacts in our hands with the Y-Ae antibody in a manner similar to Eα (52–68) complexed with wild-type $A\alpha^bA\beta^b$. Similar results were also obtained using either $A\alpha^bA\beta^{kbb}$ or $A\alpha^bA\beta^b$ with unconjugated Eα (52–68), followed by staining with Y-Ae mAb, and with $E\alpha E\beta^k$ and biotinylated pigeon cytochrome *c* peptide 88–104.

we examined the effect of mIi19-215 and mIi19-107 association on class II interaction with a bacterial superantigen whose binding involves the class II α1 domain and with antibodies specific for the class II α1 or β1 helices. Fig. 4 shows that mIi19-215 association decreases class II binding of TSST-1, in agreement with Karp et al. (29). This long form of Ii also inhibits class II interaction with the α chain–specific mAb Y-3P, without affecting interaction with the β chain–specific mAb M5/114. In contrast, mIi19-107 does not affect the binding of any of these probes to class II. Thus, the COOH-terminal lumenal region of Ii does show evidence of binding to the superficial region of the α chain portion of the class II binding domain. Nevertheless, a shortened form of Ii (mIi19-107) that is fully capable of inhibiting peptide binding and promoting proper class II structure shows no evidence of such interaction. This is inconsistent with the ability of Ii to inhibit peptide binding requiring superficial occlusion (capping) of the class II binding domain, although the more COOH terminal part of Ii chain may have this property.

The results presented here indicate that an internal segment of invariant chain encoded by a single exon (residues 83–107) plays a critical role in effective Ii interaction with class II, in promoting formation of properly folded α/β dimers, in enhancing MHC class II egress from the ER, and in regulating class II peptide binding. The more COOH-terminal region of Ii (from residues 108–215) is unnecessary for any of these secretory pathway functions. When considered with evidence that a naturally occurring 68 residue insertion near the COOH terminus of Ii preserves interaction with class II but alters processed antigen presentation by these molecules (32), our data imply that rather than acting as a

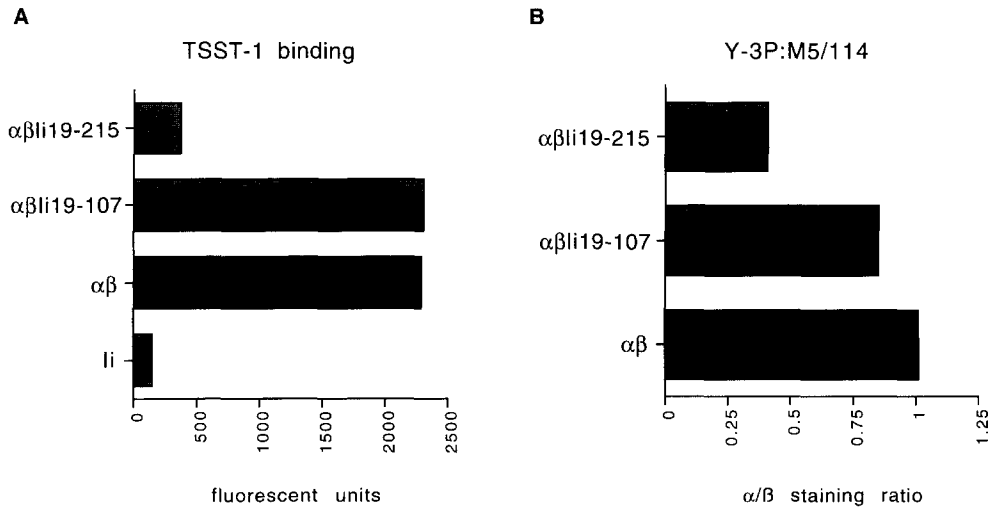

Figure 4. mIi19-215 but not mIi19-107 association inhibits binding of TSST-1 or α1 helix-specific mAb to class II molecules. (*A*) The ability of $A\alpha A\beta$, $A\alpha A\beta$ + mIi19-107, and $A\alpha A\beta$ + mIi19-215 to bind TSST-1. COS cells expressing either $A\alpha^bA\beta^{kbb}$, $A\alpha^bA\beta^{kbb}$ plus mIi19-107, $A\alpha^bA\beta^{kbb}$ plus mIi19-215, or mIi31 alone were examined for TSST-1 binding to surface class II using biotinylated TSST-1 (Toxin Technologies, Madison, WI) and PE-streptavidin. The results are expressed in fluorescence units. (*B*) Ability of $A\alpha A\beta$, $A\alpha A\beta$ + mIi19-107, and $A\alpha A\beta$ + mIi19-215 to bind the mAbs Y-3P and M5/114. COS cells expressing either $A\alpha^bA\beta^{kbb}$, $A\alpha^bA\beta^{kbb}$ plus mIi19-107, or $A\alpha^bA\beta^{kbb}$ plus mIi19-215 were surface stained with Y-3P and M5/114. The results are expressed as ratio of the fluorescence units obtained by staining with Y-3P vs. the fluorescence units obtained by staining with M5/114.

single functional unit, the lumenal region of Ii is divided into structurally discrete subdomains with different effects on class II molecules at early and late times after biosynthesis.

