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Immunology

Invariant chain can function as a chaperone protein for class II major histocompatibility complex molecules

(protein folding/protein transport/antibody epitopes/endoplasmic reticulum retention)

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ABSTRACT **During biosynthesis, class II major histocompatibility complex molecules are intimately associated with invariant chain (Ii). The Ii–class II association has been shown to block peptide–class II binding and to affect the ultimate conformation of class II expressed on the cell surface. To assess the biochemical basis for the effects of Ii on class II, we have analyzed the biosynthesis of class II in EL4 cells transfected with I-A^d with and without Ii. In these studies, we found that Ii had a profound effect on the biosynthesis of I-A^d. In the absence of Ii, class II could form dimers efficiently, but these dimers appeared to be misfolded and this altered conformation resulted in the loss of some monoclonal antibody epitopes and inefficient transport from the endoplasmic reticulum to the Golgi. In addition, class II that was transported through the Golgi accumulated an abnormally increased molecular mass associated with N-linked glycosylation. Subsequent transfection of Ii into these cells resulted in recovery of normal class II conformation, causing a restoration of monoclonal antibody epitopes, efficient intracellular transport, and normal glycosylation. Together, these data indicate that Ii can have a profound effect on the folding, transport, and modification of class II molecules and suggest that one function of Ii may be to act as a class II-specific chaperone.**

Class II major histocompatibility complex molecules are heterodimers made up of two noncovalently associated transmembrane glycoproteins, the 34-kDa α chain and the 28-kDa β chain (1). These heterodimers are complexed intracellularly with a third glycoprotein called the invariant chain (Ii). This association with Ii occurs soon after translation of the α and β chains in the rough endoplasmic reticulum (ER) and continues during cytoplasmic transport through the Golgi apparatus (2, 3). Ii dissociates from the class II heterodimer just before the complex reaches the cell surface in a late intracellular compartment (2–4), and the vast majority of cell-surface class II is no longer associated with Ii (5).

Class II molecules function in the immune response by presenting foreign antigens to the CD4-positive subpopulation of T cells. These T cells recognize small processed peptide fragments of foreign antigens complexed with the class II molecule on the surface of an antigen presenting cell (6). Of particular interest, over recent years, has been defining both the mechanism(s) and the relevant intracellular compartments in which antigen presenting cells process and degrade antigens and then subsequently allow for class II association with the resultant antigenic peptide. Recent studies indicate that class II preferentially associates with exogenous antigens in an acidified late intracellular compartment, or endosome, where antigens are degraded into peptide fragments (6, 7). The association of class II with these exogenous antigens is thought to occur through a unique transport route for class II. This route involves an apparent delay in the intracellular transport of class II to the cell surface in a post-Golgi compartment that intersects the endocytic pathway (8). This intersection has also been detected in an electron micrograph study that colocalized class II and antigen in an endosomal compartment (9). The transport delay of class II in this endosomal compartment indicates that newly synthesized class II molecules may be critical for proper antigen loading in the antigen presenting cell.

Given the intimate intracellular association of newly synthesized class II with Ii, it has become of interest to determine the role of Ii in class II biosynthesis and transport and, ultimately, its role in class II-restricted antigen processing and presentation. Ii is not required for cell-surface transport of class II; however, in the absence of Ii, class II has an altered conformation at the cell surface (10). The goal of this study is to clarify where this conformational change occurs intracellularly and to determine any potential effects on class II transport caused by this conformational change. Here we show that synthesis of class II in the absence of Ii allows for efficient $\alpha\beta$ dimer formation, but these dimers are inefficiently transported from the ER to the Golgi, accumulate an altered glycosylation pattern, and stably lose some monoclonal antibody epitopes. Subsequent transfection of Ii into these cells results in recovery of a normal class II conformation, efficient intracellular transport, and a normal glycosylation pattern. These data indicate that one role for Ii is to function as a class II-specific chaperone, facilitating proper class II folding during assembly in the ER.

