Alteration of a Single Hydrogen Bond between Class II Molecules and Peptide Results in Rapid Degradation of Class II Molecules after Invariant Chain Removal

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Summary

To characterize the importance of a highly conserved region of the class II β chain, we introduced an amino acid substitution that is predicted to eliminate a hydrogen bond formed between the class II molecule and peptide. We expressed the mutated β chain with a wild-type α chain in a murine L cell by gene transfection. The mutant class II molecule (81βH$^-$) assembles normally in the endoplasmic reticulum and transits the Golgi complex. When invariant chain (Ii) is coexpressed with 81βH$^-$, the class II–Ii complex is degraded in the endosomes. Expression of 81βH$^-$ in the absence of Ii results in a cell surface expressed molecule that is susceptible to proteolysis, a condition reversed by incubation with a peptide known to associate with 81βH$^-$. We propose that 81βH$^-$ is protease sensitive because it is unable to productively associate with most peptides, including classII–associated invariant chain peptides. This model is supported by our data demonstrating protease sensitivity of peptide-free wild-type I-A^d molecules. Collectively, our results suggest both that the hydrogen bonds formed between the class II molecule and peptide are important for the integrity and stability of the complex, and that empty class II molecules are protease sensitive and degraded in endosomes. One function of DM may be to insure continuous groove occupancy of the class II molecule.

Key words: major histocompatibility complex class II • peptide • hydrogen bond • invariant chain • proteolysis

Occupancy of the MHC-encoded class II molecule plays a key role in its fate. Association of the surrogate peptide CLIP,[1] derived from invariant chain (Ii), with class II molecules enhances assembly and export from the endoplasmic reticulum (ER) (1–6). In the absence of Ii, class II molecules can bind other proteins in the ER via the peptide binding pocket (7, 8), thus suggesting a drive for binding site occupancy. In addition, when Zhong and co-workers engineered a class II β chain to express a covalently linked, antigenic peptide at its amino terminus, it assembled more efficiently with α chain and egressed more quickly from the ER than wild-type (WT) class II molecules (9). Hence, occupancy of the class II binding site in the ER by a tethered peptide promotes rapid transport of class II–peptide complexes into the Golgi.

The association of peptide with class II molecules also has consequences late in biosynthesis, i.e., in post-Golgi compartments. Several groups have shown that the exogenous provision of peptide increases the half-life and yield of class II molecules in both the endosomal compartments and at the cell surface (10–12). In addition, the work of Germain and co-workers suggests that in the absence of peptide, empty class II molecules aggregate in endosomes (12). Hence, peptide plays an important role in the entire life cycle of the class II molecule, from facilitating its assembly in the ER to determining its longevity in the endosomal compartments and at the cell surface.

Solving the three-dimensional crystal structures of both a class I–peptide complex and a class II–peptide complex directly demonstrated that the MHC-encoded α and β chains formed a groove occupied by peptide (13, 14). The crystal structures also provided insight into exactly how the class II molecule associates with peptide (14–18), identifying both the pockets that form stable interactions with the peptide side chains, as well as the hydrogen bonds that form between amino acid (aa) side chains of the class II

[1] *Abbreviations used in this paper:* aa, amino acids; CHO, Chinese hamster ovary; CLIP, class II–associated invariant chain peptides; cys, cystatin-C; ER, endoplasmic reticulum; GPI, glycan-phosphatidylinositol; HPAP, human placental alkaline phosphatase; Ii, invariant chain; PK, proteinase K; RT, room temperature; WT, wild-type.

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molecule and the main chain atoms of the peptide (Fig. 1, *top*). The majority of these hydrogen bonds involve aa's that are conserved in mouse and human class II molecules (14–18).

One highly conserved region of the class II β chain lies at the periphery of its antigen-binding pocket at residues 79–83. Amino acids 81 and 82 form hydrogen bonds to the peptide main chain. To characterize the importance of the histidine at position 81, we created a transfected murine fibroblast L cell line that expressed the mutant class II molecule formed by association of a WT A^d α chain with an A^d β chain in which a conservative aa substitution was introduced at residue 81, changing a histidine to an asparagine (His→Asn) (19). The mutant A^d molecule lacking the potential for a single hydrogen bond to the peptide main chain will hereafter be referred to as 81m or as $81\beta H^-$. The mutant class II β chain assembles normally with the WT α chain in the ER. The assembled mutant class II molecule then transits the Golgi, obtains mature glycosylation, and has a half-life comparable to that of WT A^d expressed in L cells (20). However, $81\beta H^-$ does not form detectable SDS-stable dimers, suggesting that $81\beta H^-$ does not stably associate with peptide. In addition, $81\beta H^-$ is not detected in the endosomes, unlike WT class II where 15% of the steady-state pool is localized to these compartments (20).

In this paper, we characterize the fate of the mutant class II molecule, $81\beta H^-$. Although coexpression of Ii redistributes $81\beta H^-$ to the endosomes (19), we show here that Ii also dramatically reduces the level of $81\beta H^-$ expressed at the cell surface and changes its fate within the cell. Upon reaching the endosomes, $81\beta H^-$ in association with Ii is rapidly degraded. Based on our experiments examining the susceptibility of $81\beta H^-$ to proteases as well as its ability to bind peptides, we hypothesize that when $81\beta H^-$ accesses the endosomes and class II–associated invariant chain peptide (CLIP) dissociates, $81\beta H^-$ is unable to productively associate with available peptides. These empty class II molecules are then susceptible to degradation. We conclude that peptide is key to the endosomal survival of both mutant and WT class II molecules and propose that a principle role of CLIP and DM is to insure continuous groove occupancy by peptide.

Materials and Methods

Cell Lines, DNA Construct Cell Lines, and Protease Treatment. Cell lines were maintained at 37°C and 5% CO_2 in DMEM containing 5% FCS and 5% BCS supplemented with 5 mM Hepes, 2 mM glutamine, and 1 mM nonessential amino acids (complete medium). All media and supplements were purchased from GIBCO BRL (Gaithersburg, MD) unless otherwise noted. The derivation of L cell transfectants expressing $81\beta H^-$, $81\beta H^-$ Ii (e.g., $81\beta H^-$Ii), WT, and WTIi was described previously (19, 20). Chinese hamster ovary (CHO) cells were transfected with genes encoding WT I-A^d molecules or I-A^d molecules that had been modified so that they were linked to the plasma membrane by the phospholipid linker derived from the human placental alkaline phosphatase (HPAP) I-E^k construct, described by Davis and colleagues (21). G418 (geneticin) and HAT were obtained from GIBCO BRL. Proteinase K (PK) and trypsin were obtained from Sigma Chemical Co. (St. Louis, MO). Trypsin-EDTA was obtained from GIBCO BRL and was used directly as supplied by the manufacturer. 100 mg of PK was resuspended in 10 ml of DMEM and filter sterilized through a 0.45-μm filter right before use. TPCK-trypsin was obtained from Sigma Chemical Co. and prepared in the same way as PK at a concentration of 50 mg/ml. The final solution of trypsin was neutralized with 1 N NaOH. To treat the L cells with proteases, we first adapted them to petri dishes to grow them nonadherently. The cells were pelleted from culture media, rinsed once with DMEM-Hepes, and resuspended in 1–2 ml of the protease. CHO cells were treated similarly, but before protease treatment they were maintained adherent in tissue culture dishes, and harvested immediately before enzyme treatment by brief incubation with EDTA (Versene; GIBCO BRL). After treatment for 0.5 h at room temperature (RT) for PK or 10 min at 37°C for trypsin, 1 ml of calf serum was added, and the cells were pelleted, washed with complete medium, and analyzed by flow cytometry. In experiments where trypsin-EDTA was used, enzyme treatment was for 0.5 h at RT. Ammonium chloride was obtained from Sigma Chemical Co. and used at a final concentration of 20 mM. The protease inhibitor, Z-phe-ala was obtained from P. Morton (Searle, Chesterfield, MO) and used at a final concentration of 10 μM.

