

Invariant Chain Controls H2-M Proteolysis in Mouse Splenocytes and Dendritic Cells

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Abstract

The association of invariant (Ii) chain with major histocompatibility complex (MHC) class II dimers is required for proper antigen presentation to T cells by antigen-presenting cells. Mice lacking Ii chain have severe abnormalities in class II transport, T cell selection, and B cell maturation. We demonstrate here that H2-M, which is required for efficient class II antigenic peptide loading, is unexpectedly downregulated in splenocytes and mature dendritic cells (DCs) from Ii^{-/-} mice. Downregulation reflects an increased rate of degradation in Ii^{-/-} cells. Degradation apparently occurs within lysosomes, as it is prevented by cysteine protease inhibitors such as E64, but not by the proteasome inhibitor lactacystin. Thus, Ii chain may act as a lysosomal protease inhibitor in B cells and DCs, with its deletion contributing indirectly to the loss of H2-M.

Key words: invariant chain • H2-M • DM • cathepsin • dendritic cell

Introduction

MHC class II molecules are heterodimeric cell surface glycoproteins that bind exogenously derived antigenic peptides and present them to CD4⁺ T cells (1, 2). Class II α and β chains are translocated into the endoplasmic reticulum (ER), where they form nonamers with invariant (Ii) chain (3). Ii chain prevents the binding of immunogenic peptides due to the presence of a 14-amino acid domain (CLIP) that occupies the peptide-binding groove of α/β dimers (3). After Ii degradation in the endocytic pathway, the MHC-encoded molecules HLA-DM (or H2-M in the mouse) and HLA-DO (H2-O) facilitate the removal of CLIP from α/β dimers, allowing peptide binding (4–6).

Ii chain has been implicated in functions such as ER export, endosome targeting, and even B cell maturation (3, 7). Two alternatively spliced Ii isoforms exist (p31 and p41), distinguished by a 64-residue domain in the luminal por-

tion of p41 (8). The isoforms are expressed differently in various APCs and regulate the presentation of certain antigen epitopes in B cells (9). This difference may reflect protease inhibition by the amino acid insertion in p41, as it has been shown to inhibit the lysosomal cysteine protease cathepsin L both in vitro and in vivo (9, 10). Therefore, Ii chain may contribute to the modulation of the proteolysis in the endocytic pathway and thus modulate antigen processing indirectly (11, 12). We demonstrate here that Ii chain deletion leads to the lysosomal degradation of H2-Mb in APCs, suggesting that Ii chain is required to prevent the proteolysis of H2-M and perhaps of other proteins. This feature may help explain how Ii chain expression affects T cell selection and B cell maturation independently from its effect on MHC class II traffic (13–15).

Materials and Methods

Mice and Cell Culture. C57BL/6 (control) and Ii^{-/-}, Ii p31^{lo} (14, 16), class II^{-/-}, and class II/Ii^{-/-} mice (the gift of P. Marrack, University of Colorado Health Sciences Center, Denver, CO) were kept in a pathogen-free environment for 7–8 wk before killing. Splenocytes were obtained as described (7). Bone marrow-derived dendritic cells (DCs) were cultured as described (17). After purification, immature DCs were characterized by im-

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munofluorescence and processed in parallel with the LPS-treated DCs. Epidermal sheets from mouse ears were explanted and fixed with 3.5% paraformaldehyde (17).

RNA Analyses. Poly(A)⁺ RNA purification and PCR were performed as described (18). The primers used here to detect I-A^b α, H2-Mα, and H2-Mβ are identical to the primers described previously (19).

Pulse-Chase Radiolabeling Experiments. 3 × 10⁷ late DCs were pulse labeled with 7.5 mCi/ml of [³⁵S]methionine Translabel (ICN) and chased as described (17). α/β₂ H2-M was immunoprecipitated with the rat mAb 2C3A (a gift of L. Karlsson, R.W. Johnson Research Institute, San Diego, CA) and protein A-Sepharose. Quantification was performed using a PhosphorImager[®] (Molecular Dynamics).

