Defective Major Histocompatibility Complex Class II Assembly, Transport, Peptide Acquisition, and CD4+ T Cell Selection in Mice Lacking Invariant Chain Expression

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Summary

We used gene targeting techniques to produce mice lacking the invariant chain associated with major histocompatibility complex (MHC) class II molecules. Cells from these mice show a dramatic reduction in surface class II, resulting from both defective association of class II α and β chains and markedly decreased post-Golgi transport. The few class II α/β heterodimers reaching the cell surface behave as if empty or occupied by an easily displaced peptide, and display a distinct structure. Mutant spleen cells are defective in their ability to present intact protein antigens, but stimulate enhanced responses in the presence of peptides. These mutant mice have greatly reduced numbers of thymic and peripheral CD4+ T cells. Overall, this striking phenotype establishes that the invariant chain plays a critical role in regulating MHC class II expression and function in the intact animal.

The class II membrane glycoproteins encoded in the MHC expressed by B cells, macrophages, dendritic cells, and thymic epithelial cells present antigenic peptides to helper T lymphocytes and guide thymic selection of CD4+ T lymphocytes. Mature class II molecules are heterodimers comprised of the products of two closely linked genes, the α and β chains. Highly polymorphic residues located in the NH_2-terminal domains of both chains are accessible to ligands bound within the peptide binding groove (1). Recent studies have shown that this site on class II molecules can accommodate peptides 15–20 amino acids in length lacking precisely defined NH_2- and/or COOH-terminal ends (2–5). It has been known for some time that presentation of exogenous protein antigens in association with MHC class II molecules involves antigen uptake, internalization, and proteolytic degradation in endocytic vesicles. Recent observations have strengthened the idea that class II molecules selectively acquire peptides through the endocytic pathway. Thus, sequence analysis of the predominant peptides recovered from purified class II molecules indicates that these are derived from integral membrane proteins, surface receptors, and exogenous serum proteins (2–5).

The class II α/β heterodimers assembled shortly after synthesis in the endoplasmic reticulum (ER) are associated with a third polypeptide chain, the invariant chain, a highly conserved glycosylated type II membrane protein encoded by an unlinked gene (6–9). There is evidence that the invariant chain can associate with free α and β chains before dimer assembly (10–12). However, recent studies suggest that the formation of nine-subunit α/βI complexes (13) involves the sequential addition of α/β dimers to preexisting invariant chain trimers (14). The α/βI complexes are extensively modified during export through the Golgi complex (7, 8), and subsequently transported to a peripheral endocytic compartment (12, 15–18), where exposure to acidic pH and/or proteolytic enzymes (19, 20) results in the dissociation of invariant chain before expression of fully mature class II heterodimers at the cell surface. Recent experiments have identified a targeting signal in the cytoplasmic tail of the invariant chain that is responsible for localization to endocytic vesicles (12, 21). The association with invariant chain has been shown to prevent peptide occupancy (11, 20, 22, 23), suggesting that removal of the invariant chain may be necessary for the acquisition of

[1] This work was undertaken as a collaborative project between three senior investigators, who made equally important contributions to the various technical and intellectual aspects of these experiments.

peptide ligand. On the other hand, class II molecules never previously exposed to the invariant chain possess the ability to bind peptide ligands in vitro (24).

Although early studies demonstrated substantial surface expression of class II molecules in the absence of invariant chain (25, 26), subsequent work indicated that coexpression of the invariant chain can markedly enhance class II transport (27, 28). Over recent years, there have been conflicting reports regarding a requirement for invariant chain for class II–restricted antigen presentation (29–34). The extent of invariant chain dependency seems to vary according to the particular T cell clone receptor avidity and/or antigenic specificity (32, 34), and may also reflect divergent activities mediated by distinct invariant chain polypeptides that arise due to alternative mRNA splicing (34, 35). In light of these complex results, and concerns about the physiological relevance of observations made in vitro using cell lines not normally expressing class II molecules and invariant chain, a satisfactory picture of the role played by the invariant chain in regulating class II expression and/or peptide binding has not yet emerged.

Reliable methods for introducing precise mutations into the mouse germline have recently been established (36). To better understand possible contribution(s) of the invariant chain to the function of class II molecules under physiological conditions, we produced a null mutation at the invariant chain locus using homologous recombination in pluripotent embryonic stem (ES)[2] cells. The absence of invariant chain caused several striking changes in MHC class II structure and function. The present findings demonstrate that invariant chain plays a critical role in assembly, transport, and peptide acquisition by MHC class II molecules and in the development of $CD4^+$ T lymphocytes.

Materials and Methods

Construction of the Targeting Vector. The 1.2-kb SalI-XhoI fragment from pMC1neo (37) was subcloned into the SalI site of pSP73 (Promega Biotec, Madison, WI) and the resultant plasmid was digested with XhoI and HindIII. We then inserted the 1.7-kb HindIII-XhoI fragment from MC1tk (38). Genomic fragments were obtained from the AKR cosmid clone 10.7 (39). The 5′ fragment corresponding to bases −707 to +242 was isolated by PCR amplification using oligonucleotide primers containing HindIII restriction sites. The 0.95-kb 5′ Ii fragment was then inserted, in the appropriate orientation, into a unique HindIII site. Finally, the 7.5-kb 3′ SacI fragment, blunt ended, was cloned into the unique SalI site. The targeting vector containing the SacI fragment in the correct orientation was designated pSP73neotk5′3′Ii.

Generation of ES Cell Chimeras. CCE ES cells (40), maintained on STOneo feeder cells, were transfected with 10 μg of ClaI linearized plasmid, plated, and selected in G418 and gancylovir as described (41). Drug-resistant colonies were individually picked and expanded into duplicate 24-well trays. One set of clones was frozen, the remaining set harvested in 500 μl DNA lysis buffer per well, and DNA extracted from pools of lysates (five clones/pool) amplified in 50 μl containing 1 mM of each deoxynucleoside triphosphate, 1.5 U Taq polymerase (Perkin-Elmer Corp., Norwalk, CT), and primers as shown in Fig. 1 *A*. Amplification products were separated on a 0.8% agarose gel and visualized by ethidium bromide staining. Individual clones among the pools positive by PCR were then separately analyzed to identify recombinants. For Southern blot analysis, DNA (10–20 μg) was digested to completion with SacI, separated on a 0.8% agarose gel, transferred to GeneScreen, and probed using a neo fragment or the 800-bp AluI fragment derived from genomic sequences 5′ to the Ii targeting vector as shown in Fig. 1.

Recombinant ES cell clones were used to generate chimeric mice after microinjection into either MF1 or C57BL/6J blastocysts as described (42). Male chimeras were mated to C57BL/6J females to identify animals that transmitted the ES cell–derived agouti coat color marker. The F_1 progeny were screened by PCR. The heterozygotes were then intercrossed. F_2 animals were genotyped by Southern blot analysis or, more routinely, by PCR using primers shown in Fig. 1, and the products of 1,400 bp (mutant allele) and 1,108 bp (wild type allele) were visualized on agarose gels after ethidium bromide staining.

Antibodies and Peptides. Hybridomas 28-14-8 and 25-9-17 (43) were provided by Dr. Keiko Ozato (National Institutes of Health, Bethesda, MD). M5/114 (44) was from the American Type Culture Collection (Rockville, MD). B22.249 (45) was from Dr. Ulrich Hammerling (Sloan-Kettering Memorial Institute, New York). Y-Ae (46) and Y3P (47) were generously provided by Drs. Charles Janeway, Jr. (Yale University Medical School, New Haven, CT) and Donal Murphy (New York State Department of Health, Albany, NY). In-1 (48) was from Dr. Ralph Steinman (The Rockefeller University, New York). P4H5 (49) was from Dr. Pat Jones (Stanford University, Palo Alto, CA). Rabbit chain-specific antibodies directed against determinants located in the cytoplasmic tails of the α and β chains, respectively, have been described (50). The E_α 52–68 and HEL 46–61 peptides were provided by Dr. J. Coligan (Biological Resources Branch, National Institute of Allergy and Infectious Diseases, NIH). The HEL 74–88 peptide was kindly provided by Dr. N. Shastri (University of California, Berkeley, CA).