Monoclonal Antibodies. The hybridomas producing mAb reactive with I-A^d (MKD6, M5/114) and with class I K^k (16-1-11N) were obtained from American Type Culture Collection (Rockville, MD). The specificity of the anti–class II mAbs has been described previously (22). ID4B is a rat mAb that recognizes murine LAMP-1 and was provided by Dr. Thomas August (Dept. of Pharmacology and Molecular Sciences, Johns Hopkins, Baltimore, MD). The rabbit anti–I-A^d β chain cytoplasmic tail peptide was produced by HTI Bioproducts, Inc. (Ramona, CA) using the peptide AB described below. The rat mAb In-1, which recognizes the amino terminus of Ii was provided to us by Jim Miller (University of Chicago, Chicago, IL). Its characterization is described in reference 23.

Peptides. The AB and cystatin-C (cys) peptides were synthesized by Dr. Giri Reddy of the University of Chicago Amino Acid and Protein Core Labs., Chicago, IL. The cys derived peptide is DAYHSRAIQVVRARKQ (aa 40–55), and the AB peptide is CQKGPRGPPPAGLLQ, which corresponds to the cytoplasmic tail of I-A β. The concentration of cys derived peptide used in the experiments (20.75 μM; 43.75 μg/ml) was determined to be the optimal concentration of peptide that increased surface expression of $81\beta H^-$ but did not increase staining with the fluoresceinated, second step GAM reagent.

Flow Cytometry Analysis. MHC cell surface expression was measured by staining with mAb followed by a secondary staining reagent FITC-labeled goat anti–mouse Ig (FITC-GAM) (Cappel Laboratories, Cochranville, PA) as described previously (24). The samples were analyzed on a FACScan® cytofluorometer (Becton Dickinson, Mountain View, CA).

Metabolic Labeling, Immunoprecipitation, and SDS-PAGE. For the pulse-chase experiments, 5×10^5 cells per time point were plated out the night before in complete media in 60-mm tissue culture dishes. The next day, the cells were prelabeled for 1.5 h in leucine-free DMEM supplemented with 5% dialyzed FCS. The cells were then labeled in the same medium containing 300–350 μCi/ml of [^{3}H]leucine (Amersham Corp., Arlington Heights, IL), at 37°C, 5% CO_2 for 30–45 min. The pulse plates were washed once in cold complete media and lysed in 0.5% NP-40 lysis buffer with the protease inhibitors TPCK (50 μg/ml), PMSF

(200 μg/ml), leupeptin (0.5 μg/ml) (Boehringer Mannheim Biochemicals, Indianapolis, IN), and 20 mM iodo-acetamide. The chase point plates were washed and incubated in prewarmed medium containing a twofold excess of unlabeled leucine for the indicated chase times. The radiolabeled cells were lysed on the dishes on ice. Postnuclear supernatants were precleared for 1 h with 60 μl of packed PAS (Pharmacia Biotech AB, Uppsala, Sweden) for the mouse mAbs and rabbit antisera or with PGS (Pharmacia Biotech AB) for the rat mAbs. The lysates were then incubated with PAS or PGS prebound with the appropriate antibody for at least 2 h. The immunoprecipitated material was washed three times in lysis buffer, resuspended in sample buffer counting 2% 2-ME, boiled, and analyzed by SDS-10% PAGE. Gels were treated with En^{3}Hance (NEN Research Products, Boston, MA) or Fluor-Hance (Research Products International, Mount Prospect, IL) and subjected to autoradiography at −80°C. The overnight labeling was done in 300 μCi/ml of leucine in 3 ml of leucine-free media (ICN Biomedicals, Inc., Costa Mesa, CA).

CLIP Analysis. CLIP analysis was performed essentially as described above except for the following changes. The cells were labeled for 0.5 h in 250 μCi/ml of [^{35}S]methionine (Amersham Corp.) in methionine-free DMEM. The immunoprecipitated proteins were resolved on a 12.5% SDS polyacrylamide gel which was run until the dye front was ~2 inches from the bottom of the glass plates. The gels were treated sequentially, for 20 min each, with fixative (30% methanol, 10% acetic acid), water, and then Fluor-Hance (Research Products International) before drying at 75°C for 2 h.

Cell Surface Biotinylation and Western Blot Analysis. The cells were plated the night before at a density of 5×10^5/2 ml on 60-mm tissue culture dishes. The next day the plates were rinsed six times with cold PBS-CM (PBS with 1 μM CaCl and 250 μM MgCl) and then incubated 30 min with 1.5 μg/ml NHS-SS-biotin (Pierce Chemical Co., Rockford, IL) in PBS-CM on ice with gentle rocking. The biotinylation reaction was stopped by washing the cells six times with 50 mM glycine in PBS-CM. Cells were lysed on the dishes on ice in lysis buffer containing 0.5% CHAPS with the protease inhibitors and iodo-acetamide as described above. The postnuclear supernatants were precleared with PAS as described above and then sequentially immunoprecipitated with PAS prebound to 16-1-11N, followed by immunoprecipitation with PAS prebound to the rabbit antiserum (10 μl/sample). The immunoprecipitates were washed two to three times with lysis buffer, resuspended, boiled in sample buffer lacking 2-ME, and resolved by SDS-PAGE. The proteins were transferred on to nitrocellulose membranes at 250 milliamps for 2 h. The membranes were blocked with 3% BSA in a buffer composed of 0.5% Tween 20, 1 M d-glucose, and 10% glycerol. The membranes were then incubated for 1 h in a 50-ml solution containing 10% of the solution described above plus 15 μl of streptavidin conjugated to HRP (GIBCO BRL) and then washed extensively with Tris-buffered saline containing 0.05% Tween 20 (TBST) and developed by chemiluminescence using ECL (Amersham Corp.). Western blot analysis of the p12 fragment was carried out as described elsewhere (25). In brief, the nitrocellulose containing the immobilized proteins was blocked for 1 h in water containing 5% powdered nonfat milk, 0.1% sodium azide, and 0.028% antifoam (Sigma Chemical Co.). The membranes were then probed sequentially for class II and Ii with M5114 and In-1, respectively, for at least 3 h. A 1:5,000 dilution of a species-specific secondary antibody coupled to horseradish peroxidase was added to the blot for 1 h before extensive washes in TBST. The blots were then developed using chemiluminescence using LumiGLO™ (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD).