Based on in vitro data using synthetic peptide versions of the CLIP region, other investigators have suggested a role for this portion of Ii in controlling peptide binding to class II (16, 18). Our results here with membrane anchored forms of Ii provide clear support for this model, but perhaps more significantly, they also reveal that this same segment of Ii is involved in the control of class II heterodimer folding and ER to Golgi transport. We regard as striking the functional similarities between these coordinate CLIP-dependent effects of Ii on class II early postsynthetic behavior and the effects of peptide binding on class I in the ER. In both cases, the interaction promotes conformationally correct MHC subunit interaction, inhibits chaperone association, promotes secretory pathway transport, and interferes with binding of other ligands (3).

How does Ii, and in particular the CLIP region, mediate these various effects? Our data do not rule out an allosteric model in which Ii interaction with class II outside the binding groove is the key event in regulating all of these aspects of the ER/Golgi behavior of class II molecules. It is, however, notable that the complete CLIP segment is just slightly longer than a typical antigenic peptide occupying the class II binding site (14, 15, 17), peptides corresponding to this region of Ii can be eluted from purified class II molecules under the same conditions that release antigenic peptides, free peptide versions of sequences within this region bind to many different class II alleles and isotypes (14–18), and at least some forms of CLIP can promote the formation of SDS-stable class II molecules in the same manner as known groove-binding antigenic peptides (15). In contrast to many antigenic peptide ligands, long peptide analogues of CLIP (16, 18) and all membrane-anchored forms of Ii containing this region ([13] and our unpublished observations) fail to generate the SDS-stable form of class II. We have recently found, however, that conventional antigenic peptides also can interact with class II in a low affinity mode that stabilizes class II structure at 37°C without promoting the internal molecular changes involved in SDS-denaturation resistance (18a). Finally, molecular modelling based on the recent crystal structure of class II (33, 34) indicates that an extended chain version of the Ii1-107 segment could reach and enter the binding site, and the type II membrane orientation of Ii would permit this occupancy to occur in the NH_2 to COOH orientation typical of antigenic peptides. These various pieces of data and the obvious parallels to class I–antigenic peptide interaction make attractive a model in which the CLIP region plays the role of a low to moderate affinity, "generic" class II groove–occupying peptide while it is still a part of the intact Ii structure. This could promote heterodimer stability in the organelles of the secretory pathway while precluding interaction with other potential ligands until Ii is removed by proteolytic processing in endosomes/-prelysosomes.

Other parts of Ii, including possibly a portion of CLIP that extends out of the binding site proper, may affect the ability of class II to undergo the intramolecular rearrangements involved in very stable peptide binding (35, 36), helping to assure that CLIP does not become too tightly associated with class II. Distinct regions of intact Ii clearly contact class II, which may help strengthen the low affinity association of the CLIP segment with diverse binding sites, yet allow effective dissociation after partial Ii proteolysis in the endocytic pathway. CLIP control of binding site availability would affect where within the endosomal pathway class II mole-

cules can be loaded with antigenic peptides, as it is necessary to remove this region prior to effective association with processed antigen. This event appears to be critically dependent on the expression of the DMA and DMB genes of the MHC (37, 38).

The MHC class II binding site allows occupancy with long peptides (35) and even unfolded segments of intact proteins (39). Thus, the ability of Ii to interfere with binding site function may be critical to the export of useful class II molecules to the relevant antigen processing compartment. Class II might otherwise interact unproductively with exposed peptide segments of the incompletely folded proteins present in high concentration in the ER lumen, in a manner analogous to the presumed binding of class II to denatured protein antigens in endosomes/lysosomes (3). This interference with unproductive ER binding to class II to large proteins seems to be the relevant consequence of Ii inhibition of binding site function, not the prevention of binding of the short peptides that are the ligands of class I (5).

MHC molecules lacking binding site ligands behave within cells as incompletely folded proteins. The dichotomy in preferred sites of antigenic peptide acquisition by class I and class II molecules, despite initial formation of both classes of binding sites in the ER, demands distinct solutions to the occupancy requirement for folding compatible with secretory pathway transport. Previous studies have shown that Ii provides for class II the ER functions performed by short antigenic peptides for class I (5). The present study suggests that this similarity may be more than superficial, possibly involving the direct occupancy of the class II binding site with an internal Ii segment.

We wish to thank M. Marks, E. Long, P. Roche, J. van Meerwijk, and S. Sadegh-Nasseri for helpful comments on this manuscript; L.-Y. Huang and J. van Meerwijk for assistance with PCR methods; and L. Stern for helpful structural discussions.

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Received for publication 2 May 1994.

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