Immunofluorescence Analysis. For single-color analysis, spleen cell suspensions were freshly prepared or harvested after overnight culture at 37°C (5×10^6 cells/ml) in complete RPMI 1640 supplemented as described (51) alone or in the presence of peptides (100 μM). Cells were incubated with saturating amounts of biotin-conjugated anti-I-A^b antibodies followed by FITC-labeled avidin D, or with the anti-Ii chain mAb In-1 followed by FITC-conjugated goat $F(ab')_2$ anti-mouse IgG (H + L), as indicated in the figure legends. Before staining with the In-1 antibody, cells were treated with 10% formalin for 10 min at room temperature and extensively washed with PBS containing 0.1% saponin as described (33). In this case, incubations and all washing steps were carried out at room temperature in the presence of saponin (0.1%). Fluorescence was analyzed on an Epics C flow cytometer (Coulter Electronics Inc., Hialeah, FL) (Figs. 2 and 3), or using a FACScan® flow cytometer (Becton Dickinson & Co., Mountain View, CA) (Fig. 7), and data were displayed as cell number vs. log fluorescence. Dead cells and erythrocytes were eliminated from the analysis by appropriate gating.

For three-color analysis, suspensions of thymocytes or lymph node cells were incubated on ice with anti-CD8-FITC, anti-TCR-β-PE, and biotinylated anti-CD4, followed by Streptavidin-Cy-Chrome™ (all antibodies and Streptavidin-Cy-Chrome™ were from Pharmingen, San Diego, CA). Listmode data were collected for all three fluorescence channels using a FACScan® flow cytometer

[2] *Abbreviations used in this paper:* ER, endoplasmic reticulum; ES, embryonic stem; nt, nucleotide.

and analyzed with Lysys II software (Becton Dickinson & Co.). Dead cells and nonlymphoid cells were excluded from analysis using gates based on scatter. Contour plots for CD4 vs. CD8 were generated from the scatter-gated data. Single histogram plots of TCR-β expression for cells with defined levels of CD4 and CD8 expression were generated using the gates indicated on the CD4 vs. CD8 contour plots.

RNAse Protection Assay. A riboprobe vector suitable for the analysis of Ii mRNA was previously described (33). Briefly, a 277-bp PstI-BglII fragment comprising coding sequences from exon 6 (92 nucleotides [nt]) and exon 6b (185 nt) was inserted into PstI-BamH1-digested pGEM4 (Promega Biotec, Madison, WI). The resultant plasmid (pGEMIi41) was linearized with EcoRI and transcribed using T7 polymerase to yield the 321-nt Ii41 probe. Total cellular RNA was hybridized overnight at 45°C in the presence of ^{32}P-labeled antisense RNA. Unprotected RNA was digested using RNAse A (40 μg/ml) and RNAse T1 (2 μg/ml) for 60 min at 30°C. Samples were treated with SDS and proteinase K, twice extracted with phenol/chloroform, ethanol precipitated, redissolved in buffer containing 80% formamide, and analyzed by electrophoresis in a 6% polyacrylamide denaturing gel.

Radiolabeling and Immunoprecipitation Experiments. Biosynthetic and surface labeling, immunoprecipitations, and SDS-PAGE were carried out as described (52). Briefly, spleen cells pretreated with Tris-ammonium chloride to lyse red blood cells were washed with warm HBSS containing 2% FCS and antibiotics, and resuspended (2×10^7 cells/ml) in warm leucine-free RPMI 1640 supplemented with 4 mM glutamine and 5% dialyzed FCS. After 1 h at 37°C, [^{3}H]leucine was added (250 μCi/ml) for 40 min. Cells were either immediately harvested, washed twice with ice-cold PBS, and the cell pellet was lysed in buffer containing 1% NP-40, 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, and 10 μg/ml aprotinin. After incubation on ice for 15 min extracts were cleared of nuclei and debris by centrifugation for 30 min at 15,000 rpm. Alternatively, labeled cells were resuspended in a fivefold excess volume of warm RPMI 1640 supplemented with 15% FCS and 10× cold leucine, incubated at 37°C a further 4 h, harvested, and extracts prepared as above. Lysates were routinely precleared once with rabbit anti-mouse IgG (H + L) antibodies, twice with rabbit anti-rat IgG (H + L), and twice with protein A-agarose (Bethesda Research Laboratories, Gaithersburg, MD), before the addition of specific antibodies. In the case of rat mAbs M5/114 and In-1, secondary anti-rat IgG (H + L) antibodies were added to facilitate recovery of immune complexes. Immunoprecipitates were washed three times with buffer containing 0.05 M Tris-HCl, pH 8, 0.45 M NaCl, 0.5% NP-40, 0.05% Na azide, and 1 μg/ml aprotinin, and then solubilized in Laemmli buffer containing 2% SDS and 2-ME by treatment either for 60 min at room temperature or by heating at 100°C for 10 min as indicated in the figure legends. Samples were analyzed by SDS-PAGE using 10% polyacrylamide gels subsequently treated with EnHance (DuPont-NEN, Wilmington, DE), dried, and exposed to x-ray film. Alternatively, for analysis of surface proteins, freshly isolated spleen cells were kept at 4°C and surface labeled with ^{125}I using lactoperoxidase and glucose oxidase. Iodinated cells were extensively washed with ice-cold 10 mM KI in PBS, and lysates prepared and analyzed as above.

Antigen Presentation Assays. I-A^b-restricted T cell hybrids included BDK 11.1 specific for KLH (53), provided by Dr. P. Marrack (Howard Hughes Medical Institute, National Jewish Center, Denver, CO); 171.3 L3T4$^+$ stimulated by the HEL peptide 74–88 (54), given to us by Dr. N. Shastri (University of California, Berkeley, CA), and the E_α 52–68-specific hybridoma 1H3.1 (5), kindly provided by Dr. A. Rudensky (University of Washington, Seattle, WA). IL-2 production was assessed by incubating T cells (5×10^4/well) with irradiated (2,000 rad) spleen cells (10^5/well) in 200 μl complete RPMI 1640 supplemented with 10% FCS, 0.1 mM nonessential amino acids, 5×10^{-5} M. 2-ME, and 2 mM glutamine, and increasing concentrations of antigen as indicated in Fig. 8. Supernatants were collected after 24 h and assayed for IL-2 content in a secondary culture using CTLL indicator cells. [^{3}H]Thymidine incorporation was measured in the presence of diluted (1:4) primary supernatant. Results shown are mean cpm of duplicate cultures.

The B61-34 T cell clone specific for IgG2a of the *a* allotype has been described (51). Briefly, T cells were maintained by repeated antigen stimulation in the presence of irradiated (2,000 rad) syngeneic C57BL/6 spleen cells followed by short periods of rest. To measure antigen-specific proliferation, rested T cells (2×10^4 cells/well) were incubated with irradiated (3,300 rad) spleen cells (10^6 cells/well) in 200 μl of complete RPMI 1640 alone or in the presence of LPC1 myeloma protein (500 μg/ml) as described (51). After a 48-h culture period, clonal expansion was assessed by a 16–18-h exposure to 1 μCi [^{3}H]thymidine. Results are expressed as mean cpm of triplicate cultures.