Results

Ii Coexpression Alters Fate of 81βH$^-$. Although the His→Asn substitution at position 81 of the class II β chain is considered a conservative aa substitution, this mutation is predicted to disrupt a hydrogen bond between class II and the peptide backbone (Fig. 1). When the gene encoding the substituted β chain is introduced into L cells, the mutated β chain assembles with WT A^d α chain and is expressed at the cell surface at levels comparable to WT (19). Surprisingly, coexpression of Ii causes a dramatic decrease in the amount of 81βH$^-$ expressed at the cell surface (Fig. 2 *A*, *right*). These data are in contrast to studies done in our

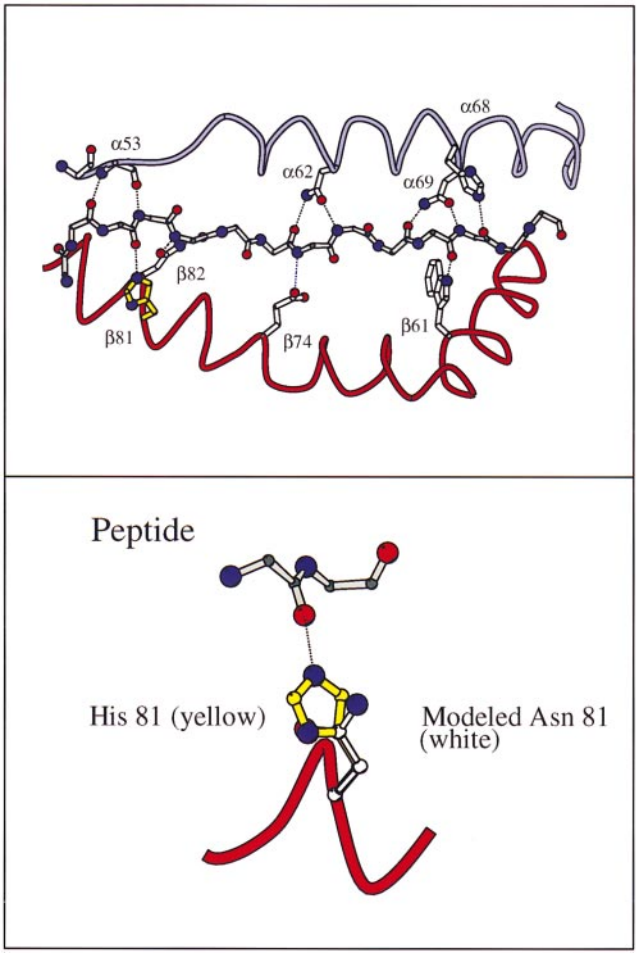

Figure 1. Model of class II–peptide interactions. (*Top*) Hydrogen bonds formed between the I-A^d molecule and the ovalbumin peptide backbone (18). The α chain is shown in blue and the β chain is shown in red. The histidine at aa 81 of the β chain is shown in yellow with the nitrogen atoms colored blue. Oxygen atoms are shown in red and the peptide bonds and carbon atoms are colored gray. The hydrogen bonds are indicated by the dashed lines. (*Bottom*) Close-up view of the hydrogen bond formed between the histidine at position 81 of the β chain and peptide backbone (distance, ~2.7 Å). Asparagine was substituted for histidine and a common rotamer conformation was chosen to overlap the histidine side chain. In this conformation the distance from the peptide carbonyl to the asparagine nitrogen is 4.2 Å. The program O (50) was used to model the histidine to asparagine mutation and to select a common rotamer for asparagine. The figure was made with the program MOLSCRIPT (51).

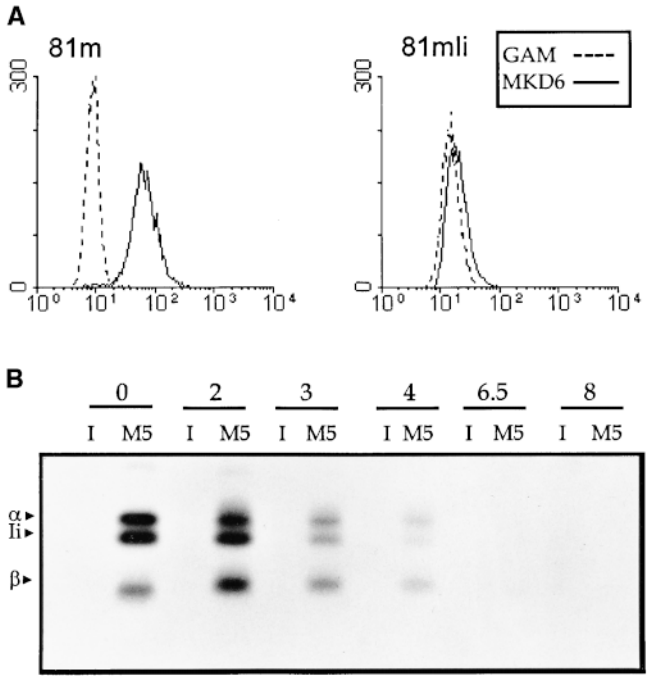

Figure 2. Coexpression of Ii results in loss of 81βH⁻ expression at the cell surface. (*A*) Class II surface expression on stably transfected L cells expressing 81βH⁻ (*left*) and 81βH⁻ supertransfected with the murine Ii (*right*) was determined using mAb MKD6. Background staining detected with the goat anti–mouse Ig conjugated to FITC (GAM) is shown as a dashed line. (*B*) [^{3}H]Leucine pulse-chase experiment of 81βH⁻ Ii shows synthesis of the α, β, and Ii proteins. The cells were pulsed for 45 min (*0*) followed by chase times of 2, 3, 4, 6.5, and 8 h. The cells were lysed in 0.5% NP-40 lysis buffer and immunoprecipitated sequentially with a rat mAb recognizing LAMP-1, ID4B (*I*), as negative control, followed by a rat anti–murine class II β chain, M5114 (*M5*). Location of the α, β, and Ii chains are shown on the left.