MATERIALS AND METHODS

Cell Lines and Antibodies. EL4 thymoma cells were cotransfected by electroporation with cDNA clones encoding I-A^d α and β chains (11) and class II-positive cells were selected and cloned as described (10). The resulting clone, 7.2.6, expressing I-A^d without Ii was then transfected with cDNA encoding the p31 form of murine Ii (12). The clone 15.3.3, expressing both I-A^d and Ii, was selected for Ii expression by Northern blot analysis and by staining with monoclonal antibody 40B (10). Cells were stained with a panel of I-A^d-reactive monoclonal antibodies—MKD6, M5/114, K24-199, and 40B—as described (10). The hamster monoclonal antibody P4H5 is specific for murine Ii (13).

Radiolabeling. Cells were metabolically labeled at 37°C at 2×10^6 cells per ml in leucine-free MEM (GIBCO) containing 300 μCi of [^{3}H]leucine per ml (1 Ci = 37 GBq; Amersham). For the continuous label experiments, cells were labeled for 6 hr at 37°C. For the pulse–chase analysis, cells were preincubated for 30 min in leucine-free medium, pulse labeled for 30 min, and then chased in complete medium containing unlabeled leucine for various times. For cell-surface labeling,

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Abbreviations: Ii, invariant chain; ER, endoplasmic reticulum; endo H, endoglycosidase H.

5×10^6 cells (>95% viable) were labeled with ^{125}I by the lactoperoxidase method.

Immunoprecipitation and Electrophoresis. Labeled cells were solubilized at 2×10^6 cells per ml in 0.5% Nonidet P-40/0.15 M NaCl/0.05 M Tris·HCl/5 mM EDTA/200 μg of phenylmethylsulfonyl fluoride per ml/25 μg of aprotinin per ml. Postnuclear lysates were precleared in normal rabbit serum and *Sac* I immunoprecipitin (BRL), followed by *Sac* I alone, and finally with protein A-Sepharose CL-4B (Pharmacia) or protein G-Sepharose fast flow (Pharmacia). Immunoprecipitations were carried out overnight at 4°C with immunoconjugates of MKD6, 40B, and P4H5 previously bound to protein A-Sepharose or M5/114 previously bound to protein G-Sepharose. Immunoprecipitates were washed in 0.2% Nonidet P-40/0.15 M NaCl/0.05 M Tris·HCl/5 mM EDTA, eluted by boiling in 2% SDS/62.5 mM Tris·HCl, pH 6.8/20% (vol/vol) glycerol/2% 2-mercaptoethanol and analyzed on SDS/10% polyacrylamide gels. Gels were processed with Enhance (NEN), dried, and autoradiographed at −70°C.

Enzymatic Treatments. For N-glycanase digests, washed immunoprecipitates were eluted with 0.1 M $NaPO_4$, pH 8.6/0.5% SDS/0.1 M 2-mercaptoethanol and heated at 90°C for 3 min; Nonidet P-40 was added to a final concentration of 0.5%. The eluates were incubated overnight at 37°C with and without N-glycanase (20 units/ml) (Genzyme) and analyzed by SDS/PAGE. Endoglycosidase H (endo H) (Genzyme) digests were performed similarly, except that the elution buffer contained 0.05 M $NaPO_4$ (pH 6.0) and 0.25% SDS, and the enzyme concentration was 50 milliunits/ml.

RESULTS

Ii Increases the Rate of Class II Transport. We have previously observed that Ii affects the conformation of class II at the cell surface in transfected L cells (10). To verify that this effect was not cell-type dependent, EL4 thymoma cells that do not express their endogenous class II and Ii genes (data not shown) were first transfected with I-A^d to generate clone 7.2.6, and then clone 7.2.6 was transfected with Ii to generate clone 15.3.3. This approach provided two class II-positive cell lines that differed only in Ii expression. These two clonal lines were stained with a panel of class II-specific monoclonal antibodies (Fig. 1). MKD6 and M5/114 react similarly with Ii-negative and Ii-positive transfectants, reflecting equivalent levels of cell-surface class II. In contrast, 40B and K24-199 bound Ii-negative cells less well than Ii-positive cells, consistent with our previous results (10). Because the majority of class II at the cell surface is not associated with Ii (5), the conformational change in class II caused by Ii coexpression is probably imparted by Ii intracellularly.

