

The Invariant Chain Is Required for Intracellular Transport and Function of Major Histocompatibility Complex Class II Molecules

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Summary

The major histocompatibility complex (MHC) class II-associated invariant chain (Ii) is thought to act as a chaperone that assists class II during folding, assembly, and transport. To define more precisely the role of Ii chain in regulating class II function, we have investigated in detail the biosynthesis, transport, and intracellular distribution of class II molecules in splenocytes from mice bearing a deletion of the Ii gene. As observed previously, the absence of Ii chain caused significant reduction in both class II-restricted antigen presentation and expression of class II molecules at the cell surface because of the intracellular accumulation of α and β chains. Whereas much of the newly synthesized MHC molecules enter a high molecular weight aggregate characteristic of misfolded proteins, most of the α and β chains form dimers and acquire epitopes characteristic of properly folded complexes. Although the complexes do not bind endogenously processed peptides, class II molecules that reach the surface are competent to bind peptides added to the medium, further demonstrating that at least some of the complexes fold properly. Similar to misfolded proteins, however, the α and β chains are poorly terminally glycosylated, suggesting that they fail to reach the Golgi complex. As demonstrated by double label confocal and electron microscope immunocytochemistry, class II molecules were found in a subcompartment of the endoplasmic reticulum and in a population of small nonlysosomal vesicles possibly corresponding to the intermediate compartment or *cis*-Golgi network. Thus, although α and β chains can fold and form dimers on their own, the absence of Ii chain causes them to be recognized as "misfolded" and retained in the same compartments as bona fide misfolded proteins.

MHC class I and II molecules bind peptide antigens and present them to T cells (1-4). Whereas class I molecules predominantly bind peptides generated from endogenously synthesized cytosolic proteins before their release from the endoplasmic reticulum (ER)¹ (4-6), class II MHC molecules present internalized processed antigen (7-9).

Class II molecules are expressed on the cell surface as heterodimers composed of 34-kD α and 28-kD β chains (10). The α and β chains also interact with a third glycoprotein, the invariant chain (Ii), after their translocation into the ER (11, 12). Recently, it has been shown that the Ii and $\alpha\beta$ heterodimers form a nonamer structure consisting of three Ii and three $\alpha\beta$ heterodimers before their exit from the ER (13). The $(\alpha\beta Ii)_3$ complex is then transported through the Golgi

apparatus to an acidic compartment where the Ii is proteolytically removed before class II expression on the cell surface (11, 14).

Current models suggest that Ii may have several functions in class II-restricted antigen presentation. First, Ii may serve as a chaperone that plays an essential role in the initial folding and oligomerization of newly synthesized α and β chains in the ER. Second, Ii chain may act to target the transport of class II molecules from the ER to the *trans*-Golgi and then to an endosomal compartment where the complexes encounter exogenous proteins that have been internalized and processed to peptide antigens (4, 15). In support of this possibility is the recent finding that the Ii cytoplasmic domain may contain a specific endosomal targeting or retention signal (15, 16). Third, Ii may prevent the premature association of peptides with "empty" class II molecules while in transit to endosomes (17-20). Evidence to support this possibility comes from examination of the peptides bound *in vivo* to HLA-DR $\alpha\beta$ and $\alpha\beta Ii$ complexes. Whereas peptide fragments were

¹ Abbreviations used in this paper: CGN, *cis*-Golgi network; EM, electron microscopy; ER, endoplasmic reticulum; Ii, invariant chain; PAS, protein A-Sepharose; TGN, *trans*-Golgi network.

found to be associated with purified $\alpha\beta$ dimers, no peptides were detected with $\alpha\beta$ Ii complexes (19, 20). Similarly, free peptides have been shown to bind only to $\alpha\beta$ and not to $\alpha\beta$ Ii complexes (19, 20). By affecting the folding, assembly, and competence for peptide binding or intracellular transport of class II molecules, Ii directly affects class II-restricted antigen presentation.

Attempts to define the precise function or functions of the Ii by analyzing the behavior of class II molecules with or without Ii in transfected cells have yielded conflicting results depending on the type of cells transfected and/or the protein antigens tested (17, 21–24). More recently, it has become possible to analyze the role of Ii chain in authentic class II-positive cell types by using homologous recombination to delete the Ii chain gene in murine embryonic stem cells (25, 26). Deletion of the Ii chain gene was found to greatly diminish the ability of splenic B cells to present exogenous antigen in a class II-restricted fashion in vitro and to reduce the maturation of CD4⁺ T cells in vivo. These effects reflected an inability of class II molecules to acquire antigenic peptides from endogenously processed proteins. Surface expression was also markedly reduced, with class II molecules accumulating intracellularly early in the secretory pathway. However, the actual site of accumulation remains to be established. Moreover, it is unclear whether intracellular accumulation of the α and β chains reflected a role for Ii chain in folding and assembly of newly synthesized class II molecules or a role in facilitating the transport of properly assembled α/β dimers.

In this paper, we examine in greater detail the mechanisms responsible for the cellular phenotype associated with deletion of the Ii chain gene. Using a series of independently constructed recombinant mice, we have performed double label immunocytochemistry to precisely define the site at which α and β chains accumulate in the absence of Ii chain. Moreover, we have analyzed the kinetics of synthesis, assembly, and transport of MHC class II molecules in these mice. Our results show that although the α and β chains are capable of significant assembly and folding, they are largely retained by the quality control system of the ER/intermediate compartment/cis-Golgi network (CGN) as typical "misfolded" proteins. Since the class II molecules also fail to acquire endogenous peptides, this defect is more likely to reflect their inability to be delivered to the intracellular site(s) where suitable peptides are encountered, as opposed to a direct role of Ii chain in regulating assembly or peptide binding.

Materials and Methods

Gene Targeting and Embryonic Stem Cells. A 2.1 kb BglII-EcoRI fragment encoding the promoter and exon 1 was subcloned into pSK Bluescript (Stratagene, La Jolla, CA). The internal 0.4 kb StuI fragment was deleted and the neomycin resistance cassette (pMC1neo PolyA, Stratagene) was introduced in place of the StuI fragment. An additional 3.0-kb EcoRI-KpnI fragment encoding exons 2 and 3 was added to the 3' end of the BglII-EcoRI subclone. Finally, two copies of the HSV-1 thymidine kinase gene were subcloned into the NotI polylinker site 5' of the Ii gene. ES-D3 cells (10⁷) in 0.7 ml of media were transfected with 25 μ g of NotI linearized DNA with a gene pulser (25 μ F, 0.32 kV; Bio-Rad Laboratories, Cambridge, MA). After electroporation, the cells were plated on

10 100-mm plates in media (DMEM with 15% FCS, 2 mM glutamine, and 0.1 mM 2-ME) supplemented with leukemia inhibitory factor (Gibco BRL, Gaithersburg, MD). Media with geneticin (170 μ g/ml; Gibco BRL) and gancyclovir (2 μ M; Syntex, Palo Alto, CA) was added 24 h after transfection. All transfectants were maintained on a monolayer of 1^o embryonic fibroblasts during the 10-d drug selection. Genomic DNA was purified from individual clones and analyzed by Southern blot with a 5' probe (see *solid box*, Fig. 1 A). This probe is a 0.6-kb BglII-XbaI fragment from the 5' end of the Ii gene. It hybridizes to a 3.0-kb EcoRI fragment of the endogenous Ii gene and to a 2.5-kb fragment of the disrupted allele.