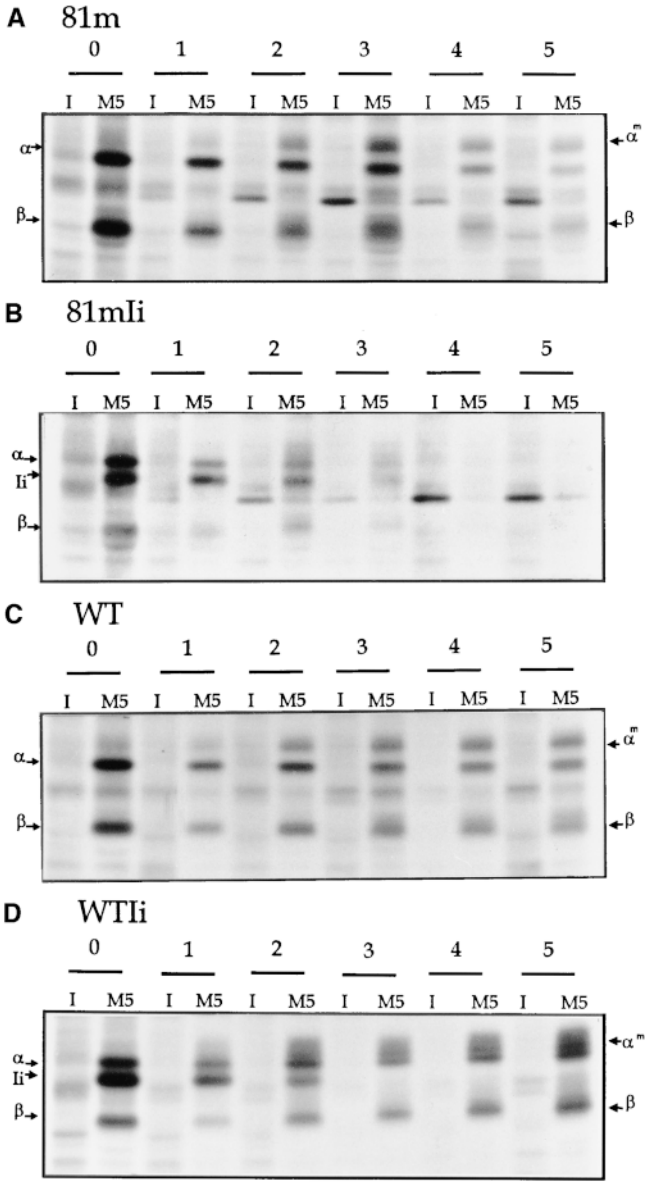

Figure 3. The effect of Ii on 81βH⁻ is a late event in biosynthesis and specific for 81βH⁻. L cells expressing 81βH⁻ (*A*), 81βH⁻Ii (*B*), WT (*C*), and WTIi (*D*) were labeled with [^{3}H]leucine for 30 min (*0*) and chased for 1, 2, 3, 4, and 5 h. The lysates were sequentially immunoprecipitated with ID4B (*I*) followed by M5114 (*M5*). At later time points, the class II molecules transit the Golgi complex to attain mature glycosylation (α^m).

lab and others (2, 4) where coexpression of Ii with WT class II molecules facilitates transport and cell surface expression of class II.

The low levels of class II surface expression observed on 81βH⁻Ii-expressing cells are not caused by the loss of class II gene expression, but rather correlate with rapid degradation (Fig. 2 *B*). At time 0, α, Ii, and β chains are present. By 2 h, the β chain has increased in size, as has the α chain, indicating that the αβIi complex has left the ER and has undergone additional glycosylation in the Golgi complex. At 3 h, there is noticeable attrition of the entire αβIi complex, which is nearly gone by the 4-h chase point. We conclude that the deficiency in cell surface class II expression on 81βH⁻Ii-expressing cells cannot be accounted for by low class II protein synthesis; rather, its loss occurs relatively late in its biogenesis.

We compared the fate of 81βH⁻Ii with that of 81βH⁻, WT, and WTIi in a pulse–chase experiment (Fig. 3). All of the cell lines have similar maturation kinetics early in the pulse–chase experiment where mature α and β chains are present after 2 h. Cells expressing WT class II with Ii lose Ii between 2 and 3 h, indicating that the class II–Ii complex has accessed the endosomal compartments. A strikingly different pattern is observed in cells expressing 81βH⁻Ii (Fig. 3, *D* and *B*). In Fig. 3 *B*, the mature 81βH⁻Ii complex begins to disappear at 3 h and is undetectable by 4 h. In contrast, mature class II molecules persist in cells expressing 81βH⁻, WT, or WT class II and Ii after 5 h of chase (Fig. 3, *A*, *C*, and *D*). We conclude that the loss of 81βH⁻ in the presence of Ii is unique to the mutant class II molecule, as it is not seen in cells expressing 81βH⁻, WT class II, or WT class II and Ii.

Loss of the 81βH⁻Ii Complex Occurs in the Endosomes. Because degradation of 81βH⁻ occurs late in biosynthesis after addition of N-linked glycans in the Golgi and because

our previous intracellular staining data indicates that 81βH⁻Ii accesses the endosomes (19), we hypothesized that the loss of class II molecules occurs when the 81βH⁻Ii complex reaches the endosomes. To test this, we used the weak base, NH_4Cl, to raise the pH of the endosomal compartments. In addition, because sulfhydryl proteases have been implicated in Ii degradation (26–32), we tested the effects of a sulfhydryl protease inhibitor, Z-phe-ala, on 81βH⁻Ii. Cells expressing 81βH⁻Ii were biosynthetically labeled overnight in the presence or absence of either NH_4Cl or Z-phe-ala, and class II immunoprecipitates were prepared. In Fig. 4 *A*, the short exposure clearly shows that the 81βH⁻Ii complex is preserved by NH_4Cl treatment. The same experiment (Fig. 4 *A*; *right*) shows that mature class II molecules in 81βH⁻Ii-expressing cells can also be preserved by a sulfhydryl protease inhibitor. Our results suggest that when 81βH⁻ has been transported with Ii into the endocytic compartments, the complex is degraded by endosomal proteases.

In Fig. 4 *B*, we compared the fate of 81βH⁻Ii with that of WTIi in the presence or absence of NH_4Cl. Ammonium chloride treatment profoundly preserved Ii in both cell lines, indicating successful neutralization of the endosomal compartment. In addition, the amounts of mature α and β chains are greatly increased in 81βH⁻Ii in the presence of NH_4Cl. We also observed some preservation of WT class II molecules by NH_4Cl. We conclude that the entire 81βH⁻Ii complex is degraded, whereas only a small amount of WTIi is degraded in the endocytic compartments upon proteolysis and release of Ii.

Finally, to show that the class II–Ii complexes in cells expressing either WTIi or 81βH⁻Ii access the same endosomal environment, we assayed for the Ii-derived p12 fragment in association with class II (Fig. 4 *C*, *p12*) (33, 34). This 12 kD degradation intermediate is derived from the amino terminus of Ii (aa 1∼102) and contains the transmembrane and CLIP region. Fig. 4 *C* shows that when similar amounts of class II are immunoprecipitated from 81βH⁻Ii and WTIi, comparable amounts of p12 are present. This result suggests that the pool of p12-associated class II molecules is the same in cells expressing either WTIi or 81βH⁻Ii. Taken together, the data in Fig. 4 suggest that the class II attrition observed in 81βH⁻Ii occurs endosomally but at a point after the Ii-p12–81βH⁻ complex is generated.