Results

Generation of a Null Mutation at the Invariant Chain Locus. To disrupt the invariant chain gene in ES cells, a positive–negative selection strategy was used. The targeting vector (Fig. 1 *A*) contained 8.45 kb of genomic homology interrupted by the MC1neopA cassette in the same transcriptional orientation. The HSV thymidine kinase gene was placed at the 5′ end of the genomic homology to facilitate selection against random integration events (38). Replacement of the wild-type locus by homologous recombination should result in a 3.8-kb deletion removing the first intron and 11 nt of exon 2.

CCE ES cells were transfected with linearized plasmid DNA by electroporation and transformants selected in the presence of G418 and gancyclovir. Surviving colonies were screened for homologous recombinants using PCR primers specific for the unique 5′ junctional fragment in the targeted locus. From 1,600 drug-resistant colonies analyzed, we recovered six PCR-positive clones. Homologous recombination at the invariant chain locus and the absence of randomly integrated copies of the targeting vector were verified by Southern blot analysis using a probe external to the 5′ Ii sequence and also using a neo probe (data not shown). All six clones showed the restriction pattern predicted for the correctly targeted allele. As the gancylovir counterselection provided an eightfold enrichment, the frequency of homologous recombination was ~1 in 2,000 integration events.

Three independent recombinant ES cell clones were injected into blastocysts and gave rise to male chimeras that transmitted the mutation to their offspring. The resultant heterozygous progeny were intercrossed to generate mice homozygous for the mutation. In experiments described below, we analyzed invariant chain–deficient mice derived from two independent ES cell clones. Genotyping of intercross progeny indicates that live-born homozygotes are recovered at the expected frequency (representative data shown in Fig. 1). For the most part, homozygous mutants raised under con-

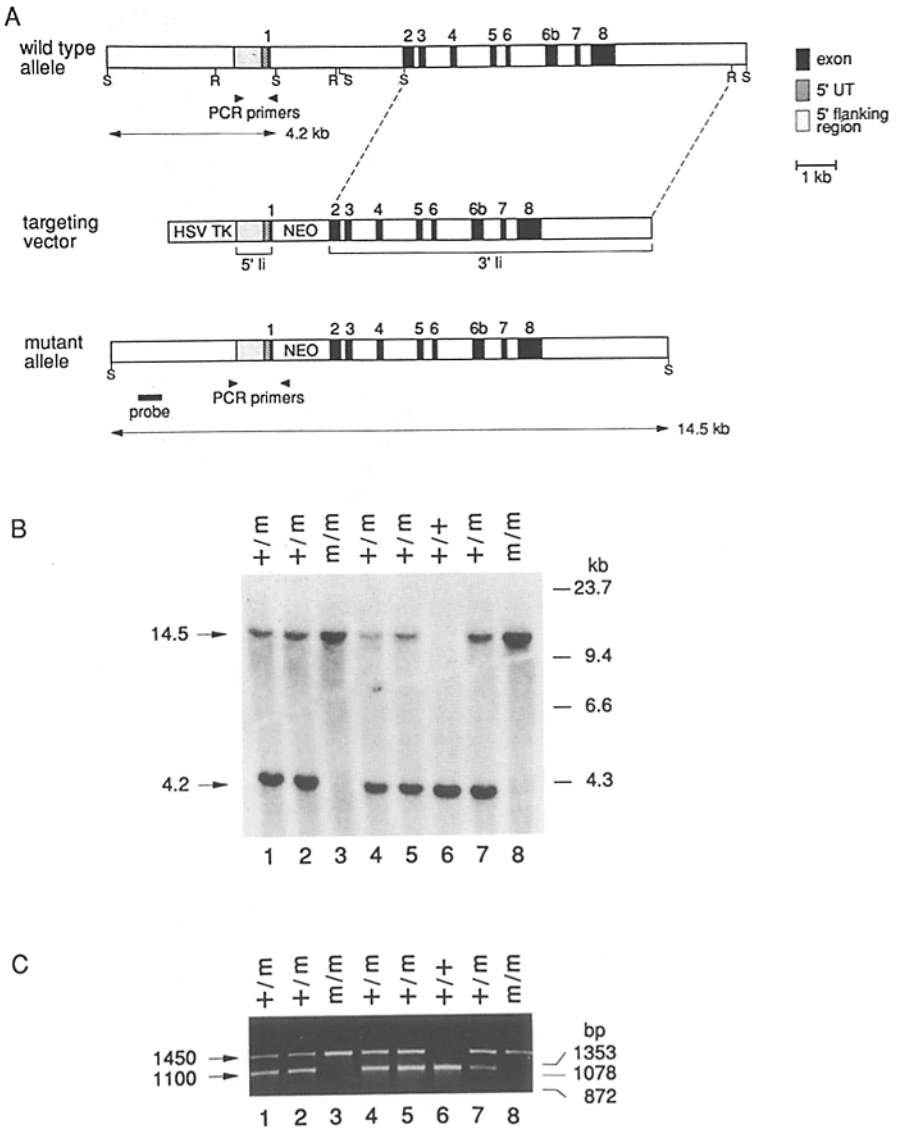

Figure 1. Homologous recombination at the invariant chain locus. (*A*) Schematic representation of the wild-type and mutant alleles and the targeting vector. (*R* and *S*) Positions of EcoRI and SacI restriction sites, respectively. (*B*) Southern blot analysis of representative tail biopsies. Male and female mice carrying the targeted allele were intercrossed and representative F_2 progeny analyzed. Genomic DNA was digested with SacI and hybridized with the 5′ flanking probe. Positions of the 4.2-kb fragment derived from the wild-type locus and the 14.5-kb fragment derived from the targeted allele are indicated. (*C*) PCR screen genotyping the same individuals as shown in *B* using oligonucleotide primers specific for a 5′ junction fragment or a wild-type sequence contained within the deletion to generate 1,450- or 1,100-bp products derived from the mutant or the wild-type locus.

ventional conditions appeared healthy. Occasionally they showed symptoms of severe wasting, and homozygotes were generally smaller than littermates.

Absence of Invariant Chain Expression in Homozygous Mutants. To establish that the mutation generated a null allele, invariant chain expression was examined in cytoplasmic staining experiments using the In-1 mAb reactive with an epitope located near the NH_2 terminus. As shown in Fig. 2 *A*, a discrete strongly positive subpopulation comprised of ~40% of wild-type spleen cells was clearly observed. In contrast, there was no significant staining of mutant spleen cells. As documented below, the absence of invariant chain does not simply reflect decreased numbers of B cells. Similarly, immunoprecipitation experiments using In-1 (Fig. 2 *B*) or the P4H5 mAb (data not shown) directed against the COOH-terminal segment demonstrated abundant levels of invariant chain produced by wild-type cells and the absence of detectable expression in cells from homozygous mutants. Precipitations with anti–class I (H-2D^b) antibodies showed equivalent amounts of class I molecules were present in mutant and wild-type cells.