To investigate the effect Ii may have on the processing and maturation of class II intracellularly, the biosynthesis of class II was followed by pulse–chase analysis (Fig. 2). The assembly of class II heterodimers appears to be Ii-independent (Fig. 2*A*), as both α and β chains are detectable at equivalent levels in both transfectants at the 0 time point with the β-chain-specific monoclonal antibody M5/114. However, Ii does influence the maturation of class II. In Ii-negative cells, the precursor forms of α and β chains (indicated by α′ and β′) chase very slowly into larger molecular mass forms (indicated by brackets). In addition, the absence of Ii appeared to affect the glycosylation of class II, as the mature α chain in the Ii-negative transfectant increased in apparent molecular mass by ≈3 kDa compared to the mature α chain in the Ii-positive transfectant (Fig. 2*A*).

Transfection of Ii into these cells allows for synthesis of Ii (Fig. 2*B*) and association of Ii with newly formed αβ dimers (Fig. 2*A*, 0 time point). The association of class II with Ii dramatically increases the maturation rate of the αβ dimer

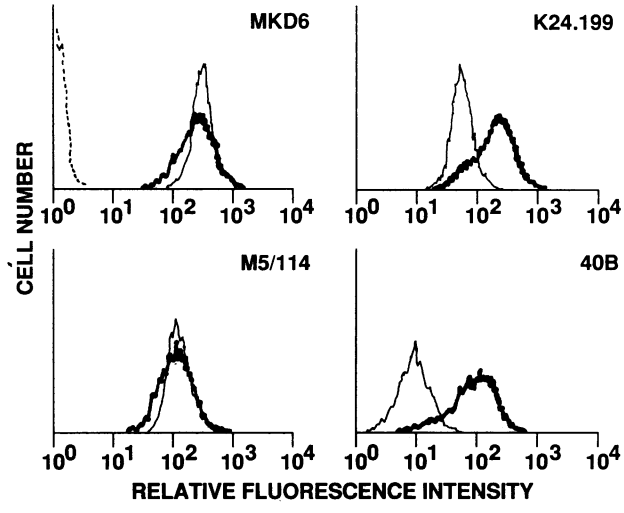

FIG. 1. EL4 transfectants express class II at the cell surface in an altered conformation in the absence of Ii. Ii-negative (7.2.6; thin line) and Ii-positive (15.3.3; thick line) transfectants were stained with a panel of I-A^d-reactive monoclonal antibodies and analyzed by flow microfluorimetry. Dashed line, staining profile for both transfectants stained with the secondary fluorescein isothiocyanate goat anti-mouse antibody reagent only. Mean fluorescence intensity (7.2.6, 15.3.3): MKD6 (300, 293), M5/114 (139, 114), K24.199 (62, 239), and 40B (10, 102).

(Fig. 2*A*) and the majority of class II chases into larger molecular mass forms by 1 hr. Because this increase in molecular mass during the intracellular maturation of cell-surface proteins is typically associated with complex sugar additions in the Golgi, these data suggest that Ii plays an important role in the efficient transport of class II from the ER to the Golgi.

Excessive N-Linked Glycosylation of Class II α Chain in the Absence of Ii. To assess the nature of the apparent increase in molecular mass of class II in Ii-negative cells, class II immunoprecipitates were digested with N-glycanase (Fig. 3, lanes 1–6). This enzyme removes all N-linked oligosaccharides from glycoproteins, whether or not they have been modified in the Golgi. After digestion with N-glycanase, all detectable α and β chains from both transfectants migrate at α_o and β_o (lanes 3 and 6). This suggests that the differences observed in the molecular mass of the α and β chains in the Ii-negative and Ii-positive transfectants are due to differences in processing of the N-linked oligosaccharide addition sites on the class II heterodimer and not to abnormal O-linked addition sites caused by the altered conformation of class II in Ii-negative cells.