Cell Surface Expression by Flow Cytometric Analysis. 5 \times 10⁵ freshly prepared splenocytes were incubated on ice with saturating amounts of biotin-conjugated anti-I-A^b antibodies for 20 min in staining buffer (PBS with 1% FCS) in a total volume of 100 μ l. The wells were washed twice with staining buffer and incubated with the appropriate avidin FITC-conjugated second step reagent. Finally, the cells were washed, fixed with 1% paraformaldehyde, and analyzed by flow cytometry on a FACS Star[®] (Becton Dickinson & Co., Mountain View, CA). The primary Ab Y-Ae was generously provided by Dr. Charles Janeway. M5/114 (27, 28) was from American Type Culture Collection (Rockville, MD) and AF6-120.1 was from PharMingen (San Diego, CA).

Pulse-chase Labeling. Before radiolabeling, Ii⁺ or Δ Ii cells were incubated for 1–2 h in methionine-deficient α -MEM supplemented with 5% dialyzed FCS. The cells were then pelleted and resuspended in the same medium at 4 \times 10⁷/ml and metabolically labeled by a 20-min pulse incubation with 1 mCi/ml [³⁵S]methionine trans-label (ICN Biomedicals Inc., Costa Mesa, CA). After the pulse, the cells were pelleted, unincorporated radiolabel removed by washing, and the cells were resuspended at 2 \times 10⁶ cells/ml in ice-cold (0 chase) or prewarmed α -MEM containing 5% FCS. The cells in the prewarmed media were then incubated for 0.5, 1, 2, 4, or 6 h at 37°C in a 5% CO₂ atmosphere.

Immunoprecipitations. After the pulse-chase, the cells were pelleted and lysed in 0.5% Triton X-100, 300 mM NaCl, 50 mM Tris, pH 7.4, 1 mM PMSF, leupeptin, 10 μ g/ml aprotinin, 10 μ g/ml pepstatin, 10 μ g/ml chemostatin, and 20 mM N-ethylmaleimide (NEM). Nuclei and debris were eliminated by centrifugation. The cell lysates were precleared first, by an incubation with rabbit anti-mouse IgG (Zymed Laboratories, S. San Francisco, CA) and Staph A (Pharmacia, Piscataway, NJ) overnight at 4°C, second by a 1-h incubation with Staph A, and finally, 1 h with protein A-Sepharose (PAS) at 4°C. MHC class II molecules were then precipitated by incubating the lysates for 1 h at 4°C with PAS which had been previously bound to either goat anti-rat IgG and M5/114 mAb or to Y3P mAb. PAS/Ab/class II aggregates were washed three times in 0.5% NP40, 30 mM NaCl, 50 mM Tris, pH 7.4. The beads were then resuspended in Laemli sample buffer containing 3% SDS and either incubated for 1 h at room temperature or boiled for 3 min.

The immunoprecipitates were analyzed by electrophoresis using 11% SDS-PAGE mini-gels (Hoefer Scientific Instruments, San Francisco, CA). The gels were treated with salicylate, dried, and exposed to autoradiography film (Eastman Kodak Co., Rochester, NY) and quantified by image digitization using an image processor (Bio-Image 2000; Eastman Kodak Co.).

Confocal Immunofluorescence Microscopy. Splenocytes were treated for 48 h with 10 μ g/ml LPS (Sigma Chemical Co., St. Louis, MO). After stimulation, cells were washed twice, attached to Alcian blue-coated cover slips, fixed with 2% formaldehyde, and permeabilized with PBS containing 0.1% gelatin and 0.01% saponin. The cells were stained with primary Ab and affinity-purified secondary Ab and then imaged on a confocal microscope (model MRC600;

Bio-Rad Laboratories). The primary Abs used were, rabbit anti-serum raised to affinity-purified I-A^d (Rb anti-I-A^d, a generous gift of Dr. Ralph Kubo), the FcγRII-specific mAb 2.4G2 (29), the Ii-specific mAb In-1 (a generous gift of Dr. Jim Miller) (30), anti-Igp110 mAb GL2A7 (31), rabbit antimannosidase II sera (a generous gift of Dr. Kelley Moremen) (32), rabbit anti-TGN-38 (33) (a generous gift of Dr. George Banting), and an anti-BiP mAb (34) (a kind gift of Dr. David Bole).

Electron Microscopy Immunocytochemistry Using Ultrathin Frozen Sections. Since the morphology of isolated splenocytes was judged to be inferior, all immunoelectron microscopy was performed using sections prepared directly from intact spleens. Organs were fixed by in situ perfusion using 4% paraformaldehyde in PBS, minced, and processed for ultracryomicrotomy by established methods described in detail elsewhere (35, 36). Briefly, tissue pieces were cryoprotected in 2.1 M sucrose in PBS for at least 1 h, mounted onto specimen pins, and frozen by immersion in liquid nitrogen. Tissues that did not hold together well during manipulation were embedded in 10% gelatin before cryoprotection and freezing. Ultrathin sections for electron microscopy (EM) were cut at -100°C using a Leica/Reichert Ultracut-FC4 system (Leica, Deerfield, IL) with either a diamond or a glass knife. Sections were retrieved from the dry surface of the knife with a drop of 2.3 M sucrose and transferred onto formvar-carbon coated copper or nickel grids (Gilder grids; Electron Microscopic Supplies, Ft. Washington, PA) and immunolabeled using previously described procedures (35). The sections on the grids were then washed with distilled water, floated for 10 min on ice-cold drops of 1.8% aqueous methyl cellulose containing 0.3% uranyl acetate, and finally dried in a film of methyl cellulose-uranyl acetate mixture. The sections were examined and electron micrographs taken using an electron microscope (model EMM410; Philips Electronic Instruments Inc., Mahway, NJ) operating at 80 kV.

Results

Generation of Invariant Chain-deficient Mice. A germline transmitting line of mice deficient in expression of Ii was generated using gene targeting and embryonic stem cell technology. A total of 10⁷ ES-D3 cells were electroporated with a gene targeting construct, pliKO (Fig. 1 A). After selection, 1.4 × 10³ G418-resistant colonies were recovered; of these, 3 × 10² transfectants were also resistant to gancyclovir. A total of 60 doubly resistant clones were tested by Southern blot analysis. Two clones were identified as having incorporated the targeting vector by homologous recombination. Both clones were tested with a series of restriction enzymes to confirm that they contained a single insertion of the targeting construct. One clone, containing a disrupted Ii allele, was injected into C57BL/6 blastocysts. A total of eight male chimeras were born and then bred. One of the male chimeras transmitted the ES cell genotype to 100% of its offspring. As determined by Southern blot analysis of tail DNA (Fig. 1 A), approximately one half of the offspring contained one disrupted Ii allele (+/-). Of the F2 animals derived from heterozygous brother × sister mating, one quarter of the progeny were homozygous for the Ii gene mutation (-/-).

To confirm the loss of Ii protein expression, we examined LPS-treated splenocytes from heterozygous (Ii⁺), or mutant (ΔIi) mice by immunofluorescence confocal microscopy using

the Ii-specific Ab, In-1 (30). As shown in Fig. 1 B (In-1), the Ii⁺ splenocytes expressed normal levels of Ii. As expected, no Ii expression was detected either on the plasma membrane or in any intracellular compartments in LPS-treated splenocytes from the ΔIi mice. These results establish that the deletion of exon 1, of the Ii gene, results in the loss of protein expression.

Ii Gene Disruption Leads to Decreased Expression of Class II Molecules on the Cell Surface. To determine whether the loss of Ii expression had any effect on the expression of class II α and β chains, we next performed flow cytometric analysis of splenic lymphocytes. As detected by a panel of mAbs specific for either Aβ^b or Aα^b, or a polyclonal antisera, splenocytes from the ΔIi mice exhibited a 5–10-fold reduction in the level of class II staining on the plasma membrane (Fig. 2, A and B, Ii⁺, dashed line; ΔIi, solid line). Since the Abs used (M5/114 and a polyclonal anticlass II Ab) do not distinguish different conformations of class II molecules (17, 37), the observed decrease in staining was likely to reflect an actual decrease in number of cell surface class II epitopes. This was surprising given suggestions that the Ii is not required for cell surface expression of class II molecules in transfected fibroblasts (18, 23, 37), but is in accord with transfection experiments that have implied a role for Ii in exit from the ER (12, 15, 17), and is also in agreement with the results obtained for other ΔIi mice (25, 26).