Antibodies and Immunofluorescence. Murine I-A was detected using Rivoli, a rabbit polyclonal antibody directed against the conserved class II I-A β chain cytoplasmic tail (17); H2-Mb was detected by immunofluorescence and immunoblots with the rabbit polyclonal antibodies anti-H2-Mb Ulm (17), K553 (19; a gift of L. Karlsson) and anti-H2-Mb cytoplasmic tail (a gift of S. Amigorena, Institut Curie, Paris, France). Rabbit polyclonal anti-human cathepsin B was from Calbiochem. Murine Igp-B/lamp-2 and Ii chain were detected using the rat mAbs GL2A7 (17) and In1 (20). For immunofluorescence, cells were fixed in 3.5% paraformaldehyde (in PBS) and permeabilized as described (17). All secondary antibodies were purchased from Jackson ImmunoResearch Laboratories.

Protease Inhibitors. The cathepsin S-specific inhibitor LHVS (a gift of Hidde Ploegh, Harvard Medical School, Boston, MA) was added to the culture medium of late DCs (preincubated with LPS for 18 h) at the concentration of 2 nM and 1 μM. E64 was obtained from Sigma Chemical Co. and used at 20 μM. The cathepsin B-specific inhibitor CA074me used at 5 μM was obtained from Bachem.

Results and Discussion

Ii chain is responsible for the correct folding and transport of MHC class II in APCs. However, discrepancies between class II transport and T cell selection in Ii^{-/-} mice suggested that Ii chain deletion may epistatically affect another critical component of antigen processing, such as H2-M. To investigate a possible relationship between Ii chain deletion and H2-M expression, we isolated splenocytes from wild-type and Ii^{-/-} mice and probed for H2-M by Western blot. As shown in Fig. 1 A, greatly decreased steady-state levels of H2-Mb (<5% of control) were detected in Ii^{-/-} splenocytes, whereas similar levels of cathepsin B were detected.

We next asked if the decrease in H2-M might somehow reflect the ER accumulation of misfolded class II molecules that occurs in the absence of Ii chain (16). Misfolded class II might interact with H2-M resulting in ER retention and degradation. However, H2-M levels were reduced in splenocytes derived from Ii^{-/-} and class II^{-/-} double deficient mice (Fig. 1 B). Thus, Ii chain affected H2-M expression independently of its interaction with MHC class II.

To determine if H2-M disappearance correlated with the amount of Ii chain expressed, we quantified H2-Mb protein in splenocytes from Ii^{-/-} mouse reconstituted with the p31 isoform of Ii chain at very low level of expression