Although these results using In-1 and P4H5 mAbs strongly argue that disruption of the invariant chain locus created a null mutation, these serological reagents might fail to detect aberrant forms of the protein. Therefore we examined steady-state levels of Ii mRNA using an RNAse protection assay that distinguishes alternatively spliced forms of Ii mRNA. Thus, mRNA molecules encoding pIi41, in which exon 6b sequences are present, protect the full-length 277-nt fragment, whereas mRNA encoding pIi31 protects a 92-nt fragment. As shown in Fig. 2 *C*, wild-type lymph node and thymus tissues express high levels of both Ii41 and Ii31 transcripts. In contrast, we observed no significant signal using RNA from homozygous mutants (Fig. 2 *C*, and data not shown). As judged by strong hybridization to a mouse actin probe, all these samples contained approximately equal amounts of

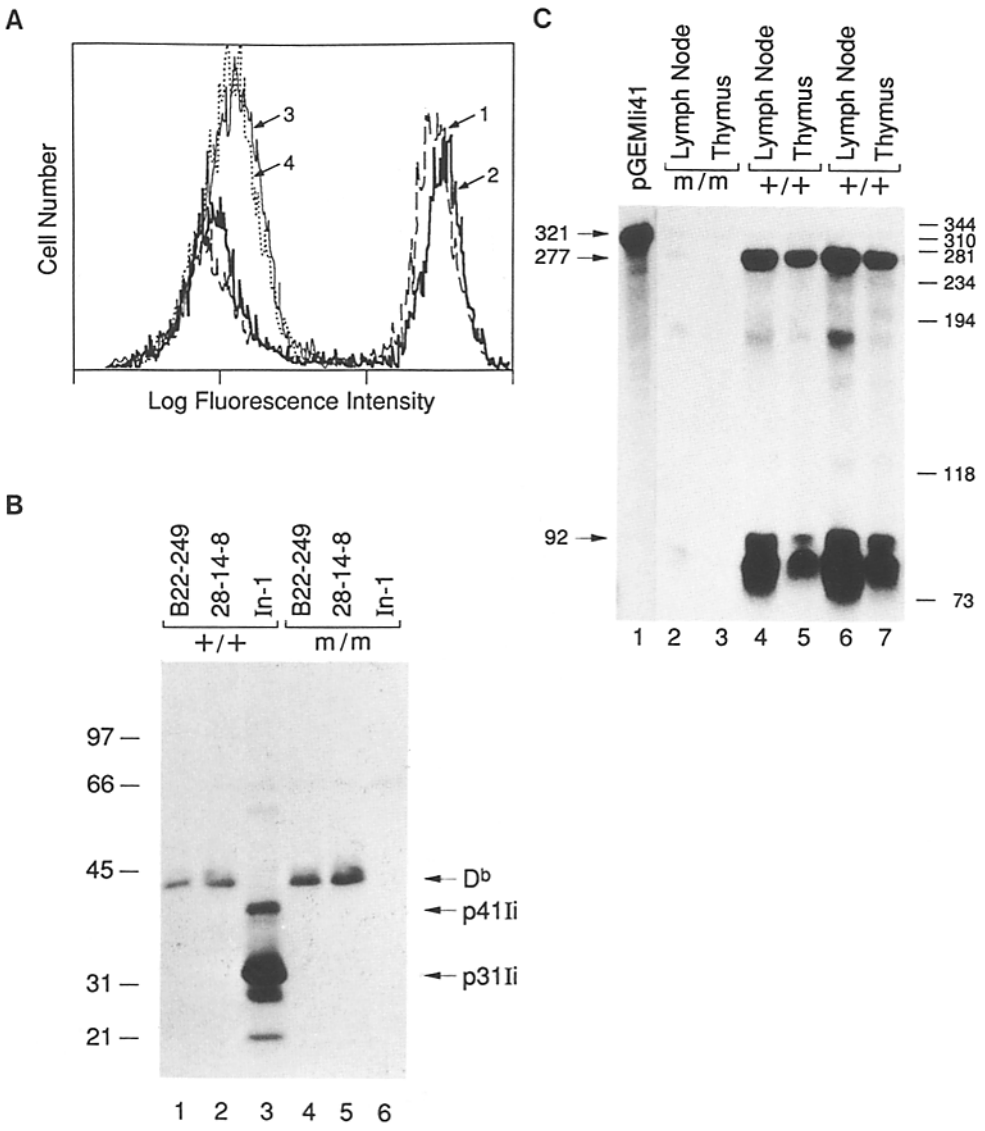

Figure 2. Gene targeting results in the absence of invariant chain expression. (*A*) Cytoplasmic staining using the In-1 mAb (48). Saponin-treated spleen cells from individual wild-type (*1* and *2*) or homozygous mutant mice (*3* and *4*) were incubated with In-1 mAb followed by FITC-conjugated goat anti-rat IgG and analyzed by FACS®. (*B*) Biosynthetic labeling and immunoprecipitation analysis. Lysates of spleen cells labeled for 40 min with [^{3}H]leucine and immunoprecipitated with the indicated mAbs were analyzed by SDS-PAGE under reducing conditions. (*C*) RNAse protection analysis. The pGEMli41 probe was hybridized with total RNA (10 μg) prepared from the indicated tissues. The arrows indicate the probe size and the positions of the predicted full-length protected fragments. Additionally, a smaller product is observed due to over-digestion at a short (5 nt) AT-rich sequence located at the 3′ end of exon 6 (33).

intact mRNA (data not shown). Thus, we conclude that homozygous mutants show drastically reduced Ii transcripts extending to this region of the locus. Taken together, these data demonstrate that disruption of the invariant chain gene results in the absence of expression.

Decreased Levels of MHC Class II Surface Expression. Although not absolutely required for MHC class II surface expression (25, 26), the invariant chain can enhance intracellular transport (27, 28) and affect serological reactivity of class II molecules (28, 31, 33). To determine whether the invariant chain regulates class II surface expression under physiologic conditions, mutant and wild-type spleen cells were stained using a panel of anti-I-A^b mAbs and analyzed by flow microfluorimetry. As shown in Fig. 3, *a–c*, mutant spleen cells exhibited markedly reduced levels of I-A^b surface antigens as detected using several mAbs, including M5/114 (β chain specific), 25-9-17 (β chain specific), and Y-3P (α + β specific) (55–57). Because similar results were obtained with all these antibodies, a strong argument can be made that this reflects decreased amounts of total surface I-A^b rather than a serological change. H-2D^b class I surface molecules (Fig. 3 *d*) and surface Ig (data not shown) were expressed equally well by mutant and wild-type spleen cells. These results demonstrate that the invariant chain is necessary for optimal class II surface expression by normal splenic B cells.

Defective Class II Assembly and Transport in the Absence of Invariant Chain. Decreased levels of class II surface antigens could reflect the requirement for invariant chain at any one of several steps during class II biosynthesis and maturation. To test whether the invariant chain acts at an early stage in the class II biosynthetic pathway, we analyzed chain synthesis and subunit assembly. Both mutant and wild-type cells produce similar amounts of class II α and β chains (Fig. 4). To specifically evaluate the ratios of free chains and stably assembled dimers, lysates prepared from pulse-labeled spleen cells were precleared using rabbit chain-specific antibodies directed

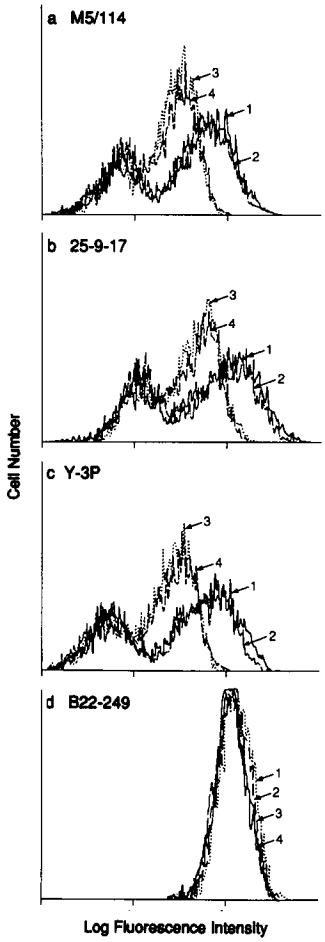

Figure 3. FACS® analysis showing reduced levels of MHC class II surface expression. Splenocytes from individual wild-type (*1* and *2*) or homozygous mutant (*3* and *4*) mice were stained with biotin-conjugated mAbs followed by FITC-conjugated avidin.

against determinants located in the cytoplasmic tails of the α and β chains, respectively (Fig. 4 *A*), or in the experiment shown in Fig. 4 *B* with M5/114 mAb to remove preexisting α/β heterodimers. As expected in wild-type spleen cells, these antibodies all precipitated substantial amounts of α/β dimers associated with invariant chain. Newly synthesized free α and β chains were also observed. In contrast, mutant spleen cells showed markedly reduced amounts of α/β heterodimers reactive with the M5/114 mAb. Increased amounts of free α and β chains were present in these cells, reflecting a decreased rate of subunit assembly or the formation of unstable dimers. These results demonstrate that invariant chain plays a critical role in promoting or maintaining MHC class II subunit interactions.