The Small Molecular Mass Forms of α and β Chains in the Ii-Negative Transfectant Are Trapped in a Pre-Golgi Compartment. The slow maturation rate of precursor forms of α and β chains in Ii-negative cells suggests that these forms are retained in the ER. To test this possibility, class II molecules from Ii-negative cells were assayed for sensitivity to endo H (Fig. 3, lanes 7 and 8). Endo H cleaves high mannose N-linked sugars and resistance to the enzyme is imparted after transport through the Golgi (15). The large molecular mass forms of the α chain (α2) are resistant to endo H digestion, while the smaller forms of α and β chains are sensitive, suggesting that these latter forms have been retained in the ER. In addition, cell-surface labeling experiments (Fig. 4) demonstrate that only the large molecular mass forms of α and β chains reach the cell surface. These data suggest that Ii is required for efficient intracellular transport of class II heterodimers and, in the absence of Ii, a large proportion of class II appears to be retained in the ER, and

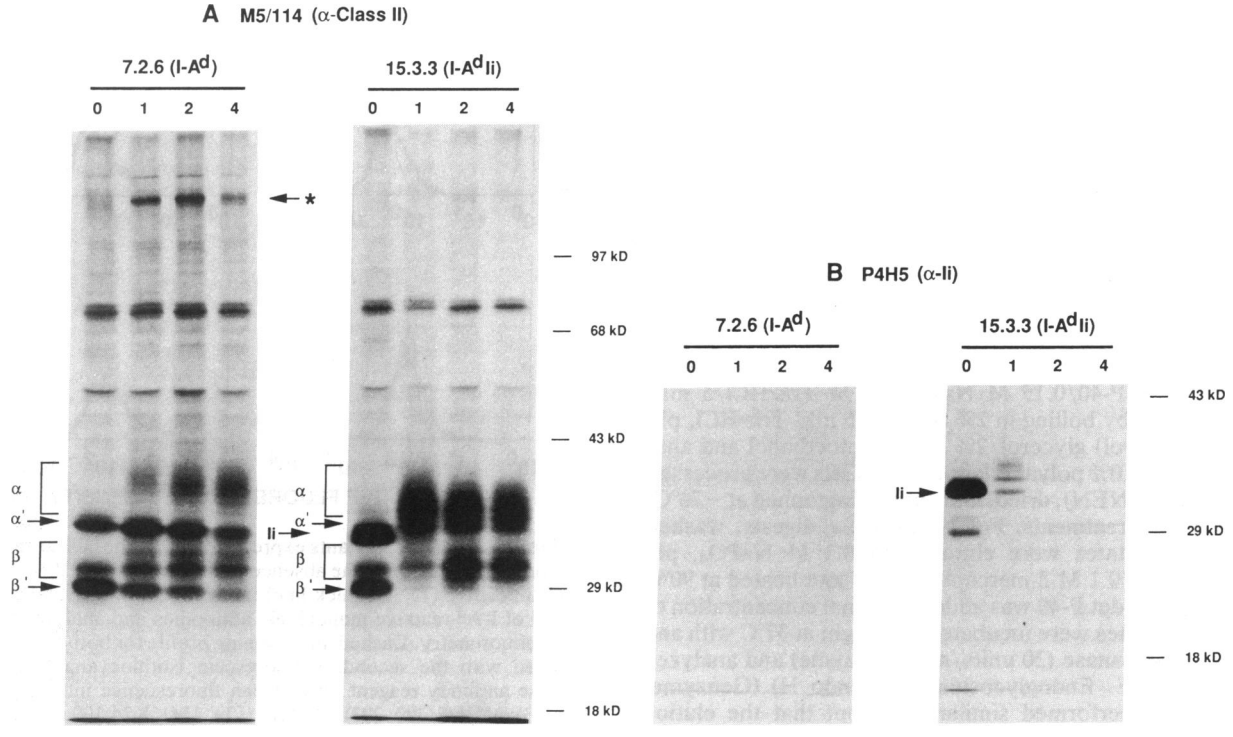

FIG. 2. Ii increases the rate of class II maturation from the ER to the Golgi. EL4 transfectants expressing either I-A^d (7.2.6) or I-A^d and Ii (15.3.3) were pulse labeled for 30 min with [^{3}H]leucine and chased for 0, 1, 2, or 4 hr. Class II molecules were immunoprecipitated with M5/114 (*A*) and Ii with P4H5 (*B*) and analyzed by SDS/PAGE. For clarity, the positions of the immature α and β chains (α' and β'), the mature α and β chains (brackets), and the position of Ii are indicated. The class II chain assignments were confirmed by two-step immunoprecipitations using chain-specific antisera on denatured class II heterodimers (as described in ref. 14; data not shown). The 1-hr chase point of the 15.3.3 cell is also slightly underloaded, causing less total class II to appear in this lane (this was confirmed by comparing several repeated pulse–chase experiments with these cells). The position of a band that preferentially coprecipitates with class II in the absence of Ii is indicated by the asterisk at ≈200 kDa. In addition, the positions of molecular mass standards are indicated.

the I-A^d dimers that do transit the Golgi to the cell surface have an altered glycosylation pattern.

Additional evidence for ER retention of class II dimers in the absence of Ii is shown in Fig. 2*A*. ER retention by retrieval from the salvage compartment between the ER and cis-Golgi allows access of the retained protein to glucosidase and mannosidase I (16). Trimming of the high mannose sugars by these enzymes results in a slight decrease in apparent molecular mass of the retained protein, as shown for the class II β chain in Fig. 2*A* and for the α chain in other experiments. These results suggest that, in the absence of Ii, the immature forms of class II are retained in the ER via retrieval from the salvage compartment.

The Ii-Dependent, Class II-Specific 40B Monoclonal Antibody Epitope Is Dependent on Proper Folding of Class II in the ER. The results described above indicate that Ii can influence the efficiency of intracellular transport, the glycosylation pattern, and the cell-surface conformation of class II. To determine whether these phenomena were related, immunoprecipitations with class II-specific antibodies that distinguish Ii-positive and Ii-negative cell lines were analyzed (Fig. 5). Ii-independent antibodies precipitate similar amounts of