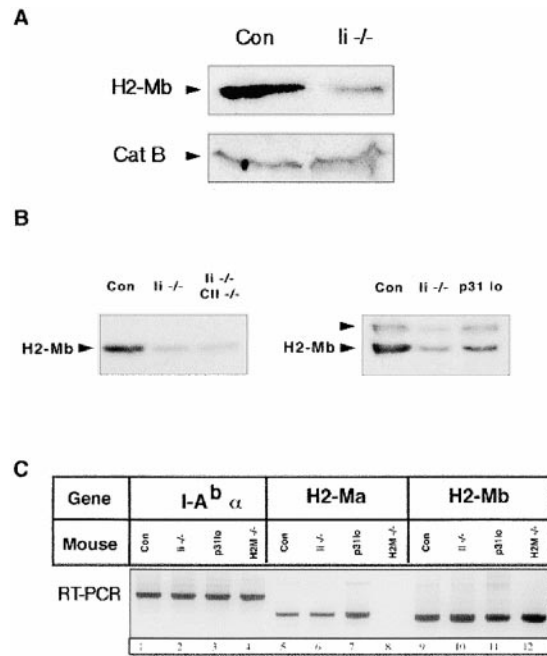


Figure 1. H2-M is downregulated in Ii^{-/-} mouse splenocytes. (A) Splenocytes were isolated from control (C57BL/6, Con) or Ii^{-/-} mice. After separation by SDS-PAGE, samples were immunoblotted and H2-Mb was detected using the rabbit polyclonal antibody K553. A severe depletion of H2-M (95%) can be observed in the Ii-deleted mice. (B) Splenocyte extracts from control (C57BL/6), Ii^{-/-}, Ii/MHC class II (CII)^{-/-}, or p31^{lo} mice were immunoblotted for H2-Mb with an anticytoplasmic tail or K553. H2-Mb1 (right) appears as a doublet due its single N-glycosylation detected by the cytoplasmic-tail antibody used here instead of K553, which does not detect this form (left). In Ii chain-deleted mice and double knockout mice, the H2-M level was strongly reduced compared with the control mice (left). However, in p31^{lo} mice (right), H2-M levels were found to be intermediate between control and knockout mice, suggesting a direct correlation between Ii chain levels and H2-M disappearance. (C) To determine if H2-M downregulation was occurring at the transcriptional level, RT-PCR was performed on control, Ii^{-/-}, p31^{lo}, and H2-Mα^{-/-} mice. All levels of transcription for I-A^b α, H2-Mα, and H2-Mβ are found to be identical except in the control mice lacking H2-Mα.

(p31^{lo} [14]). Surprisingly, even small amounts of p31 (1% of wild-type) disproportionately protected against H2-M disappearance, allowing its accumulation at much higher levels (20% of control; Fig. 1 B), despite the fact that Ii expression in p31^{lo} splenocytes was insufficient to restore proper class II transport (14).

mRNA levels of MHC class II and H2-M molecules were determined by reverse transcription (RT)-PCR (Fig. 1 C). No differences in the levels of I-A^b, H2-Mα, or H2-Mβ mRNA could be detected in mice lacking Ii chain (Ii^{-/-}), mice expressing low levels of the p31 (p31^{lo}) chain (14), or control C57BL/6 mice (Fig. 1 C). Therefore, the absence of Ii chain had no effect on the transcription of MHC class II and H2-M, suggesting that H2-Mb downregulation occurred posttranslationally.

DCs activate Ii chain degradation during maturation (20), suggesting that the absence of Ii chain might somehow render H2-M more susceptible to degradation. H2-M distribution was examined in immature and mature (LPS-