In addition, the subsequent transport of the newly formed α/β heterodimers was examined using spleen cells pulsed for 40 min and chased for 4 h. As expected, under these conditions virtually all the I-A^b molecules produced by wild-type spleen cells are comprised of mature α and β chains that have undergone N-linked glycan maturation during transport through the medial-Golgi (Fig. 4, *A* and *B*). In contrast, the majority of class II molecules in mutant spleen cells, like those produced in invariant chain–negative cell lines (27, 28), are comprised of immature α and β chains showing no biochemical evidence of having been exported past the *cis*-Golgi. Confocal immunofluorescence microscopy confirmed that class II molecules are predominantly located within the endoplasmic reticulum (ER) of mutant B lymphocytes (data not shown). Thus, efficient transport and posttranslational maturation of newly formed α/β dimers requires the invariant chain under normal physiological conditions.

Structurally Distinct α/β Heterodimers at the Cell Surface. There is evidence that class II molecules undergo a structural transition during acquisition of peptide ligand, such that newly formed, invariant chain–associated α/β heterodimers are unstable in the presence of SDS, whereas a substantial fraction of mature, self-peptide-occupied class II molecules are resistant to SDS-induced denaturation (24, 52, 58–60). To examine the formation of compact SDS-resistant dimers, we evaluated the structure of mature I-A^b molecules produced by mutant spleen cells pulsed for 40 min and chased for 4 h. As shown in Fig. 5, virtually all the I-A^b molecules produced by wild-type spleen cells migrate as compact dimers at ~56 kD. In marked contrast, no mature compact SDS-resistant dimers were produced by mutant spleen cells. Instead these experiments revealed the presence of a novel species of I-A^b molecules having a reduced mobility in SDS-PAGE, comprised of α and β chains with mature complex glycans. This population seems to correspond to the so-called "floppy" α/β heterodimers previously described by Dornmair et al. (61, 62). Because they were not previously observed in metabolic labeling experiments, and are generated upon exposure of compact dimers to low pH in the absence of peptide (59), these floppy molecules were thought to represent partially denatured compact dimers. An interesting possibility is that floppy dimers possess an empty peptide groove that adopts an extended conformation, and this may account for their decreased mobility.

To directly evaluate whether the structurally distinct heterodimers produced by mutant spleen cells are expressed at the cell surface, and the proportion of surface molecules exhibiting this novel conformation, surface proteins were directly ^{125}I labeled and analyzed by SDS-PAGE. As shown in Fig. 6, virtually all the I-A^b surface molecules expressed by wild-type spleen cells migrated in the position expected for compact SDS-resistant dimers. In contrast, mutant spleen cells exhibited undetectable levels of stable compact dimers reactive with either Y-3P mAb (Fig. 6) or rabbit anti-β chain antibodies (data not shown). Instead the few class II α/β heterodimers that reached the cell surface exhibited a slightly reduced mobility in SDS-PAGE, as seen above in metabolic labeling experiments. These results confirm the conclusion that the invariant chain plays a critical role in guiding class II export and directly demonstrate that invariant chain affects the structure and peptide occupancy of mature class II molecules.

Class II Molecules Produced by Invariant Chain–deficient Mice Show Enhanced Peptide Binding. Structurally distinct SDS-resistant α/β heterodimers produced by mutant spleen cells closely resembled floppy dimers generated in vitro by partial denaturation of compact dimers with accompanying peptide loss (59, 61, 62). It was therefore of considerable interest to

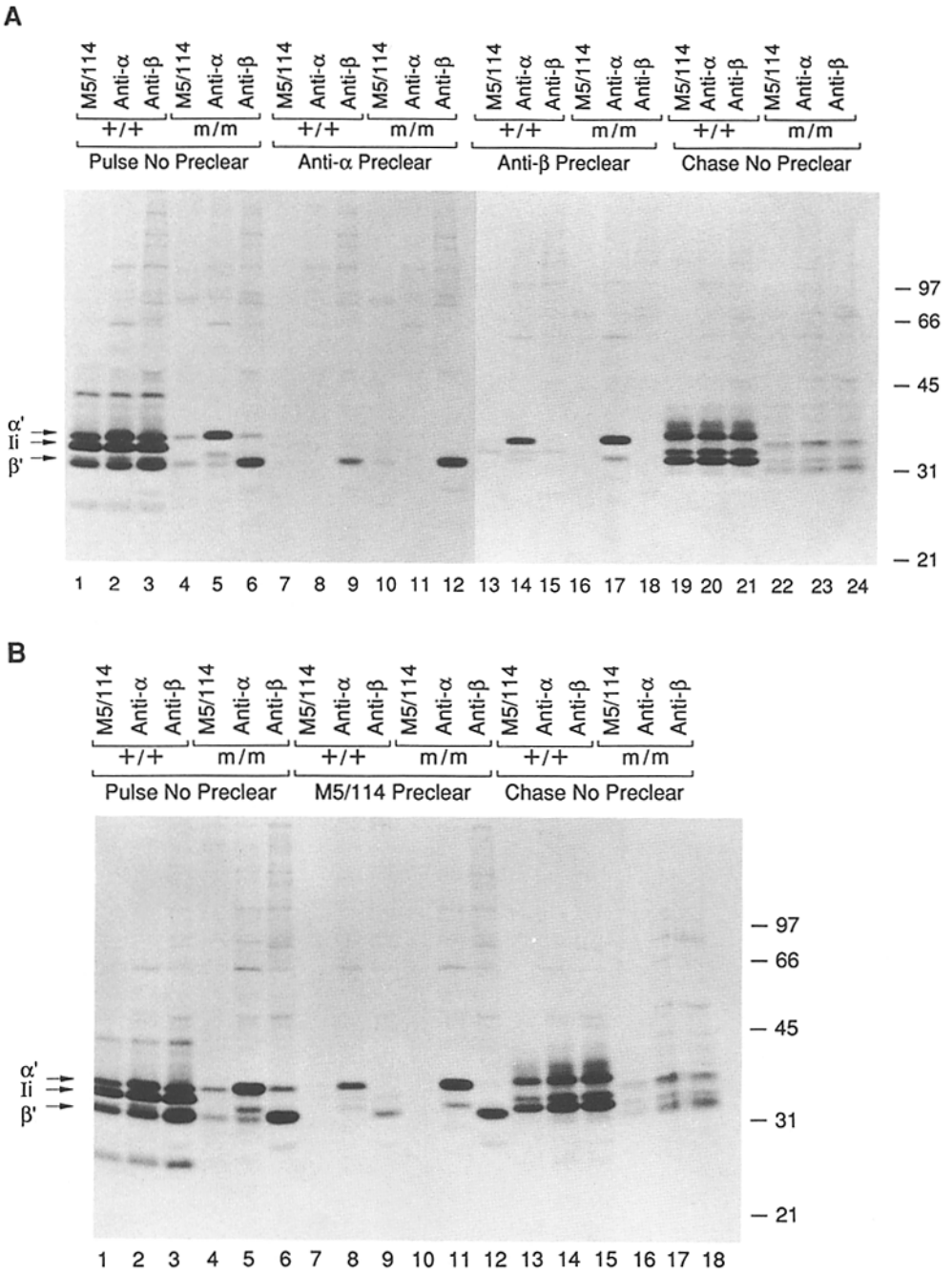

Figure 4. Defective assembly of MHC class II molecules in the absence of invariant chain. Cytoplasmic extracts were prepared from spleen cells pulse-labeled for 40 min with [^{3}H]leucine and in some cases after a 4-h chase period. Class II molecules were immunoprecipitated with the M5/114 mAb or chain-specific rabbit antibodies added directly to untreated lysates or lysates prepared from pulse-infected spleen cells and precleared as indicated.