Incubation with Peptide Protects 81βH⁻ from Proteolysis and Increases the Amount of 81βH⁻ at the Cell Surface. Based on Figs. 1–4, we concluded that when the 81βH⁻Ii complex reaches the endocytic compartments, it is degraded. To explain why 81βH⁻ in association with Ii is susceptible to endosomal degradation, we hypothesized that after Ii is removed, 81βH⁻ is unable to productively bind peptides available in the endocytic compartment. In support of this hypothesis, we showed earlier that 81βH⁻ was unable to form SDS-stable dimers (20). In addition, when we compared the peptide binding capacity of the 81βH⁻ molecule to the WT A^d molecule in an in vitro translation system, we found that 81βH⁻ binds very poorly to most peptides. An exception was found, however, in a cys derived peptide (aa 40–55), which did bind 81βH⁻ (Wolf Bryant, P., H. Ploegh, and A.J. Sant, manuscript in preparation). This same cys derived peptide was one of the five predominant peptides eluted from I-A^d molecules purified from A20 cells (35).

To test whether the 81βH⁻ molecule is inherently more protease sensitive than WT class II molecules, we treated

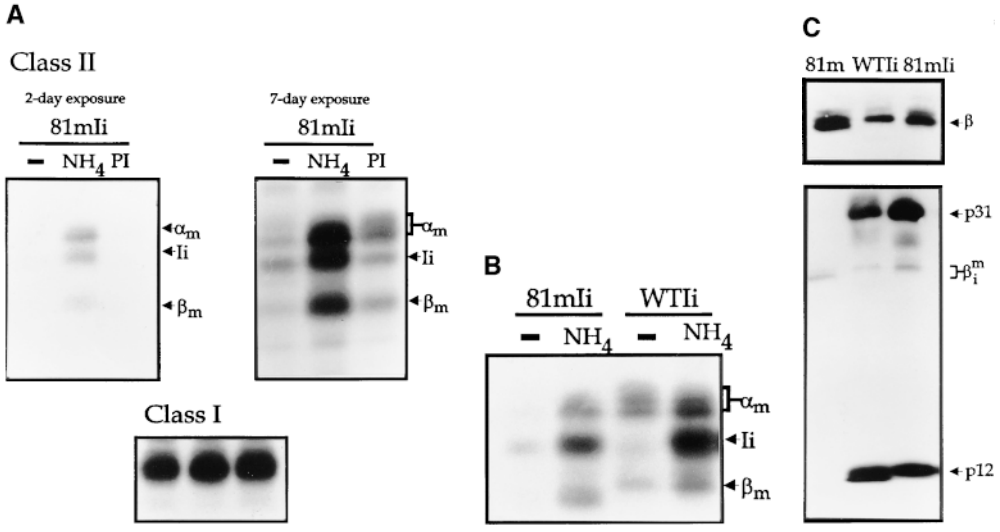

Figure 4. The 81βH⁻Ii complex is degraded in the endocytic pathway. (*A*) 81βH⁻Ii was labeled in [³H]leucine overnight in the absence (−) or presence (+) of NH_4Cl or the sulfhydryl protease inhibitor, Z-phe-ala (*PI*). The cells were immunoprecipitated sequentially with a mAb recognizing class I (16-1-11) (*bottom*) and then with M5114, a mAb that recognizes the class II β chain. This experiment is shown as two exposures of the autoradiograph: a 2-d exposure on the left and a 7-d exposure on the right. Mature α is indicated as α_m; Ii and β chain are also shown. (*B*) Cells expressing either 81βH⁻ or WTIi were labeled overnight with [³H]leucine in the absence (−) or presence (+) of NH_4Cl. Ii is preserved by NH_4Cl in both cells but mature α chain is profoundly rescued in 81βH⁻Ii. The β chain in 81βH⁻Ii migrates as a smaller size than WT because of the aa substitution at position 81. (*C*) 81βH⁻Ii and WTIi-expressing cells were lysed and immunoprecipitated with the rabbit antisera that recognizes the cytosolic tail of the class II β chain. The proteins were resolved on a 12.5% gel, transferred to nitrocellulose, and probed first for class II β chain with M5114 (*top*) and then for the amino terminus of Ii, with the rat mAb, In-1 (*bottom*). Immature β chain ($_i$) in 81βH⁻-expressing cells is indicated by the bottom of the bracket; mature β (^{m}) in 81βH⁻Ii and WTIi-expressing cells is indicated by the top of the bracket. The p31 form of Ii is also noted by the arrow.

intact cells with either the broadly reactive protease, PK or with a more restricted enzyme, trypsin, which cleaves after arginines and lysines (Fig. 5). Treatment with PK reduces surface 81βH$^-$ class II expression approximately twofold as detected by the monoclonal antibody MKD6 (Fig. 5 *A*, panel *1*), but had no effect on WT expression (Fig. 5 *A*, panel *3*). Treatment of 81βH$^-$ and WT A^d-expressing cells with trypsin gave a similar result (Fig. 5 *A*, panels *5* and *7*). These experiments support our hypothesis that the 81βH$^-$ molecule is protease sensitive compared with WT. Our data are in contrast to the inherent protease resistance of the class II molecule demonstrated by a number of groups in the early studies of Ii association with class II (36–39). These workers showed that protease treatment of class II–Ii complexes to release Ii left the class II molecules intact. One possible explanation for the observed protease sensitivity of 81βH$^-$ compared with WT, which we proposed earlier, is that 81βH$^-$ fails to acquire peptide, leading to enhanced protease sensitivity. We hypothesize that empty class II molecules are protease sensitive.