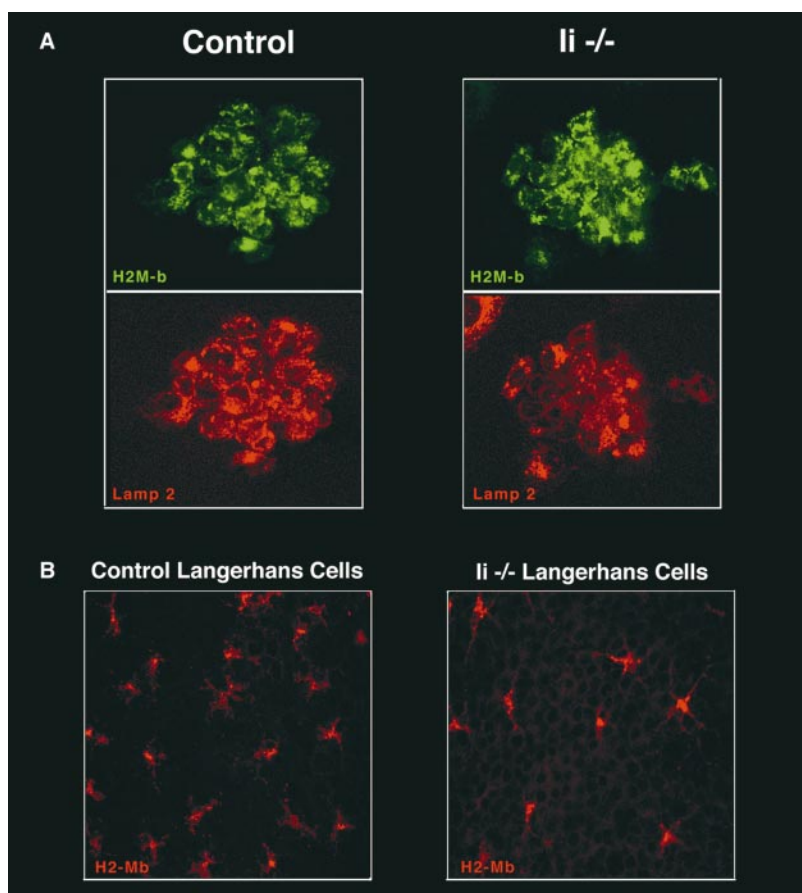


Figure 2. H2-M is not affected in *Ii*^{-/-} immature DCs. (A) Immunofluorescence microscopy of bone marrow-derived purified clusters of immature DCs stained for H2-Mb (green) and lamp-2 (red). Control cells (left) and *Ii*^{-/-} cells (right) do not display any difference for H2-M levels. (B) Confocal immunofluorescence microscopy of immature Langerhans cells from control (left) and *Ii*^{-/-} mice (right) stained for H2-M (red). No difference in H2-M staining intensity could be detected between these immature Langerhans cells.

treated) bone marrow-derived DCs from control and *Ii*^{-/-} mice. DCs were stained for H2-M and the lysosomal marker protein lgp-B/lamp-2 and observed by confocal microscopy. In immature DCs, still in clusters (Fig. 2 A), the staining intensity of H2-Mb and its colocalization with lgp-B/lamp-2 were similar in cells obtained from control or *Ii*^{-/-} mice. This was confirmed by staining immature Langerhans cells in situ, in which similar amounts of H2-M were detected in *Ii*^{-/-} and control epidermis (Fig. 2 B). Thus, in immature DCs, the absence of Ii chain did not affect delivery of H2-M to the lysosomes (21) or lead to its downregulation.

However, in mature DCs H2-M was barely detectable in cells from *Ii*^{-/-} mice (Fig. 3). The lgp-B/lamp-2 staining in these cells was indistinguishable from controls and exhibited a distinct perinuclear pattern characteristic of mature DCs (17). As expected from the Western blot experiments, H2-M was not noticeably downregulated in class II^{-/-} DCs (Fig. 3).

To evaluate the half-life of H2-Mb, a pulse-chase experiment was performed. Control and *Ii*^{-/-} mature DCs were radioactively labeled for 30 min and chased for various lengths of time. H2-M was then immunoprecipitated and quantified by PhosphorImager®. Since the H2-Mb band was most readily quantifiable, we determined its half-life at 42 h in controls but only 10 h in the *Ii*^{-/-} cells (Fig. 4 B). The rapid degradation of H2-Mb in the *Ii*^{-/-} cells could be at least partially slowed by adding 1 μ M LHSV, a cysteine