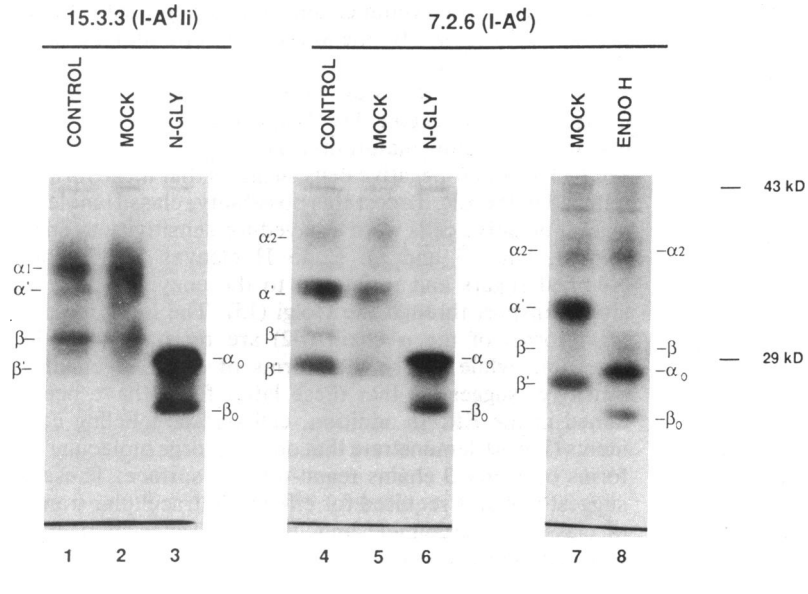

FIG. 3. The small molecular mass forms of I-Ad in the Ii-negative transfectant are retained in a pre-Golgi compartment. The EL4 transfectants 7.2.6 and 15.3.3 were labeled for 6 hr with [^{3}H]leucine, class II was immunoprecipitated with M5/114, immunoprecipitates were treated with N-glycanase (N-GLY), mock treated, or not treated at all (control) (lanes 1–6) or endo H or mock treated (lanes 7 and 8) and analyzed by SDS/PAGE. For clarity, the positions of the various α and β chains are indicated. α' and β' indicate the immature forms of α and β. α1 indicates the mature α chain(s) for 15.3.3; α2 indicates the mature α chain(s) for 7.2.6. β refers to the position of the mature β chains in both transfectants. Finally, the positions of the α and β chains after removal of N-linked oligosaccharides with N-glycanase (lanes 3 and 6) or endo H (lane 8) are indicated by α_o and β_o. The positions of molecular mass standards are indicated on the right.

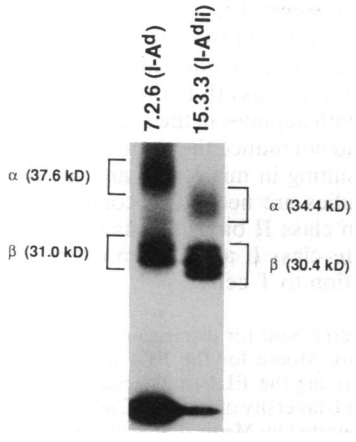

FIG. 4. Only the large molecular mass forms of α and β reach the cell surface in Ii-negative and Ii-positive transfectants. The EL4 transfectants were cell-surface labeled with ^{125}I; class II was immunoprecipitated with M5/114 and analyzed by SDS/PAGE. The positions of α and β chains for each transfectant are indicated by brackets and include the average molecular mass of the bands corresponding to the indicated chain.

class II from Ii-positive and Ii-negative cells, while the Ii-dependent antibody 40B preferentially precipitates class II from Ii-positive cells (Fig. 5*A*), consistent with the cell-surface staining. Interestingly, the 40B epitope is generated early in biosynthesis (Fig. 5 *B* and *C*) and, in Ii-positive cells, both immature and mature forms of class II are readily precipitable by 40B. In Ii-negative cells, neither the immature nor the mature forms of class II are recognized by the 40B antibody (Fig. 5 *A* and *C*). The antibody K24.199 does not precipitate newly synthesized class II in Ii-positive or in Ii-negative cell lines (data not shown) and therefore was not included in this analysis. These data suggest that the preferential reactivity of 40B is not associated with the altered glycosylation of class II in Ii-negative cells. If the altered glycosylation was responsible for the change in 40B reactivity, the immature forms of class II, which have apparently similar glycosylation patterns in both transfectants, should react with 40B equivalently. Also, expression of I-A^d in BALB/c 3T3 and Ltk$^-$ cells does not result in the same dramatic increase in class II glycosylation in the absence of Ii, yet both cell types do have an increase in 40B reactivity in the presence of Ii (refs. 10 and 17; data not shown). Therefore, the preferential reactivity of 40B appears to be associated with a conformational change of the class II molecule caused by Ii coexpression. The lack of 40B reactivity in the small molecular mass forms of α and β chains in the Ii-negative transfectants (Fig. 5*B*) suggests that this conformational change is imparted early during the assembly of the $\alpha\beta$ heterodimer in the ER and provides evidence for Ii having an effect on the proper assembly of class II heterodimers.