directly compare mutant and wild-type spleen cells for their ability to bind peptides. To measure bound peptide, we used the Y-A$_e$ mAb described by Murphy, Janeway, and their associates (46, 63) directed against a determinant present on I-A^b molecules associated with the E$_\alpha$ 52–68 peptide. Mutant and wild-type spleen cells were cultured at 37°C overnight in the presence of E$_\alpha$ 52–68, or as a control HEL 46–61 peptide, and then stained with biotin-labeled antibodies followed by FITC-labeled avidin, and analyzed by flow microfluorimetry. As shown in Fig. 7 *a*, despite reduced amounts of surface I-A^b, mutant spleen cells bound significantly higher amounts of E$_\alpha$ 52–68 peptide as detected with the Y-A$_e$ mAb. This treatment had no effect on total surface I-A^b as detected using 25-9-17 mAb (Fig. 7 *b*), nor was surface I-A^b expression enhanced in the presence of another I-A^b binding peptide (Ii 85–99; data not shown). Thus, in contrast to the class II α/β heterodimers produced by wild-type spleen cells that are stably occupied with self- and serum-derived peptides, class II molecules expressed in the absence of the invariant chain behave as if empty or occupied by an easily displaced peptide.

Defective Presentation of Native Protein Antigens and Enhanced Responses in the Presence of Peptides. Considering that class II molecules produced by mutant spleen cells fail to acquire

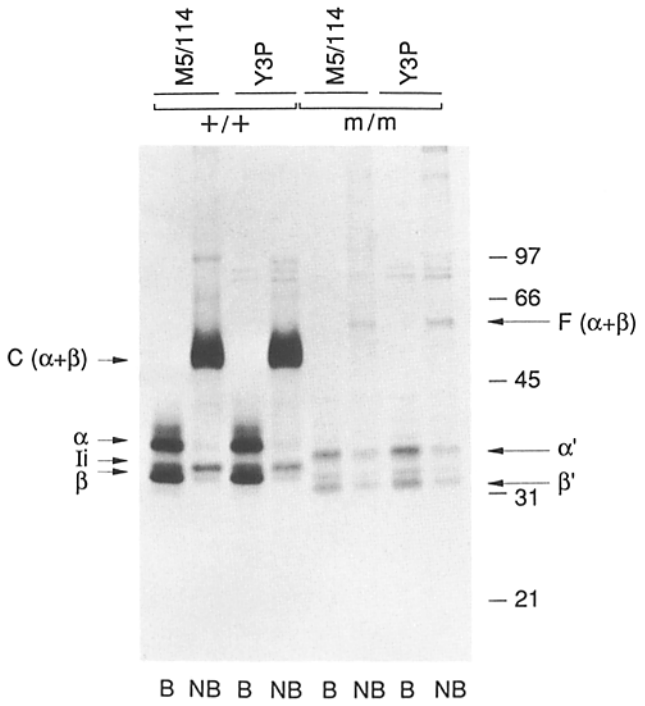

Figure 5. Absence of mature compact stable SDS-resistant dimers. Lysates of spleen cells labeled for 40 min with [^{3}H]leucine and chased for 4 h in the presence of cold leucine were immunoprecipitated with the indicated antibodies. Complexes solubilized at 100°C (*B*) or at room temperature (*NB*) were analyzed by SDS-PAGE under reducing conditions. *C* and *F* indicate the positions of compact and floppy α/β heterodimers, respectively.

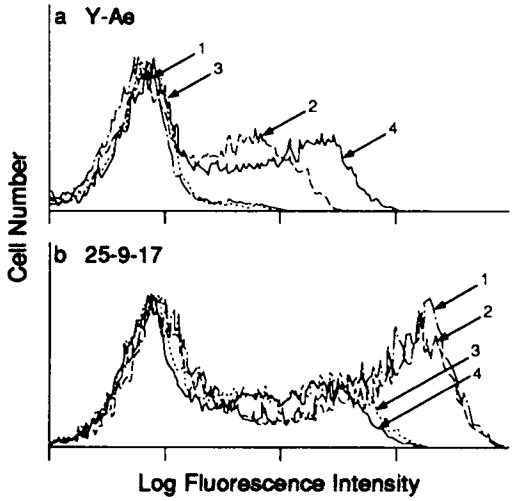

Figure 7. Invariant chain–deficient mice produce empty class II molecules. Wild-type (*1* and *2*) or mutant (*3* and *4*) spleen cells were cultured at 37°C overnight in the presence of E_α 52–68 (*2* and *4*) or, as a control, HEL 46–61 (*1* and *3*) peptides and then stained with the biotin-labeled antibodies followed by FITC-labeled avidin and analyzed by FACS®.

tightly bound peptides during transport to the cell surface, it was of interest to evaluate the functional consequences with respect to class II–restricted antigen presentation. Irradiated spleen cells were assessed for their ability to stimulate IL-2 production or T cell proliferation in the presence of a panel of I-A^b-restricted T cells. As shown in Fig. 8, mutant spleen cells stimulated stronger responses in the presence of the already processed peptides, HEL 74–88 and E_α 52–68, but they failed to present the native protein antigens, KLH and IgG2a^a. We observed no significant response directed towards endogenous IgG2a^a (Fig. 8 *B*). These findings support the conclusion that MHC class II molecules produced in the absence of invariant chain do not efficiently acquire peptide ligands in endocytic vesicles during transport to the cell surface, and are more accessible to exogenously added peptides.

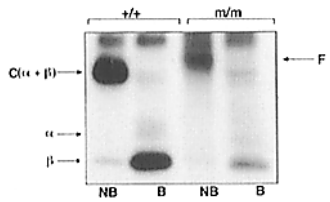

Figure 6. Structurally distinct class II molecules on the cell surface. Extracts of ^{125}I surface-labeled spleen cells were immunoprecipitated with Y3P mAb. Complexes were solubilized at 100°C (*B*) or at room temperature (*NB*) and analyzed by SDS-PAGE under reducing conditions. *C* and *F* indicate the positions of compact and floppy α/β heterodimers, respectively. Please note cell surface murine α chains label poorly with iodine.

Decreased Numbers of CD4$^+$8$^-$ T Cells in the Thymus and Periphery. MHC class II molecules play a critical role in both thymic selection and peripheral expansion of CD4$^+$ T lymphocytes, as clearly demonstrated by recent experiments analyzing mice lacking MHC class II molecules (64, 65). As shown above, invariant chain–deficient mice exhibited markedly decreased levels of class II expression and a dramatic change in peptide occupancy of class II surface antigens. We therefore investigated possible effect(s) on CD4$^+$ T cell maturation. When thymocytes from homozygous mutant mice were triply stained with anti-CD4, anti-CD8, and anti-TCR mAbs, we observed a striking reduction in the numbers of mature CD4$^+$8$^-$TCR^{hi} T cells (Fig. 9 *A*). The residual CD4$^+$CD8$^-$TCR^{hi} population included fully mature cells expressing low levels of heat-stable antigen (data not shown). In contrast, mature thymocytes with a CD4$^+$CD8$^-$TCR^{hi}HSA^{lo} phenotype have not been detected in class II–deficient mice (64, 66). The number of immature CD4$^+$CD8^{lo}TCR^{intermediate} cells (67) present in invariant chain–deficient mice did not differ significantly from that seen in wild-type controls. We found that CD4$^+$CD8$^+$ thymocytes expressed slightly increased levels of TCR, as is the case for class II–deficient mice. Invariant chain–deficient mice also show a marked reduction in the number of CD4$^+$CD8$^-$ T cells present in the periphery (Fig. 9 *B*). Although these results were obtained using lymph node cells, decreased numbers of mature CD4$^+$ T lymphocytes were similarly observed in experiments analyzing splenic T cells (data not shown), and in all the mutants analyzed (a total of five animals in independent experiments). These results extend previous work