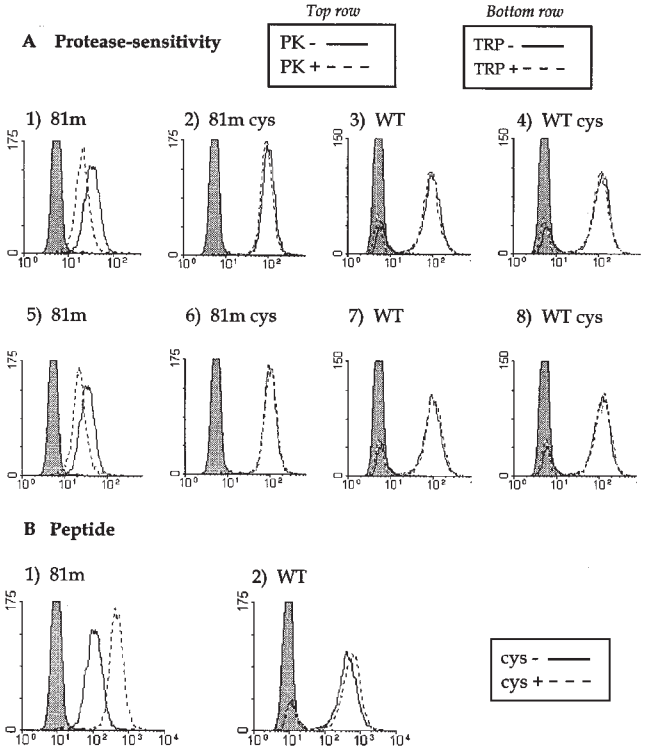

Figure 5. Protease sensitivity of 81βH$^-$. (*A*) 81βH$^-$ or WT A^d-expressing cells were incubated overnight with or without cys derived peptide and then treated with either PK (*panels 1–4*) or trypsin (*TRP*; *panels 5–8*). (*Panel 1*) 81βH$^-$-expressing cells stained with the second step goat anti–mouse FITC reagent alone (*shaded histogram*) or with the mAb, MKD6, which recognizes the murine A^d class II molecule (*solid line*). After PK treatment, the mean channel fluorescence of 81βH$^-$ is reduced from 130 to 70 (*dashed line*). The MKD6 staining of cells after protease treatment is shown as a dashed line; staining after mock treatment is shown as a solid line. (*Panel 2*) 81βH$^-$-expressing cells incubated overnight with cys derived peptide and then treated with or without PK. (*Panel 3*) WT A^d-expressing cells stained with MKD6 after treatment with or without PK. (*Panel 4*) WT A^d-expressing cells incubated overnight with cys peptide and then treated with or without PK. A subpopulation of the WT cells have lost expression of the A^d transgenes and therefore do not stain with the MKD6 mAb. These cells are overlapping with the GAM FITC control (*shaded histogram*). Panels 5–8 show the MKD6 staining of 81βH$^-$ or WT A^d-expressing cells incubated alone or with the cys peptide and then mock-treated (*solid line*) or treated with trypsin (*dashed line*). MKD6 staining of 81βH$^-$ expressing cells was reduced from a mean channel fluorescence of 130 to 76 by trypsin treatment (*panel 5*). (*B*) Peptide. Panel 1 shows the MKD6 staining of 81βH$^-$ incubated alone (*solid line*) or with cys peptide (*dashed line*). Panel 2 shows the MKD6 staining of WT Ad-expressing cells incubated alone (*solid line*) or with the cys peptide (*dashed line*). Staining of the cells with only the second step GAM FITC reagent is shown as a shaded histogram on the left.

To determine if 81βH$^-$ is protease sensitive because it is empty, we asked whether peptide loading could protect 81βH$^-$ from proteolysis. L cells expressing 81βH$^-$ were incubated overnight with the cys derived peptide and then treated with proteases. Strikingly, addition of cys peptide to 81βH$^-$-expressing cells not only protected the cells from protease treatment (Fig. 5 *A*, panels *2* and *6*), but also increased the amount of surface 81βH$^-$ expression (Fig. 5 *B*). Fig. 5 *B*, panel *1* shows that incubation of 81βH$^-$ with cys peptide increases the cell surface expression of 81βH$^-$ nearly fourfold, increasing the mean channel fluorescence from 130 to 530. This increase in surface expression of 81βH$^-$ after culture with peptide was detected by a panel of different mAbs (data not shown) indicating an overall increase in cell surface class II expression. In contrast, WT class II expression is increased only 1.3-fold by incubation with cys peptide (Fig. 5 *B*, panel *2*). These data support our model that the primary mechanism underlying the protease sensitivity of 81βH$^-$ is its underoccupancy by peptide. Because exogenous addition of a peptide able to bind 81βH$^-$, i.e., cys, confers protease resistance, we conclude that the 81βH$^-$ molecule is protease sensitive when it is underoccupied by peptide.

To show that the increase in cell surface 81βH$^-$ expression by peptide treatment is due to an increase in the yield of class II molecules rather than the restoration of mAb epitopes, we used a biochemical assay that did not rely on reactivity with mAbs. Cell surface molecules were biotinylated after three different culture conditions: cells expressing 81βH$^-$ incubated alone, with irrelevant peptide, AB, or with cys derived peptide. Immunoprecipitation with an anti–class I mAb shows that equal numbers of cells were biotinylated (Fig. 6 *A*). Class II molecules were isolated from the same lysates with a rabbit antiserum reactive with the cytoplasmic tail of the I-A class II β chain. When we compare 81βH$^-$ molecules on cells incubated alone (or with a control peptide) with those incubated with cys peptide, the amount of recovered 81βH$^-$ αβ dimers increases approximately three- to fourfold in the presence of cys peptide. We conclude that the increase in 81βH$^-$ detected both by several different mAbs (Fig. 5 and data not shown) and by cell surface biotinylation is not due simply to a subtle conformational change, but rather reflects an increase in the amount of 81βH$^-$ expressed at the cell surface.

81βH$^-$Ii Does Not Accumulate CLIP. The rapid degradation of 81βH$^-$Ii complexes in the endosomes predicts that either Ii digestion products such as CLIP do not re-

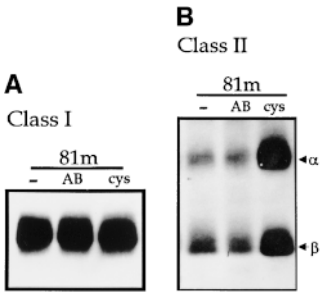

Figure 6. Incubation with cys peptide increases the yield of 81βH⁻ class II molecules at the cell surface. 81βH⁻ incubated alone or with AB peptide or cys peptide were cell surface biotinylated, lysed, and then immunoprecipitated sequentially with an anti–class I reagent (*A*) followed by a rabbit antisera raised against the cytosolic tail of class II I-A^d β chain (*B*). The α and β chains are indicated by the arrows. The Western blot was developed with streptavidin-HRP.

main associated with 81βH⁻ or that CLIP binding does not confer protease resistance to 81βH⁻. To explore the first possibility, we isolated class II molecules from WTIi and 81βH⁻Ii expressing cells in a pulse–chase experiment and looked for the appearance of the p12 fragment and CLIP (Fig. 7). After 2 h of chase, abundant levels of p12 are found associated with class II molecules in both WTIi and 81βH⁻Ii-expressing cells. After 3 h of chase, we can detect CLIP ahead of the dye front in cells expressing WTIi: however, there is no CLIP found associated with class II at any time points in 81βH⁻Ii-expressing cells. Since we cannot detect CLIP associated with 81βH⁻ either in a pulse–chase (Fig. 7) or in a continuous label (data not shown), we conclude that loss of the hydrogen bond at His 81 causes CLIP to rapidly dissociate from 81βH⁻. Exogenous addition of either human or murine CLIP peptides to 81βH⁻-expressing cells does not increase cell surface class II expression (data not shown). These data are consistent with our in vitro data indicating a failure of this molecule to stably associate with CLIP. Thus, we conclude that when 81βH⁻ reaches the endocytic pathway in association with Ii, the Ii molecule is removed by proteolysis, leaving a p12–class II complex. When this complex is further processed to CLIP–class II, CLIP rapidly dissociates, and the empty 81βH⁻ molecule is degraded.