The ability of the Ii⁺ and ΔIi splenocytes to process/present intact Eα-containing fusion protein versus exogenously added Eα peptide was examined by antigen presentation assays in vitro. Ii⁺ cells were proficient at presenting both exogenous peptide as well as intact protein antigen. In contrast, APCs from our Ii KO mice showed a defect in their ability to present intact protein while being proficient at presenting exogenous peptide antigens (data not shown). Since both live and fixed splenocytes were capable of presenting peptide, these results demonstrate that those class II molecules that reach the plasma membrane are capable of productively binding antigenic peptide despite the absence of Ii chain.

Moreover, it was apparent that a significant fraction of class II molecules on the surface of ΔIi splenocytes were “empty”, i.e., devoid of bound peptide. We compared the ability of Ii⁺ and ΔIi splenocytes to bind an Eα peptide by measuring cell surface staining with Y-Ae after incubation with Eα peptide. Y-Ae is a monoclonal alloantibody that detects a determinant expressed on a subset of class II I-A^b molecules when complexed with an Eα-derived peptide Eα56-73 (38–40).

ΔIi and Ii⁺ splenocytes were incubated with Eα peptide for 4 h and then stained for the Y-Ae determinant. Both Ii⁺ (Fig. 3, dashed line) and ΔIi (Fig. 3, solid line) cells expressed the Y-Ae epitope. However, the ΔIi cells showed a substantially higher degree of staining with Y-Ae Ab than did the Ii⁺ cells which is striking given that the ΔIi cells actually express 5–10-fold fewer class II molecules on their cell surface than the Ii⁺ cells. This finding is consistent with the prediction that a greater fraction of surface class II molecules on ΔIi cells were empty and therefore capable of binding a greater amount of Eα peptide. This suggestion is also consistent with the antigen presentation results in which the ΔIi

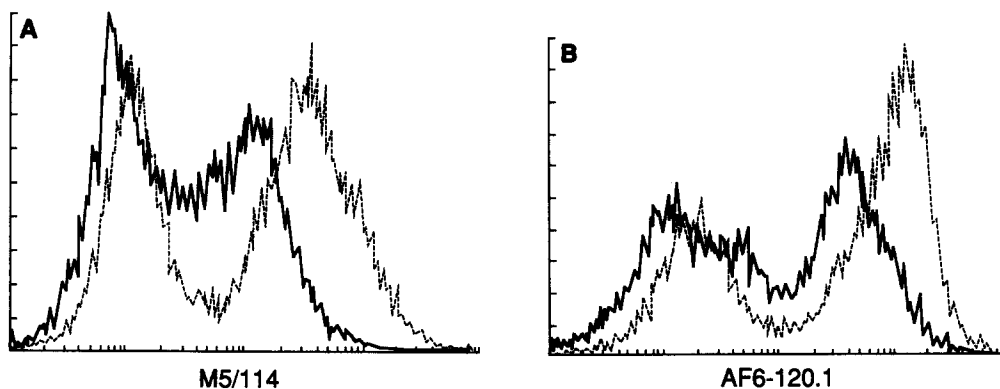


Figure 2. Flow cytometric analysis of MHC class II expression on splenocytes of Ii -deficient mice. Splenic single cell suspensions were made from individual Ii^+ (dashed line) and ΔIi (solid line) animals. 5×10^5 cells were stained with class II-specific Abs M5/114 (A) and AF120.6 (B). In addition to M5/114 and AF120.6, other class II-specific Abs (Y3P, Y248, Y219, Y237, and a polyclonal rabbit anti- $I-A^d$) (40) were used to stain splenocytes. All Abs tested gave similar results.

a more complete kinetic analysis of the synthesis and assembly of class II molecules in the presence or absence of Ii chain.

Splenocytes were briefly pulse labeled (20 min) in [^{35}S]methionine and then chased in medium containing excess unlabeled methionine for 0.5–6 h. At various time points, cells were harvested and class II molecules immunoprecipitated using mAbs that selectively detect either total or assembled complexes. Immunoprecipitates were analyzed by SDS-PAGE under nonreducing conditions with or without boiling before electrophoresis, to determine the rate at which peptide loaded, and thus SDS stable, complexes formed.

In agreement with previous results (25, 26), splenocytes from Ii -deficient (ΔIi) and Ii -heterozygous (Ii^+) mice appeared to synthesize comparable amounts of class II molecules. As can be seen in Fig. 4 A (boiled), similar amounts of β chain were immunoprecipitated from Ii^+ and ΔIi cells using M5/114, a mAb that detects all conformations of the $I-A^b$ β chain (27, 28). Moreover, the fact that M5/114 coprecipitated α chain from both cell types indicated that a significant fraction of the newly synthesized class II molecules formed dimers shortly after synthesis in both Ii^+ and ΔIi cells. The α chain band appeared denser than the β chain band in the immunoprecipitates from the Ii^+ cells because newly synthesized Ii chain comigrates with α chain under the conditions used for these experiments.

Interestingly, in the ΔIi cells most of the α/β dimers were found in a high molecular weight complex (Fig. 4 A, top). Such aggregates are characteristic of many misfolded proteins and indicate noncovalent and disulfide-stabilized interactions

among newly synthesized proteins together with components of the ER lumen that are thought to be involved in protein folding (41, 42). Indeed, it appeared that the high molecular weight complex was stabilized by both covalent and noncovalent interactions. Although the α and β chains were partly dissociated by boiling, a significant fraction of the labeled class II molecules remained associated with a narrow band of ~ 250 kD even after treatment at 100°C (Fig. 4 A). Since all procedures were performed under nonreducing conditions, this was likely to reflect the existence of disulfide bonds between at least the β chain and itself and/or with resident ER components.

In Ii^+ cells, only a small fraction of the α/β dimers were detected in high molecular weight complexes although a significant but minor amount of newly synthesized molecules were transiently associated with a band of 90 kD at early time points (Fig. 4 A, bottom). After 1–2 h, however, an increasing fraction of α and β chains migrated as a band of 52–55 kD ($c[\alpha/\beta]$, Fig. 4). By 6 h, this fraction accounted for >80% of the total immunoprecipitable protein. Since this band was completely sensitive to boiling in SDS, it was indicative of “compact” peptide-loaded α/β dimers (28, 43, 44). Compact dimers were not observed at any time point in the ΔIi cells, indicating that although α/β dimers could form in the absence of Ii chain, these dimers did not productively bind peptide. Interestingly, two polypeptides of 6–8 and 12 kD were present in the Ii^+ precipitates after boiling, which might represent Ii fragments, since they were not present in the ΔIi precipitates.

The fact that a large fraction of α and β chains entered a high molecular weight aggregate in the absence of Ii chain suggested that the quality control system of the ER recognized these chains as misfolded proteins. This represented something of a paradox since newly synthesized α and β chains were correctly folded at least to the extent that they could form stable α/β dimers. To further investigate the degree to which α and β chains could fold and assemble in the ΔIi splenocytes, we next analyzed their reactivity with another β chain-specific mAb, Y3P, which recognizes a conformation-dependent epitope. Y3P precipitates primarily $I-A^b$ α/β -dimers, including those already bound to peptide (28, 45). As found using the conformation-independent Ab M5/114, Y3P precipitated both α and β chains from ΔIi splenocytes

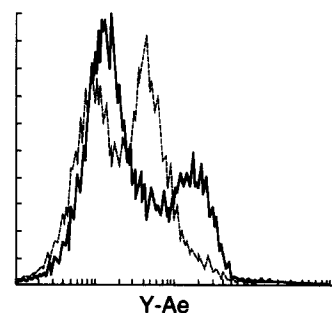


Figure 3. Enhanced peptide binding to ΔIi -cells. Flow cytometric analysis of Y-Ae expression on splenocytes of Ii -deficient mice pulsed for 4 h with E α 56-73 peptide. 5×10^5 splenocytes were stained with the alloantibody Y-Ae after incubation in the presence or absence of E α peptides. Background staining was observed for both the wild-type and mutant cells in the absence of peptide (data not shown). (Dashed line) Ii^+ ; (solid line) ΔIi .