DISCUSSION

In this study, Ii was shown to have a dramatic effect on the intracellular transport and posttranslational modification of class II molecules. The lack of Ii in our I-A^d-positive EL4 transfectants resulted in several changes in the transport and assembly of class II. Although class II could dimerize efficiently regardless of the presence of Ii, a large population of class II that dimerized in the absence of Ii appeared to be retained in a pre-Golgi compartment. Class II molecules that did exit the ER in the Ii-negative cells matured and reached the cell surface but were more heavily glycosylated during transit through the Golgi than class II in Ii-positive transfectants. In addition, the lack of Ii in these cells resulted in loss of reactivity with several I-A^d-reactive monoclonal antibodies. The subsequent transfection of Ii into these Ii-negative cells resulted in efficient class II intracellular transport, a normal class II glycosylation pattern, and restoration of class II-specific monoclonal antibody epitopes.

One interpretation consistent with these results is that class II misfolds in the absence of Ii. Because the class II-specific 40B epitope is generated early in biosynthesis in Ii-positive cells, its loss in Ii-negative cells suggests that at least some of the Ii-dependent folding events probably occur in the ER. Interestingly, this is not the case for other Ii-dependent epitopes, such as K24.199, which develops late in class II maturation. Recently, the generation of maturation-dependent epitopes has been correlated with Ii dissociation from and peptide loading onto class II (18). Together, these data suggest that Ii may influence the conformation of class II at several stages in biosynthesis. Because the 40B epitope is generated early in Ii-positive cells and is maintained at the cell surface, where Ii is no longer associated with class II, 40B

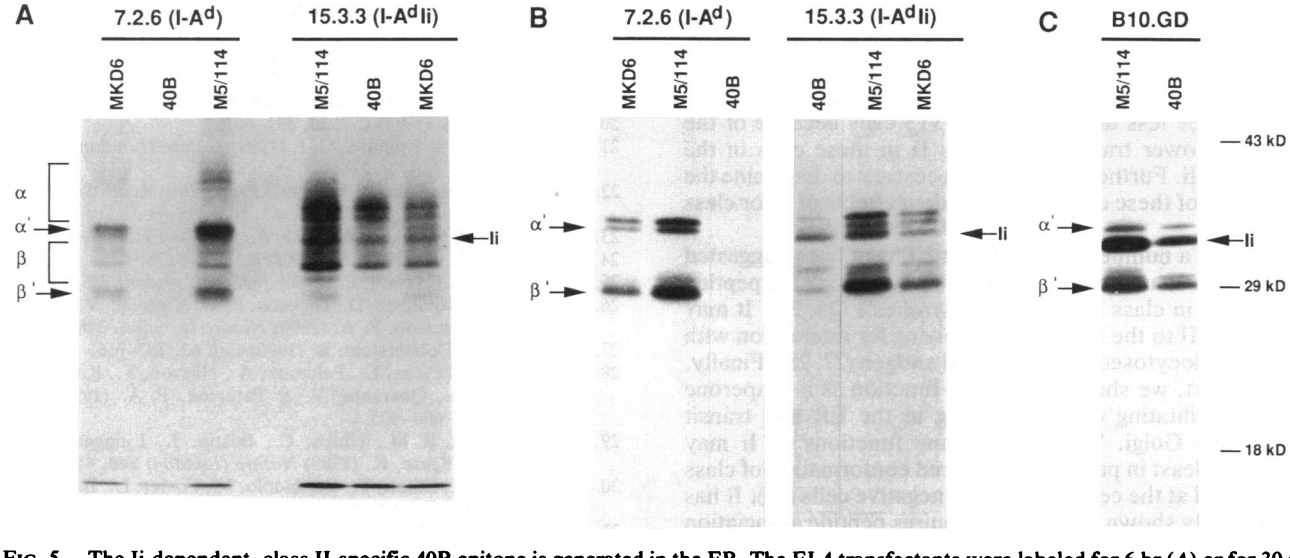

FIG. 5. The Ii-dependent, class II-specific 40B epitope is generated in the ER. The EL4 transfectants were labeled for 6 hr (*A*) or for 30 min (*B*) with [^{3}H]leucine. B10.GD spleen cells were pulse labeled for 30 min with [^{3}H]leucine (*C*). Class II was immunoprecipitated with the I-A^d-reactive monoclonal antibodies MKD6, M5/114, and 40B and then analyzed by SDS/PAGE. The positions of the immature α and β chains (α' and β'), the mature α and β chains (brackets), and the position of the Ii are marked. Molecular mass markers are indicated on the right.

appears to recognize a stable conformational feature of class II that is imparted by association with Ii in the ER.