Empty WT Class II Molecules Are Protease Sensitive. One issue raised by our preceding studies was whether the protease sensitivity of 81βH⁻ was due exclusively to its underoccupancy by peptide. The finding that peptide occupancy by a high affinity peptide confers protease resistance argues in favor of this conclusion. However, we wished to evaluate the protease sensitivity of WT I-A^d molecules to be able to generalize the conclusions we made. Empty soluble class II molecules have been shown by other workers to aggregate when they are in solution. This behavior complicates attempts to assess their protease sensitivity. Thus, we adopted an alternate strategy to examine empty WT A^d molecules. We constructed class II I-A^d molecules that would be tethered to the cell surface by glycan-phosphatidylinositol (GPI) linkage, contributed by the carboxy-terminal segment of the GPI-linked dimer HPAP. Davis and co-workers (21) have characterized I-E^k molecules constructed in such a way and conclude that these molecules

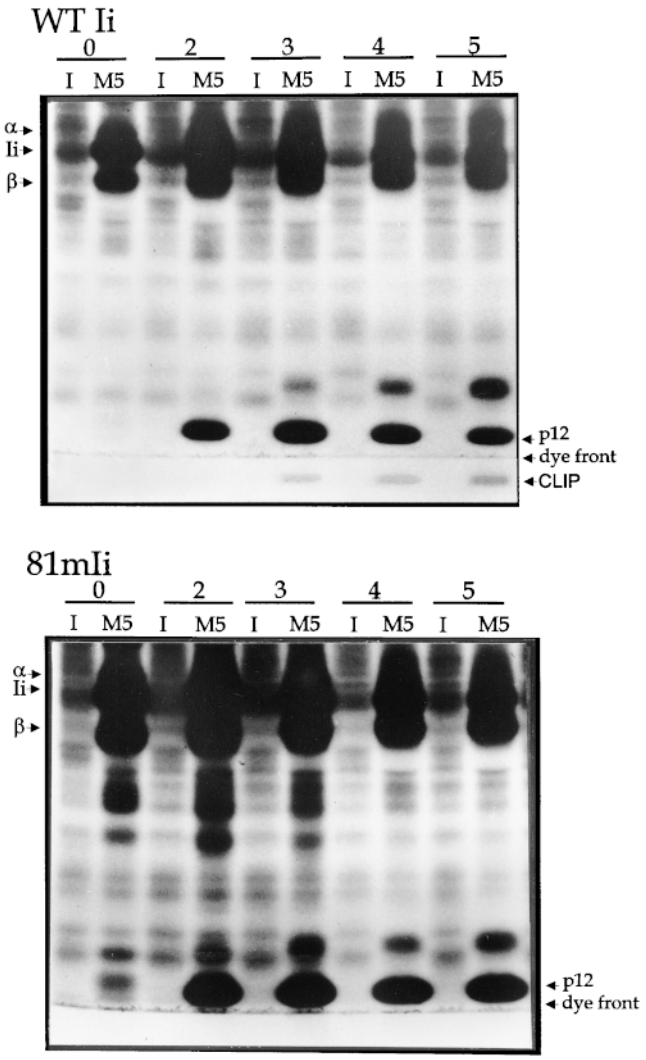

Figure 7. 81βH⁻Ii does not accumulate CLIP. Using a protocol adapted from reference 40, L cell transfectants expressing WT A^d and Ii (*top*) or 81βH⁻Ii were pulsed for 30 min with [^{35}S]methionine and then chased for 2, 3, 4, or 5 h. The cells were lysed and immunoprecipitated sequentially with ID4B followed by M5114. The proteins were resolved on a 12.5% polyacrylamide, reducing SDS gel that was run until the dye front was 2 inches from the bottom. The class II α and β chains and Ii are indicated on the left. CLIP runs just ahead of the dye front, both of which are indicated by arrows, as is the p12 fragment (*right*).

exist free of peptide at the cell surface. Recombinant I-A^d–GPI-linked dimers were engineered from I-E^k–HPAP by PCR and introduced into CHO cells. These molecules are released from the cell surface by phospholipase treatment. They do not allow presentation of antigen, but do present peptide (data not shown). Thus, we conclude that like their I-E counterparts, GPI-linked I-A^d molecules exist at the cell surface primarily in a form devoid of peptide.

When tested for their protease sensitivity (Fig. 8), GPI-linked I-A^d molecules were found to be sensitive to trypsin treatment (Fig. 8, *C*), a property protected by prebinding of peptide during overnight incubation (Fig. 8, *D*). Their protease sensitivity is comparable to 81βH⁻ (Fig. 8, *B*).

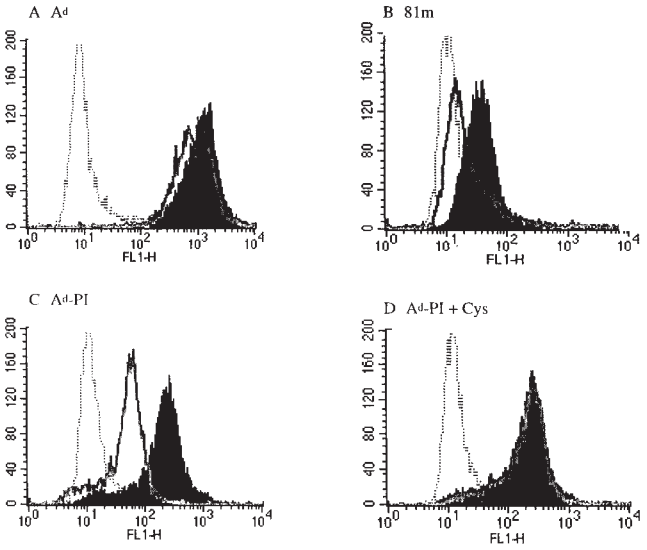

Figure 8. Empty WT class II molecules are protease sensitive. Genes encoding WT I-A^d (*A*) or a recombinant I-A^d molecule with the carboxy-terminal segments derived from the GPI-linked protein HPAP (*C* and *D*) were transfected into CHO cells. Cells were either cultured normally overnight (*C*) or cultured overnight with cys (*D*) peptide as described in Materials and Methods. Cells were harvested from culture and either left untreated (*filled profiles*) or treated for 0.5 h at RT with trypsin-EDTA (*gray lines*). Shown are the flow cytometry profiles of cells stained with the I-A^d specific antibody MKD6. Dotted lines represent background staining. L cells expressing 81βH$^-$ similarly treated are shown for comparison (*B*).

WT I-A^d with intact transmembrane segments expressed in CHO cells that presumably access peptide en route to the cell surface are not protease sensitive (Fig. 8, *A*). We conclude from these studies that peptide occupancy by class II is an essential feature to its well-known protease resistance.

Discussion

In this paper, we provide evidence that a class II molecule with a compromised ability to bind peptides is susceptible to proteolysis. A number of observations support this model. First, we observed that 81βH$^-$ in association with Ii is degraded in the endosomes. Then, we showed that the 81βH$^-$ molecule itself is protease sensitive and that addition of a cys derived peptide restores the protease-resistant character observed with WT A^d. These observations led us to propose that 81βH$^-$ is protease sensitive because it is empty. In support, earlier work in our lab showed that 81βH$^-$ was unable to form SDS-stable dimers, suggesting an inability to productively associate with peptide (20) and our more recent work showing that 81βH$^-$ displays greatly enhanced dissociation rates from peptides (McFarland, B., C. Beeson, and A.J. Sant, manuscript in preparation).