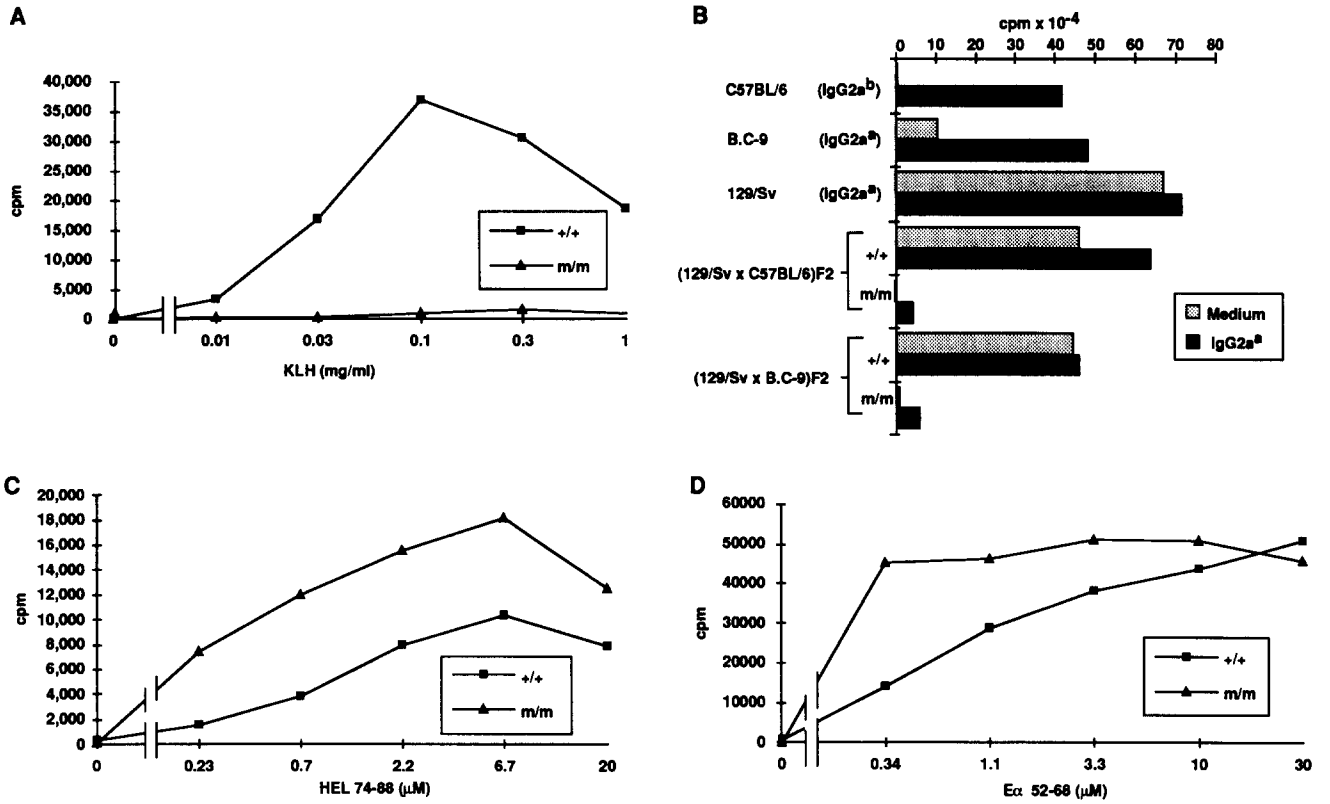

Figure 8. Defective presentation of native protein antigens and enhanced responses directed towards peptides. Irradiated spleen cells were added to cultures containing I-A^b-restricted T cells specific for the indicated antigens. (*A*, *C*, and *D*) Culture supernatants were assayed for IL-2 content in a secondary assay with CTLL cells. (*B*) Proliferation of IgG2a-specific T cells was measured as [^{3}H]thymidine incorporation. Responses in the presence of medium alone or added LPC1 myeloma protein (500 μg/ml) are shown.

on positive selection and further indicate that coexpression of the invariant chain is necessary for the normal development of CD4$^+$ T cells.

Discussion

To evaluate the role of invariant chain under normal physiological conditions, we generated an invariant chain–deficient mouse strain by targeted gene disruption in embryonic stem cells. The dramatic effects on class II structure and function described in this report are therefore specifically attributable to the absence of invariant chain. The present results demonstrate that invariant chain is a specialized and essential chaperone, necessary for efficient production of α/β heterodimers, that plays a pivotal role in peptide capture, and thus ultimately guides the performance of class II molecules as ligands for CD4$^+$ T cells in the thymus and the periphery.

Surprisingly, we found that the invariant chain functions at an early stage in class II biosynthesis promoting optimal class II assembly, though as predicted by earlier observations (25, 26), coexpression of the invariant chain is not absolutely required for subunit interactions. Indeed a likely possibility was that assembly of newly synthesized α and β chains might occur spontaneously due to the intrinsic affinity of certain α/β pairs (24, 68–70). Consistent with this, there is evidence that invariant chain trimers are assembled with preexisting α/β dimers (14). Additionally, recent experiments have demonstrated that conserved residues present in the transmembrane domains of α and β chains provide stability for subunit association (71). Unexpectedly, we observed significantly fewer dimers and consistently higher amounts of free α and β chains in mutant spleen cells. These results demonstrate for the first time that the invariant chain plays a critical role in the production and/or maintenance of α/β dimers under normal physiological conditions.

Recent experiments have shown that the ER lumen is a highly dynamic environment where oligomeric proteins undergo continuous ATP-dependent folding, unfolding, and refolding (72). Thus, the invariant chain may be necessary to prevent inappropriate disulfide bond formation and/or improper folding of the subunits. In this sense, the invariant chain may act as a true molecular chaperone promoting and stabilizing subunit interactions. Consistent with this, Schaiff et al. (73) recently found ER resident stress proteins GRP94 and ERp72 associated with HLA-DR molecules produced in the absence of invariant chain. Similarly, we observed that the majority of α/β heterodimers produced by invariant chain–deficient mice failed to acquire complex N-linked glycans and showed no evidence of export past the *cis*-Golgi complex. In contrast, α/βI complexes can escape ER resident