A

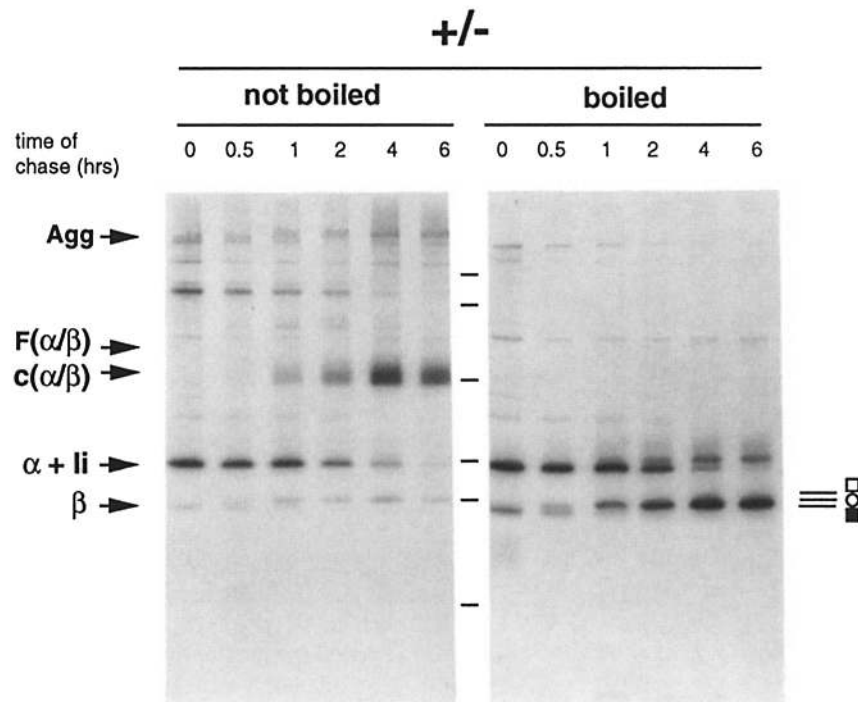
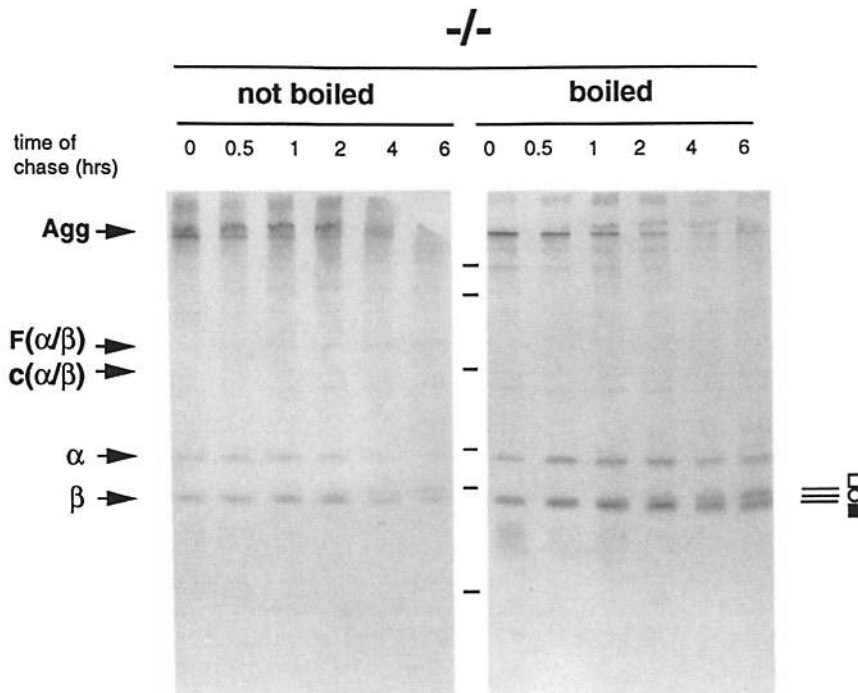
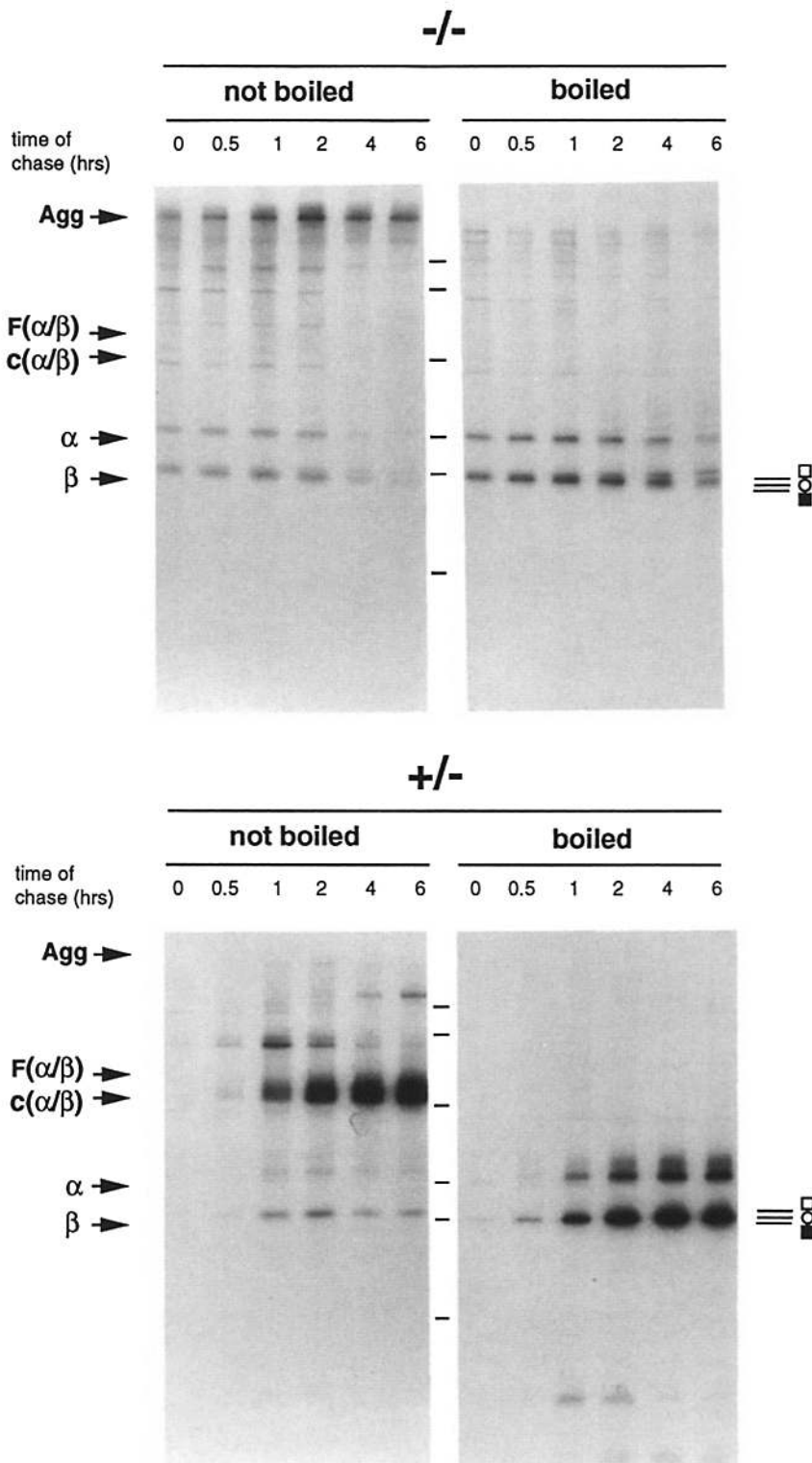