These effects of Ii on class II folding strongly suggest that Ii acts as a chaperone protein (19) during class II biosynthesis. It is now becoming apparent that the folding and assembly of many secreted and membrane proteins are dependent on a transient interaction with other cellular proteins, termed chaperones. Although a majority of known chaperones appear to bind a wide array of proteins and are related to the heat shock family of proteins (20), some are quite specific in their interactions (21). Ii appears to fall in this latter category in that its action appears to be specific for class II molecules. In addition, Ii is a unique chaperone in that it remains associated with class II during subsequent intracellular transport, suggesting that Ii may play several roles in class II biosynthesis and transport.

The misfolding of class II in the Ii-negative cells appears to cause retention of a large portion of class II in a pre-Golgi compartment. This observation is supported by a recent report indicating that Ii could facilitate the egress of haplotype-mismatched class II dimers from the ER (22). Many misfolded proteins are retained in the ER (23), often by association with the ER resident chaperone protein BiP (24). Although we have not detected BiP in class II immunoprecipitates in the Ii-negative transfectants, a band of ≈200 kDa preferentially coprecipitates with class II in the absence of Ii in the pulse–chase experiments. The identity of this protein, its potential role in ER retention of class II, and the ultimate fate of retained class II molecules remain to be determined. Class II is expressed at the plasma membrane in Ii-negative cells, but it is unknown whether only a portion of synthesized class II reaches the cell surface or whether the majority of class II eventually reaches the cell surface but is held up for a considerable period of time in the ER.

In the absence of Ii, class II is aberrantly glycosylated, resulting in an apparent increase of 3 kDa in the α chain, associated with N-linked sugar addition. One possible explanation for this increase in molecular mass is that class II may have a slower transit rate through the Golgi complex in the absence of Ii, allowing for increased access to glycosyltransferases. A recent study utilizing the identical genes for transfection in BALB/c 3T3 cells did not observe this altered glycosylation event for class II (17). In addition, the effect of Ii on the rate of class II transport was marginal in these cells. Nevertheless, the 3T3 transfectants demonstrate the same Ii dependence on the generation of certain class II-specific antibody epitopes. Close examination of the pulse–chase analyses shows that I-A^d matures much slower in the presence of Ii in the 3T3 cells (4–6 hr) than in EL4 cells (1–2 hr). Therefore, the effect of Ii on class II transport and glycosylation may be less dramatic in the 3T3 cells because of the relatively slower transport of class II in these cells in the presence of Ii. Further studies are necessary to determine the mechanism of these cell type-dependent effects of Ii for class II transport.

There are a number of functions that have been suggested for Ii in class II antigen presentation. Ii may block the peptide binding site in class II early in biosynthesis (25, 26). It may direct class II to the endosome, allowing for interaction with recently endocytosed and processed antigen (27, 28). Finally, in this report, we show that Ii may function as a chaperone protein, facilitating class II folding in the ER and transit through the Golgi. These chaperone functions of Ii may account, at least in part, for the altered conformation of class II expressed at the cell surface of Ii-negative cells (10). It has been recently shown that class I requires peptide association in the ER for efficient folding, assembly with β_2-microglobulin, and transport to the cell surface (29–31). One model that may account for some of these observations is if Ii functions as a surrogate peptide in the ER, blocking association with peptides in the ER and allowing for proper folding and efficient transport of class II. In the absence of Ii, class II may not associate with peptides in the ER, or it may associate with peptides that do not induce the same conformational features as does Ii, resulting in misfolding and inefficient transport. Additional studies are needed to correlate the biochemical effects of Ii on class II biosynthesis reported here with the specific peptide–class II association events required for antigen presentation to T cells.

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