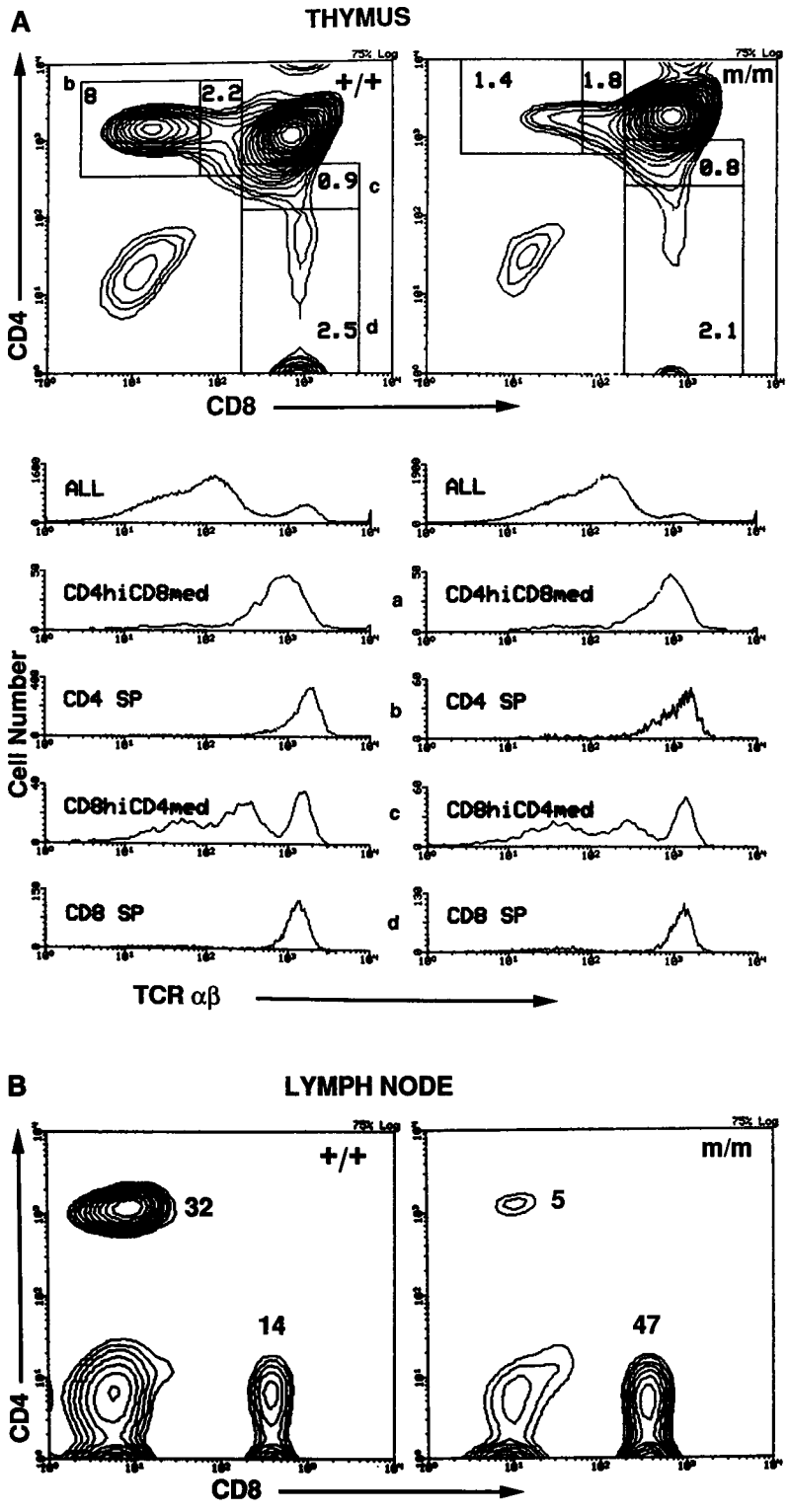

Figure 9. Absence of mature $CD4^+8^-$ T cells in the thymus and periphery. (*A*) The upper portion shows contour plots of thymocytes analyzed for CD4 and CD8 expression. Numbers refer to the percentage of total cells found in each indicated gate. The lower portion shows single-color histograms for TCR expression of the cells within each of the gates indicated on the contour map. (*B*) Lymph node cells analyzed for CD4 and CD8 expression.

chaperones that bind incompletely assembled and/or misfolded molecules.

Perhaps invariant chain serves as the functional equivalent of peptide for class II molecules to stabilize subunit interactions. It is known that peptide ligand is necessary to promote the association of MHC class I heavy chain and β_2m light chain (74–76). The invariant chain may contact residues present on both chains (10–12), perhaps located in or near the peptide binding site. This association may be necessary to protect the otherwise exposed, empty groove from being

recognized by ER resident proteins. This idea is supported by the observation that empty class II molecules produced in insect cells tend to aggregate, a property reversed upon occupancy of the peptide binding site (24). Additionally, haplotype-mismatched α/β pairs are retained in the ER/*cis*-Golgi complex in the absence of invariant chain (27), and the residues responsible for defective expression of these particular α/β combinations are located within the peptide-binding groove (10, 56, 57). The ability of the invariant chain to interact with newly formed α/β dimers in the absence of their folding into a mature structure may be essential to maintain an open conformation of the binding site ready for peptide occupancy.

Recent work has shown that MHC class II molecules can undergo a structural transition during peptide acquisition such that they become resistant to SDS-induced denaturation (24, 52, 58–60), allowing us to examine the contribution of invariant chain to peptide occupancy in a T cell–independent manner. In the absence of invariant chain, there was no detectable synthesis of mature compact SDS-resistant dimers. Surprisingly, the few α/β heterodimers inefficiently transported to the cell surface displayed a reduced mobility in SDS-PAGE. In contrast to the unstable dimers produced by somatic mutants exhibiting antigen processing defects (77–79), this novel SDS-resistant species closely resembles the so-called floppy dimers previously described by Dornmair et al. (61, 62) generated upon exposure of compact dimers to low pH in the absence of peptide (59). In the present study, these molecules were observed under physiological conditions in biosynthetic labeling experiments.

It is possible that these structural differences in part reflect the absence of transport to peripheral endosomes where prolonged exposure to acidic pH, proteolytic enzymes, and/or additional factors may potentially affect subunit associations and the shape of the peptide-binding groove. Consistent with this, exposure to low pH has been shown to increase functional antigen presentation (80), the amount of peptide quantitated in direct binding studies (81), as well as stable complex formation (59). On the other hand, previous studies suggested that floppy molecules adopt an extended conformation accounting for their slower migration in SDS-PAGE due to the absence of a tightly associated peptide (59). Consistent with this, direct peptide binding studies and functional peptide presentation assays described in this report demonstrate that surface class II molecules produced by mutant cells behave as if empty, or occupied by an easily displaced peptide.

In light of the evidence that the invariant chain serves to protect the peptide-binding groove and its removal by proteolytic cleavage in vitro promotes peptide occupancy (11, 20, 22, 23), it was surprising that class II molecules produced by mutant spleen cells showed no evidence of stable peptide occupancy. Because invariant chain–deficient mice expressed normal levels of MHC class I surface antigens, it seems clear that peptides normally bound by class I molecules during constitutive export cannot function as appropriate MHC class II ligands. Numerous reports have described an endogenous class II–restricted antigen presentation pathway (82–87), and there is evidence that presentation of self-peptides is independent of invariant chain coexpression (29, 33). On the other hand, the present data indicate that under physiological conditions, the majority of peptides presented in association with class II molecules, including self-peptides and those derived from exogenous antigens, require invariant chain for their capture. We also observed defective presentation of intact protein antigens, such as KLH and IgG2a, that depend on endocytic processing. Exceptional antigens such as RNAse, showing invariant chain–independent presentation (32), may gain access to class II molecules via a distinct pathway. Under physiological conditions, excess invariant chain (88, 89) may be necessary to insure that newly formed α/β heterodimers are quantitatively transported to endocytic vesicles, thus promoting selective interactions with peptides derived from internalized antigens. This intracellular transport route avoids surface expression of empty molecules, and selects against occupation by peptides present in the extracellular environment.

The present experiments largely examined class II expression by the predominant population of splenic B cells. Class II molecules and the invariant chain are coexpressed in diverse cell types such as B cells, macrophages, dendritic cells, and Langerhans cells (90–92), and are also coordinately upregulated in response to lymphokines (93–96). We found that invariant chain–deficient mice, like those entirely lacking MHC class II molecules, have greatly reduced numbers of $CD4^+$ T cells. The present results therefore demonstrate a contribution of invariant chain to class II expression by thymic stromal cells responsible for positive selection. Because in invariant chain–deficient mice, class II expression was affected both quantitatively and qualitatively in terms of structure and peptide occupancy, these mutations might affect thymic selection by different mechanism(s). In sum, the invariant chain–deficient mouse strain described in this report should prove useful for analyzing functional consequences of invariant chain expression in diverse Ia^+ accessory cell populations such as those responsible for thymic education, T-B cooperation, autoimmunity, and tissue graft rejection.

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