Figure 4. Kinetics of SDS-stable complex formation. Cytoplasmic extracts were prepared from spleen cells pulse labeled for 20 min. Class II molecules were immunoprecipitated with either M5/114 (*A*) or Y3P (*B*). Different glycosylation intermediates are also shown. (○) Core glycosylated; (◼) trimmed intermediate; (◻) terminally glycosylated. Molecular weight markers are indicated by the six horizontal lines between the “not boiled” and “boiled” gels for both $-/-$ and $+/-$ in *A* and *B*. As shown, the markers indicate the migration of the 106, 80, 49.5, 32.5, 27.5, and 18.5 kD, respectively.

at all time points (Fig. 4 *B*, top). This provided further confirmation that properly assembled α/β dimers can form in the absence of Ii chain (Fig. 4 *B*, top). Whereas the dimers were also largely found in a high molecular weight aggregate, the aggregate detected by Y3P was completely sensitive to boiling. Thus, Y3P only recognized the β chains that

formed noncovalent aggregates, suggesting that the covalent aggregates consist only of misfolded β chains that cannot be detected by this conformation-specific Ab.

As expected, in lysates from Ii^+ splenocytes, Y3P detected peptide-loaded compact α/β dimers (Fig. 4 *B*, bottom). These were found after 1 h of chase and thus appeared at the same

B

kinetics as compact dimers detected using the conformation-independent Ab M5/114. Since only "empty" heat-labile dimers were detected by YP3 at early time points in the ΔIi cells (Fig. 4 B, top), the Ab is not specific for peptide-loaded dimers, as has been suggested previously (28, 45). Interestingly, however, YP3 failed to immunoprecipitate any class

II molecules at earlier time points in the Ii^+ cells (Fig. 4 B, bottom), suggesting that YP3 cannot detect dimers that are still complexed with Ii chain.

Class II Molecules Are Not Terminally Glycosylated in the Absence of Ii Chain. It was also evident from Fig. 4 that although newly synthesized α and β chains formed dimers in

the absence of Ii chain, they inefficiently received terminal sugar modifications characteristic of traversal through the Golgi complex. In Ii⁺ cells, the immature high mannose form of β chain (Fig. 4, *open circles*) was rapidly converted to a lower mobility terminally glycosylated protein (Fig. 4, *open squares*). These events were initiated within 0.5 h of chase and involved the majority of newly synthesized β chain, whether immunoprecipitated with M5/114 (Fig. 4 A) or Y3P (Fig. 4 B). Similar alterations were exhibited for α chain, but its comigration with Ii chain made interpretation more difficult.

In contrast, terminal glycosylation of β (and α) chain in Δ Ii splenocytes was both slower and far less efficient. Although a significant fraction of newly synthesized β chain was converted to the higher mobility trimmed form (Fig. 4, *closed squares*), this was not evident until 2 h of chase. Only a small amount of β chain migrated as slower mobility terminally processed molecules (Fig. 4, *open squares*), and this required chase times of 4 h (Fig. 4, A and B).

Together, these results indicate that Ii chain was required to enable rapid and efficient modification of N-linked sugars. Presumably, this reflects the inability of α/β dimers associated with high molecular weight aggregates to be efficiently transported from the ER to the Golgi complex. Whereas trimming might reflect exposure to mannosidase I which is present in "early" Golgi compartments (e.g., CGN), terminal processing requires exposure to medial cisternae and the *trans*-Golgi network (TGN) (46).

Intracellular Localization of Class II Molecules in Δ Ii Splenocytes. To establish the intracellular distribution of class II molecules in Δ Ii splenocytes, we next performed double-label immunocytochemistry at the levels of both fluorescence and electron microscopy. For scanning laser confocal microscopy, splenocytes were permeabilized with saponin and stained with polyclonal or monoclonal (M5/114) anticlass II Abs and a monoclonal anti-Fc γ RII which is known to label the cell surface of lymphocytes. Strong plasma membrane staining was observed in splenocytes from Ii⁺ cells when stained with either the class II or Fc γ RII Ab (Fig. 5 A; the orange/yellow color of the merged confocal images indicates colocalization of both patterns on the cell surface). In contrast, plasma membrane staining was only observed for Fc γ RII (Fig. 5 red) in Δ Ii splenocytes, with staining for class II molecules appearing as a concentric ring contained within the FcR surface staining (Fig. 5 B).

To examine the subcellular distribution of class II molecules relative to the Ii chain, in the Ii⁺ cells, splenocytes were double labeled for class II and Ii (Fig. 5, C and D). In Ii⁺ splenocytes (Fig. 5 C), a ring of plasma membrane staining for class II molecules (*green*) was observed that encircled the intracellular staining of Ii chain (*red*). A small amount of colocalization between Ii and intracellular class II was also observed. In the double-labeled Δ Ii splenocytes (Fig. 5 D), an intracellular staining pattern for class II was again observed. As expected, no Ii staining was seen above background.

To define the intracellular structures containing class II mol-

ecules in Δ Ii cells, we next performed a series of double labeling experiments using Abs that selectively detect specific organelles. The involvement of the endocytic pathway was first analyzed by labeling cells for class II and proteins known to be localized to endosomes and/or lysosomes. Intracellular class II was not found to colocalize with Lgp110, a marker for late endosomes and lysosomes (Fig. 5, E and F), nor with functional markers for early endosomes and recycling vesicles such as transferrin receptor or internalized complexes of membrane Ig and anti-Ig (data not shown). Thus, the internal class II molecules in the Δ Ii splenocytes did not accumulate in organelles of the endocytic pathway.

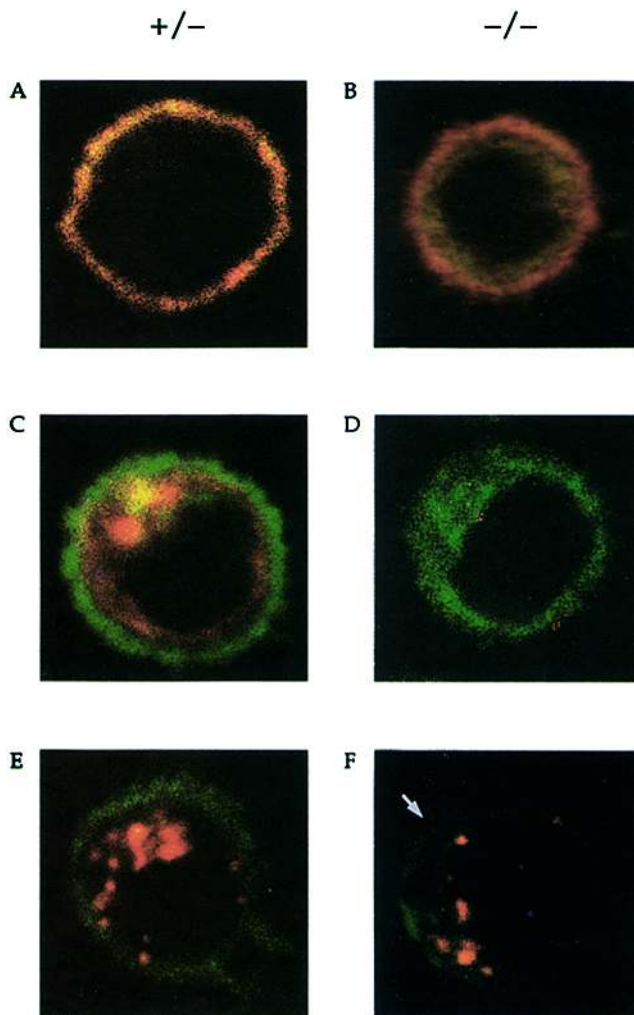


Figure 5. Distribution of class II molecules visualized by double label confocal immunofluorescence microscopy. LPS-stimulated splenocytes were double stained with: (A and B) class II mAb M5/114 (*red*) and Fc γ RII (*green*); (C and D) polyclonal class II antisera (*green*) and Ii-1 (*red*); (E and F) polyclonal class II antisera (*green*) and Igp110 (*red*). A, C, and E are Ii⁺ cells; B, D, and F are Δ Ii cells. Double staining is indicated by a yellow color in the merged images. In the case of F, a white arrow is shown to help visualize the surface of the cell.