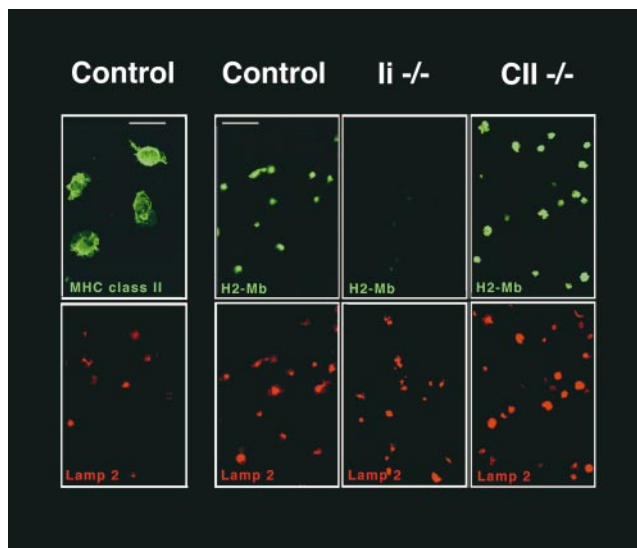


Figure 3. H2-M is downregulated in *Ii*^{-/-} but not class II^{-/-} mature DCs. Mature LPS-treated control DCs (left) were stained for class II (top, green) and lgp-B/lamp-2 (bottom, red) to demonstrate the maturity of these cells. Control and deleted mature DCs were stained for H2-Mb (top, green) and lgp-B/lamp-2 (bottom, red). In mature DCs, lysosomes converge in the cell center and appear as a unique concentrated H2-M/lamp-2-positive organelle. H2-M is severely downregulated in *Ii*^{-/-} DCs compared with the control or MHC class II (CII)^{-/-} cells. Lgp-B/lamp-2 levels (right, red) are found to be unchanged in all of the mice strains. H2-Mb downregulation is linked to the absence of Ii and to the maturation of DCs but not to the absence of MHC class II. Some contaminating granulocytes are found to be negative for H2-M in control cells and class II^{-/-}. Bars, 100 μ M.

protease inhibitor, during the chase period (Fig. 4 B). However, the effect was only evident at longer time points, possibly reflecting the time required for the LHVS to reach inhibitory concentrations within endocytic organelles. Thus, the disappearance of H2-M upon maturation of $Ii^{-/-}$ DCs appears to reflect H2-M degradation by lysosomal cysteine proteases. No differences were observed in acquisition of Endo H resistance, confirming that Ii chain was not required for transport through the Golgi complex (19, 21).

As previously observed in B cells (22), in DCs H2-M could be precipitated with Ii chain after a 30-min pulse, reflecting the presumptive interaction of these molecules in the ER (Fig. 4 C). Although both Ii chain splice forms were coimmunoprecipitated, approximately fivefold more p41 than p31 was detected, suggesting a higher affinity of p41 for H2-M. This preference is even far greater, as DCs express about three times more p31 than p41 (Fig. 4 D).

To further demonstrate the involvement of cysteine proteases in H2-Mb downregulation, early and late DCs from control and $Ii^{-/-}$ mice were incubated with cysteine protease inhibitors and analyzed by immunoblot (Fig. 5).

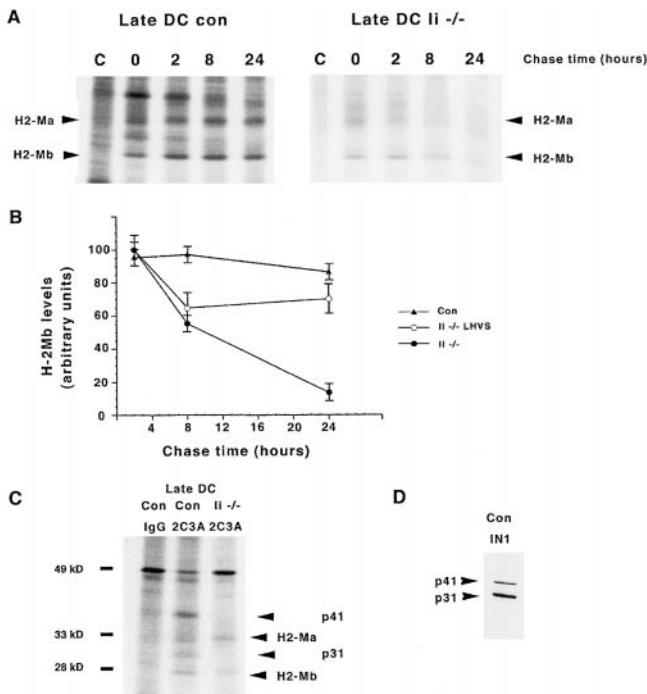


Figure 4. Quantitation of H2-M half-life. (A) Immunoprecipitation of S^{35} -pulsed and chased H2-M with the mAb 2C3A was performed. H2-Mb was found to be degraded almost completely after 24 h in $Ii^{-/-}$ DCs in contrast to the control (C) DCs. (B) Quantitation of H2-Mb immunoprecipitated with 2C3A ($n = 2$) from control (Con) or $Ii^{-/-}$ late DCs treated or not with LHVS (1 μ M) during the chase. A clear difference can be observed after 24 h of chase between the control and $Ii^{-/-}$ DCs. Interestingly, LHVS treatment during the chase prevents H2-Mb degradation, suggesting a cysteine protease involvement. (C and D) In control DCs, the p41 alternatively spliced form of Ii chain is preferentially coimmunoprecipitated with H2-M (2C3A) after 30 min of radioactive labeling, whereas p31 is the main Ii chain isoform immunoprecipitated using the IN1 mAb.