An important question raised by these studies is why changing a histidine to an asparagine at position 81 affects the ability of the class II molecule to associate with peptide. The histidine at position 81 of the β chain forms a hydrogen bond to the main chain of bound peptides (Fig. 1, *top*). This hydrogen bond is observed in three human MHC class II structures (14–16) and in the structures of murine MHC class II molecules (17, 18). Although in principle, asparagine also has a nitrogen that could form a similar hydrogen bond, the side chain of asparagine is predicted to be too short to reach the peptide backbone. All of the commonly observed rotamers of asparagine were compared to histidine in the modeling of the mutant. The asparagine rotamer with a similar orientation to the histidine was chosen to show that the shorter asparagine side chain cannot form a hydrogen bond with peptides without a large reorientation of the MHC alpha helix (Fig. 1, *bottom*). Our modeling shows that the asparagine would be too far from the peptide (~4.2 Å) to form a strong hydrogen bond. The simplest explanation for the structural consequences of the histidine to asparagine mutation is the loss of a specific peptide, class II hydrogen bond. The absence of this hydrogen bond may destabilize the binding of many peptides. Additionally, the hydrogen bond formed at position 81 may be an important first step for initiating peptide binding to the class II molecule, acting to position or dock the peptide before stable binding to the class II molecule occurs.

Additional data from both our lab and another lab support our conclusion that the hydrogen bonds between the class II molecule and peptide are important for the overall stability and integrity of the class II–peptide complex. Glimcher and co-workers demonstrated the importance of the aa's at the periphery of the class II peptide binding pocket for class II surface expression (41). They described a mutagenized murine B lymphoma that had lost cell surface A^d expression. They cloned and sequenced both the α and β chain genes and found only a single aa substitution at residue 82, changing an asparagine to a serine in the class II β chain. This single aa substitution resulted in a class II molecule (82m) which associated with Ii but was unable to access the cell surface, i.e., it was retained intracellularly. Using an L cell transfection system, we also found that coexpression of Ii reduces 82m expression at the cell surface (data not shown). In addition, 82m expressed at the cell surface in the absence of Ii is even more protease sensitive than 81βH$^-$ (data not shown). Our preliminary data also suggest very rapid endosomal degradation of 82m when it is expressed in association with Ii. Fig. 1 shows that the aa at position 82 of the β chain forms two hydrogen bonds with the peptide backbone. The Asn→Ser substitution at position 82 is predicted to eliminate both hydrogen bonds. Such a molecule may be even more impaired in its ability to bind or remain stably associated with peptide, which might explain why 82m is even more protease sensitive than 81βH$^-$.

The importance of these hydrogen bonds between conserved MHC residues and main chain atoms of the peptide has been examined for a class I–peptide complex. By substituting methyl groups for charged aa's in the peptide, Bouvier and Wiley showed that more energy was contributed by the hydrogen bonds than by the anchor residues of the peptide, suggesting that the hydrogen bonds play an important role in the stability of the complex (42). In agreement with this work, Hill and co-workers demon-

strated that a significant amount of free energy of binding arises from the hydrogen bonds formed between the class II binding site and the amide bonds of the ligand (43). In Fig. 1, aa's 53, 62, 68, 69, and 76 of the class II α chain are shown to form hydrogen bonds with the peptide backbone. Peccoud and co-workers substituted alanine at each of these positions in a class II A^k molecule and found that the ability to present peptides to a number of T cell hybridomas was detectable, although impaired. The most striking loss was observed when aa 62 was substituted (44). This work supports the idea that the hydrogen bonds formed between the class II molecule and peptide are very important and that some hydrogen bonds may be particularly critical for formation of stable peptide–class II complexes.

Our results showing the profound effects that follow from the loss in potential for a single hydrogen bond between peptide and MHC class II molecules are particularly interesting in light of the recent successful crystallization of the I-A^d molecule bound to antigenic peptides (18). The structure obtained shows that I-A^d achieves stable peptide binding with minimal pocket interactions between the class II molecule and the R groups of the peptide. The pockets within the binding groove of I-A^d appear to be either empty (P1 and P9) or only partially filled (P4). Thus, strong pocket interactions are not essential for stable peptide interactions to the class II molecules. In contrast, our results suggest that loss in potential for a single hydrogen bond can profoundly diminish the capacity of class II molecules to acquire peptide. It is not yet clear if the contribution of hydrogen bonds will be similarly great for those MHC class II peptide interactions that are characterized by strong pocket interactions. We are currently examining this issue.

Although the histidine at position 81 is highly conserved, there are some β chains which do not have a histidine at residue 81: H-2 $A\beta^u$ (I-A^u) and HLA DRw53. Both have a tyrosine at position 81, suggesting that the histidine is not required for a viable class II molecule. In describing the association of I-A^u and peptide, McConnell and Lee proposed that the tyrosine substitution at 81 would either delete the hydrogen bond or force a substantial shift in the peptide backbone around the P1 pocket (45). Based on our model that residue 81 is key for stable peptide association with class II, one might predict that class II molecules with a tyrosine at position 81 have evolved compensatory mechanisms for stable association with peptide. In support of this hypothesis, there are aa substitutions found at positions in the I-A^u molecule that are not present in other known I-A alleles.

Alternatively, it is interesting to consider the possibility that a higher proportion of I-A^u molecules may be empty. It is well documented that I-A^u has a low affinity for the immunodominant epitope of myelin basic protein, Ac1-9, which is encephalitogenic in H-2^u mice (46–48). The low affinity of I-A^u for Ac1-9 is thought to contribute to disease onset because autoreactive T cells escape self-tolerance in the thymus. One might predict that substituting a histidine at position 81 of I-A^u would result in a higher affinity for Ac1-9, and perhaps other peptides that interact with this class II molecule.

In conclusion, we describe here a class II molecule, $81\beta H^-$, that is unable to remain associated with peptide because a hydrogen bond has been altered. When $81\beta H^-$Ii complexes access the endosomes in the presence of Ii, they are degraded—presumably because the CLIP peptide immediately dissociates after removal of Ii and no other self-peptides are able to bind $81\beta H^-$. From our data, as well as the work of others (10–12), we propose that empty class II molecules in the endocytic pathway are degraded. For this reason, the cell has two chaperones to orchestrate continuous groove occupancy of the class II molecule, Ii and DM. Ii directs the class II–Ii complex to the late endosomal compartments via the strong sorting signal in its tail. Under the acidic, proteolytic conditions of the endosomes, Ii is degraded whereas the class II molecule bound by CLIP is protected. At this point, the second chaperone, DM, facilitates the exchange of CLIP for antigenic peptides. However, there are some class II molecules on which CLIP has a very fast off-rate, specifically I-A^k, I-E^d, and I-E^k (49). The existence and survival of such molecules suggest that the critical function of DM is not to remove CLIP, but rather to load peptide onto empty class II molecules which would be susceptible to proteolysis. Hence, we propose that the cooperative function between Ii and DM may be to insure continuous groove occupancy of the class II molecule by peptide.

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