We next determined the localization of the intracellular class II molecules in ΔIi cells relative to organelles of the secretory pathway. Splenocytes were first double labeled for class II (Fig. 5, *red*) and the TGN marker TGN38 (Fig. 5, *green*). As expected, little if any colocalization was observed in Ii^+ cells since virtually all of the class II molecules were present on the cell surface (Fig. 6 A). TGN staining was limited to a restricted region on one side of the nucleus, corresponding to the pericentriolar region. In the ΔIi cells, however, a significant fraction of the intracellular class II was found to costain with TGN38 (Fig. 6 B, *yellow*). Given the limited resolution of fluorescence microscopy and the limited size of the cytoplasmic compartment in splenocytes, these results do not indicate that class II molecules were found in the TGN but only that a portion of them were found in the same general region as the Golgi complex.

Splenocytes were also stained for class II and BiP, a resident of the ER and intermediate compartment that is thought to play a role in protein folding (41, 42, 46). As shown in Fig. 6 C, the patterns of surface class II labeling (*green*) and BiP labeling of the ER and nuclear envelope (*red*) were entirely distinct in Ii^+ cells. In contrast, considerable colocalization of BiP and class II was observed in the ΔIi cells (Fig. 6 D, *orange/yellow*). A region to one side of the nucleus, presumably corresponding to the pericentriolar region, also contained an area of class II staining that was relatively devoid

of BiP. This region could represent class II localized to the Golgi complex or to intermediate compartment structures that have relatively low concentrations of BiP.

Localization of Intracellular Class II Molecules by Immunoelectron Microscopy. Since even confocal microscopy was of too limited resolution to permit a definitive assessment of the intracellular localization of class II molecules in ΔIi splenocytes, we next used protein A-gold immunocytochemistry on ultrathin frozen sections. Splens were perfusion fixed *in situ* with 4% paraformaldehyde, cryoprotected in sucrose, frozen in liquid nitrogen, sectioned, and then stained using a polyclonal anticlass II Ab. To mark elements of the rough ER and intermediate compartment, the sections were also double labeled with a monoclonal anti-BiP Ab. As shown in Fig. 7, considerable labeling of morphologically identifiable ER cisternae was observed for both class II (5 nm gold) and BiP (10 nm gold) in ΔIi splenocytes (Fig. 7 A, *arrow*). As expected, some class II labeling was observed on the plasma membrane. Large class II- and BiP-positive vesicular structures corresponding morphologically to forming autophagic vacuoles were also observed (arrowhead).

In addition to conventional rough ER, class II staining was often found in a heterogeneous population of vesicles and tubules that were positive for BiP (Fig. 7 B, *arrowheads*). Based on the more extensive analysis of a variety of model systems (41, 42, 46), it is likely that these noncisternal elements represent the intermediate compartment or CGN (47) in which misfolded or ER proteins are retained or retrieved and returned back to the ER proper (41, 42, 46). Unfortunately, it proved impossible to label the splenocytes with Abs to rab2, p53, or p58, intermediate compartment/CGN markers, since none of these Abs was sufficiently reactive with murine cells. In Ii^+ splenocytes, similar patterns of BiP staining were observed, although the BiP-positive compartments were not heavily labeled for class II molecules (data not shown).

Discussion

Despite considerable interest, the precise role of *Ii* chain in the synthesis, transport, and function of class II molecules has remained unclear. In an effort to provide a new approach to this problem, we employed the recently developed technique of homologous recombination in embryonic stem cells to create a germline transmitting line of mice which contains a deletion within the coding region of the invariant chain gene. Our results concerning the immune and cellular phenotypes of *Ii*-deficient mice are in good agreement with recently published work utilizing independently generated strains of recombinant mice (25, 26). In general, it is clear that deletion of the *Ii* chain gene leads to an overall reduction in the expression of class II molecules on the plasma membrane and to their intracellular accumulation, presumably early in the secretory pathway. The few class II molecules that do reach the plasma membrane do not acquire peptide from endogenously processed antigens but can bind peptide added

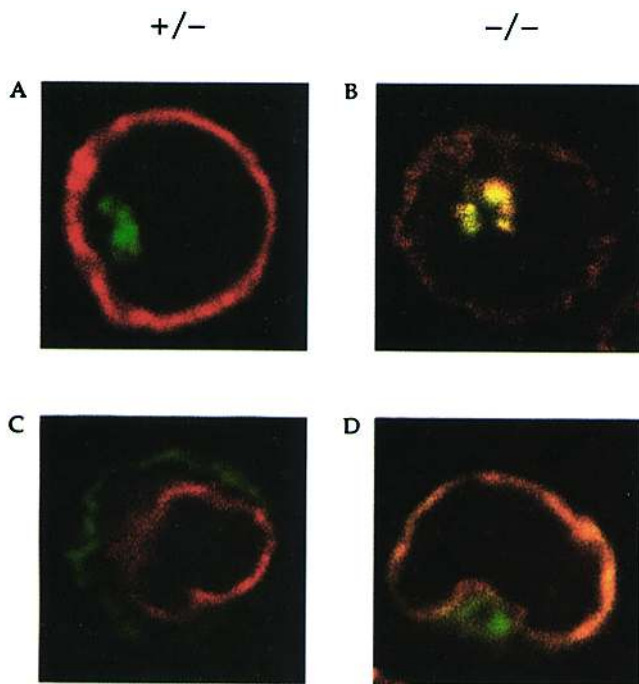


Figure 6. Distribution of class II molecules relative to the TGN and ER. (A and B) Anti-class II mAb M5/114 (*red*) and antibody to the TGN marker TGN38 (*green*); (C and D) polyclonal anti-class II antisera (*green*) and mAb anti-BiP (*red*). Coincident staining is marked by a yellow/orange color.