Although H2-M was slightly (20%) reduced in early $Ii^{-/-}$ DCs, a striking decrease was observed in the LPS-treated mature cells where H2-M was found at only 5% of the amount in controls. When the mature $Ii^{-/-}$ DCs were also treated with the inhibitor E64 (20 μ M) or LHVS (1 μ M) (11), H2-M levels were partially rescued to \sim 50% of control (Fig. 5, A and B). This protective effect was not observed at 2 nM LHVS where only cathepsin S was inhibited (20), suggesting that other cysteine proteases were responsible for H2-Mb degradation. When 5 μ M of the cathepsin B inhibitor CA074me (9) was used, a slight rescue of H2-Mb was observed, suggesting that cathepsin B might play a role (Fig. 5 C).

To rule out the possible degradation of H2-M in the cytosol, as occurs for misfolded proteins in the ER, mature $Ii^{-/-}$ DCs were incubated with lactacystin. Lactacystin is a proteasome inhibitor known to prevent degradation of free ER MHC class I (23) and MHC class II (24). 50 μ M of lac-

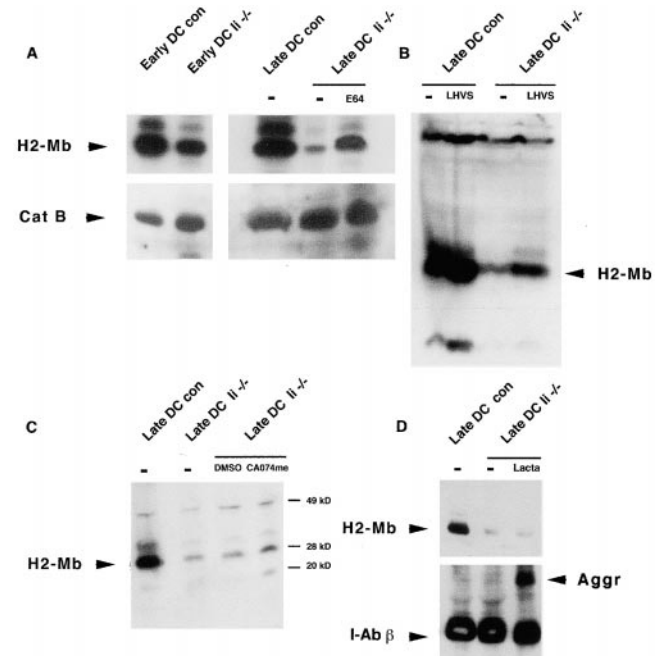


Figure 5. Cysteine proteases inhibition prevents H2-M degradation in mature DCs. (A) Early and late (LPS-treated) DCs from control (con) or $Ii^{-/-}$ mice were purified and treated with protease inhibitor before immunoblot for H2-Mb and cathepsin B. H2-Mb was mildly downregulated in $Ii^{-/-}$ early DCs and virtually absent from $Ii^{-/-}$ late DCs. Incubation of these cells with the cysteine protease inhibitor E64 for 24 h allowed for an efficient recovery of H2-M. Little variation in cathepsin B expression was observed between control and $Ii^{-/-}$ cells (bottom) or during maturation. This observation confirms that H2-M is actively degraded by cysteine proteases in the endocytic pathway of late DCs. (B) LHVS at 1 μ M but not at 2 nM (not shown) had the same effect as E64, suggesting that lysosomal cysteine proteases but not cathepsin S are degrading H2-M. (C) $Ii^{-/-}$ DCs were treated for 18 h with 5 μ M CA074me, resulting in partial H2-M rescue. The specificity of CA074 for cathepsin B suggests a role of this protease in H2-M degradation. (D) $Ii^{-/-}$ DCs were treated for 18 h with 50 μ M lactacystin, with no effect on H2-M disappearance. MHC class II (I-A^b) molecules were detected to demonstrate accumulation of class II aggregates (Aggr) in the lactacystin (Lacta)-treated cells.

tacystin somewhat enhanced the accumulation of aggregated MHC class II but had no effect on H2-M disappearance in mature $Ii^{-/-}$ DCs (Fig. 5 D).