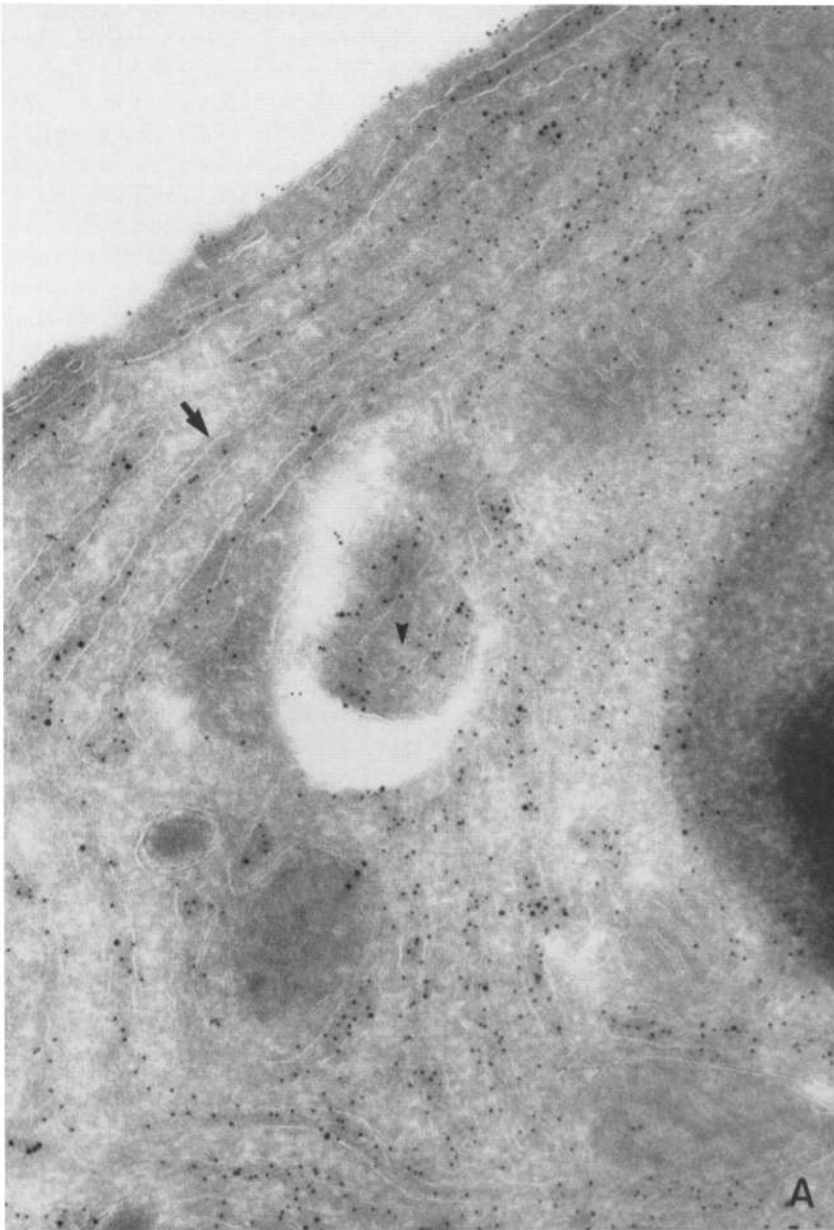
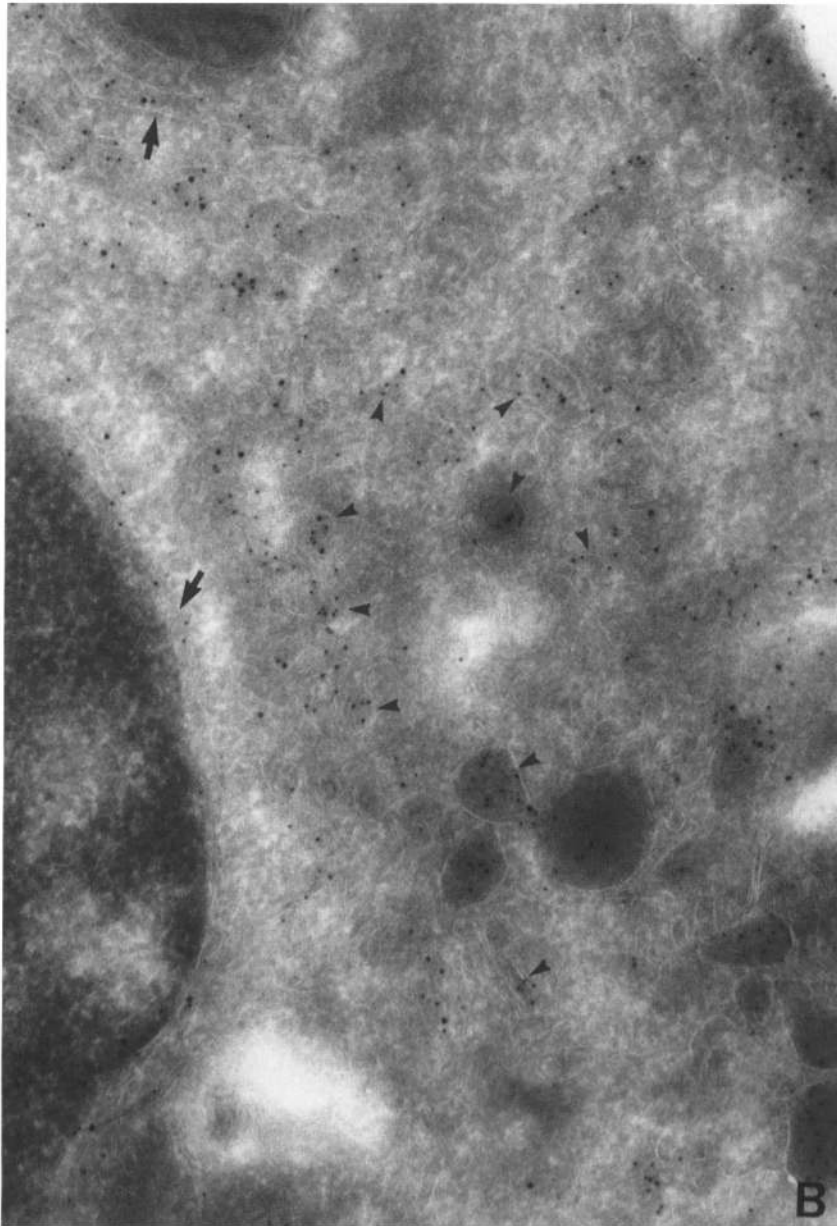


Figure 7. Localization of class II molecules and BiP to the ER of Δ Ii splenocytes by immunoelectron microscopy of frozen thin sections. After fixation by in situ perfusion with paraformaldehyde, spleens from Δ Ii mice were cryoprotected, frozen, and ultrathin sections stained with Abs to class II (5-nm gold particles) and BiP (10-nm gold particles). (A) Extensive labeling of cisternal elements of the rough ER with both anti-BiP and anti-class II reagents; (arrowhead) a likely autophagic vacuole forming from ER elements. (B) Labeling of tubular-vesicular structures characteristic of the "intermediate compartment" between the ER and the Golgi complex (or CGN-transitional elements) (47). (Arrows) Positive ER and continuous nuclear envelope elements. $\times 71,300$.

to the culture medium. Finally, these alterations are accompanied by a marked decrease in the ability of Δ Ii splenocytes to present antigen to T cell hybridomas in vitro and apparently to CD4⁺ T cells in vivo.

Together, these results demonstrate that Ii chain plays a critical role in regulating the class II-restricted immune response. Since it seemed likely that the phenotypes associated with Ii chain deletion were secondary to a defect in the folding, assembly, or intracellular transport of class II molecules, we determined the cell biological basis for the defects caused by Ii chain deletion in primary splenocytes. Surprisingly, we found that Ii chain is not required for the dimerization or at least partial folding of α and β chains, but is nevertheless required to allow newly synthesized class II molecules to escape the quality control mechanism in the ER that prevents the transport of misfolded proteins.

MHC Class II Molecules Fold and Assemble Properly in the Absence of Ii Chain. We found that splenocytes from Δ Ii mice synthesized α and β chains, and assembled them into dimers, in a fashion indistinguishable from Ii⁺ splenocytes. Abs to a conformation-independent determinant on the β chain (M5/114) were found to coprecipitate α chain with similar efficiencies from both cell types. This was in accord with the observations of Viville et al. (26) but distinct from Bikoff et al. (25) who reported that dimer formation was markedly inhibited in Δ Ii cells. Reasons for this discrepancy might reflect differences in the efficiencies or conditions of immunoprecipitation. In addition, Bikoff et al. used M5/114 as a dimer-specific reagent whereas we have found it to react well with free β chain even by Western blot (Drake, J. R., unpublished observations). In any event, it is clear that at least some dimer formation must occur since empty but func-



tional class II molecules were detected at the plasma membrane of ΔIi splenocytes from all three sets of mice.