The regulation of the degradation of H2-M, through the inhibition of lysosomal cathepsins, suggests that Ii chain may function as a protease inhibitor that may help to protect H2-M and possibly other proteins against proteolysis in DC lysosomes. This role for Ii chain has been previously suggested on the basis of two observations. First, a potent inhibitory effect on cathepsin L is mediated by the 64-amino acid segment encoded by the alternatively spliced exon of p41 (9, 10). Second, Ii chain shares significant amino acid sequence homology (40–45%) with various cysteine protease inhibitors, the cystatins (25).

Cathepsin L is present at minimal levels in DCs, and its activation by the absence of Ii or by DC maturation was not detected (not shown). However, p41 seems to be associated preferentially with H2-M, and its protease inhibitory activity could be relevant for the protection of the dimer. However, in mice expressing a low level of p31 ($p31^{lo}$) H2-M degradation was at least partially reduced. Thus, the inhibitory effect of Ii chain on degradation is not solely dependent on p41 and is consistent with the fact that p31 bears significant sequence homology to the cystatin superfamily (25). Interestingly, transfection of Ii p31 in fibroblasts resulted in the formation of enlarged endosomes and a delay in transport from endosomes to lysosomes (26). A similar phenomenon has also been observed in cells treated with the protease inhibitor E64 or leupeptin, further supporting a protease inhibitory function for Ii chain.

H2-M degradation is dramatically increased with DC maturation in $Ii^{-/-}$ mice. We demonstrated recently that members of family 2 cystatins control the proteolytic endocytic environment of immature DCs (20). In immature $Ii^{-/-}$ DCs, the lack of Ii chain might be compensated by antiprotease effects of cystatins, which normally control lysosomal cathepsins in these cells. Upon activation by LPS, downregulation of cystatins combined with the absence of Ii chain would render H2-M more susceptible to degradation. However, in splenocytes the degradation of H2-M occurs even without LPS activation. Thus, B cells may not exhibit the type of developmental regulation of protease activity seen in DCs, or B cells may express a different complement of proteases to which H2-M is more susceptible.

Ii chain has been implicated in modulating B cell maturation (7, 15) as well as T cell selection (13–15). Surprisingly, significant amounts of peptide-loaded MHC class II have been detected at the surface of $Ii^{-/-}$ APCs, especially DCs expressing the I-A^d and I-A^k haplotypes (13, 15). These observations suggest that in some strains of $Ii^{-/-}$ mice, impaired T cell selection is not the direct consequence of an abnormal transport of MHC class II molecules. Our results suggest that H2-M function and therefore the peptide repertoire presented may be altered in such mice. As a consequence, H2-M degradation could contribute to the observed decrease in CD4⁺ T cells in the thymus of all Ii chain-deficient strains and the milder phenotype

for maturation of CD4⁺ T cells in the periphery of the $Ii^{-/-}$ Balb/c (I-A^d) mice (15).

The precise contribution of H2-M disappearance to the phenotypes observed in $Ii^{-/-}$ APCs is difficult to establish because the dependency of MHC class II on both Ii and H2-M varies greatly according to the haplotype (27). The fact that mice lacking both Ii and H2-M exhibit a nearly complete inhibition of CD4⁺ T cell selection supports the idea that Ii and H2-M deletions are synergistic (28). The partial rescue of H2-M by low level Ii chain would still explain why reconstituted mice ($p31^{lo}$) have normal CD4⁺ T cell positive selection despite the fact that MHC class II traffic is still strongly inhibited (14, 15). In any event, the fact that Ii chain deletion can indirectly cause H2-M downregulation will indicate that results obtained with $Ii^{-/-}$ mice should be carefully controlled for the possible contribution of H2-M dysfunction.

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