The question of dimer formation is critical since it pertains directly to whether *Ii* has a role in the folding and/or assembly of new synthesized α and β chains, a function suggested by a variety of observations using class II-transfected fibroblasts (18). Not only did we find efficient coprecipitation of α and β chain in pulse-labeled cells, but the class II molecules formed were reactive with the dimer-specific Ab YP3. This indicated that both α and β chains were capable of extensive folding and formed "authentic" dimers in the absence of *Ii* chain. The YP3 epitope appeared very rapidly in ΔIi cells and was fully expressed by the end of the 20-min chase, in accord with the kinetics of the assembly of class II-*Ii* chain complexes in Ii^+ cells (15, 24, 48). Interestingly, α/β dimers became reactive with YP3 in ΔIi cells more rap-

idly than in Ii^+ cells in which YP3 immunoprecipitation was evident after peptide-loaded, SDS-stable complexes began to appear (>1-h chase). This also corresponded to the time at which *Ii* chain dissociation would be expected to occur (15, 19, 24, 48). Since α/β dimers in ΔIi cells remained SDS sensitive and presumably empty of peptide at all time points, it seems likely that YP3 recognizes a determinant that is masked by bound *Ii* chain and therefore is accessible immediately upon dimerization of α and β in the ΔIi cells.

Ii Chain Is Required to Evade the Quality Control Mechanisms in the ER. Since newly synthesized class II molecules exhibited extensive folding and dimerization in the absence of *Ii* chain, it was surprising to find that they failed to escape the ER mechanisms designed to prevent the forward transport of misfolded membrane and secretory proteins. Immediately after their formation, α and β chains entered a high

molecular weight aggregate that persisted for long times of chase. Such aggregates are typical of many misfolded proteins and are thought to reflect the formation of stable complexes between newly synthesized proteins and intrinsic ER components that are involved in protein folding (41, 42). Although such components typically include proteins such as BiP, calnexin (p88), and protein disulfide isomerase, the other members of the novel class II-containing aggregates observed in the Δ Ii cells remain to be identified. It is conceivable that homotypic interactions among class II molecules themselves at least in part explains the formation of the high molecular weight complexes.

Such aggregates need not reflect a "dead-end" pathway for misfolded proteins, but rather may represent the formation of folding intermediates with ER components that are involved in the normal folding pathways of many membrane proteins (41, 42). Thus, the entry of newly synthesized class II molecules into high molecular weight complexes may normally be an intermediate step in the addition of Ii chain to the nascent α/β /Ii nonamer (13). Addition of three Ii chains to the forming complex may be a prerequisite for its release from the aggregate, possibly explaining why otherwise folded α/β dimers might accumulate at this step in Δ Ii splenocytes. In favor of this possibility is the observation that a small fraction of class II molecules synthesized even in the Ii⁺ splenocytes was found in a high molecular weight complex.

It is also apparent that at least two types of interactions lead to the formation of the class II-containing complexes. One interaction is clearly noncovalent, since boiling in SDS released both α and β chains from the aggregate. Since this material was immunoprecipitated with the conformation-specific YP3 Ab, it is apparent that preformed α/β dimers must enter the noncovalent aggregate. A second interaction appeared to be covalent, since a significant fraction of M5/114-reactive material remained in a high molecular weight band even after boiling (as long as no reducing agents were included during the incubation). Since this band was not detected using YP3, it is likely to represent free and thus misfolded β chain bound via disulfide bonds to itself or possibly other ER proteins. Indeed, when transfected alone into COS cells, free β chain has recently been found to enter similar covalent aggregates (48a).

Regardless of the precise nature of the high molecular weight complex, entry of newly synthesized α/β dimers into the aggregate is likely to explain the inability of class II molecules to be efficiently transported out from the ER in the Δ Ii cells. By complexing with resident ER proteins that bear specific sequence motifs for ER retention (49–52), class II molecules would similarly be retained in the ER. ER retention may reflect either the sequestration of high molecular weight complexes in subcompartments of the ER or intermediate compartment or a dynamic retrieval process by which complexes are returned ("retrieved") back to the ER immediately after exit into the intermediate compartment or CGN (41, 42, 46). Whereas the extent to which either possibility prevails in any specific instance remains controversial, our biochemical and immunocytochemical results are entirely consistent with both proposed mechanisms.

As has been previously observed (25, 26), newly synthesized α and β chains in Δ Ii cells failed to receive terminal modifications of their N-linked sugars, consistent with their failure to reach the medial Golgi complex or TGN (47). A significant fraction of at least the β chain was subjected to outer chain trimming, but it could not be determined whether the increase in electrophoretic mobility observed reflected the activity of ER or Golgi glycosidases. More importantly, since immunofluorescence confocal microscopy of splenocytes has proved inadequate to establish the intracellular localization of class II molecules in Δ Ii splenocytes (26), the EM immunocytochemistry now provides clear and direct evidence to support a predominant localization to ER and other ER-like compartments positive for BiP (e.g., intermediate compartment).

Clearly, retention of the α/β dimers in the ER is not complete in the Δ Ii cells, since a small fraction of class II molecules does reach the plasma membrane. As illustrated by the slow kinetics by which newly synthesized α or β chains exhibit molecular weight shifts corresponding to terminal glycosylation, it is obvious that the absence of Ii chain introduces a significant kinetic inhibition of class II transport to the Golgi complex.

The Function of Ii Chain. What, then, are the functions of Ii chain in splenocytes? Clearly, Ii chain is not required for initial folding or dimer formation, but it does appear to be required to complete the formation of class II molecules that the ER interprets as completely folded, properly assembled protein complexes. So, in this sense, Ii chain can be viewed as providing an accessory folding or assembly function. Such a function must be distinguished from the activities associated with more "conventional" chaperones such as BiP or calnexin which appear to act during the initial stages of protein folding.

Whereas Ii chain has also been associated with preventing the premature loading of peptides onto class II molecules, it is evident that it is not absolutely required for this function. At no stage of biosynthesis were SDS-stable, peptide-loaded complexes observed in Δ Ii cells, although the α/β dimers formed were clearly competent to bind antigenic peptide at least after reaching the plasma membrane. Conceivably, either the length of peptides available in the ER, or the neutral pH environment of the ER lumen, was sufficient to prevent peptide loading in the secretory pathway. The loading of peptides in endocytic organelles also did not occur, but this may reflect the inefficiency of class II transport out from the ER and/or the inability of α/β dimers to reach the necessary elements of the endocytic pathway in the absence of bound Ii chain.

A substantial body of transfection data suggests that the cytoplasmic domain of Ii chain bears a targeting signal that either diverts class II molecules leaving the TGN to endosomes (or specifies the rapid internalization of surface class II-Ii complexes) (15, 17). Thus, it seems likely that the inability of those α/β dimers that do escape the ER to acquire peptide or to mediate a class II-restricted response reflects the suggested targeting function of Ii chain. We have recently found that in B cells, newly synthesized class II molecules are not only diverted from the constitutive secretory pathway

to endosomes but also appear to reach a novel compartment of MHC class II-containing vesicles (CIIV) where they may acquire peptide (Drake, J. R., et al., and Amigorena, S., et al., manuscripts submitted for publication). Conceivably, Ii

may play a role in targeting from the TGN to endosomes or from endosomes to CIIV, or both. The detailed analysis of class II transport in Δ Ii cells now provides the opportunity to address directly each of these questions.

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