HLA-DR Associates with Specific Stress Proteins and Is Retained in the Endoplasmic Reticulum in Invariant Chain Negative Cells

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Summary

The major histocompatibility complex class II molecules are composed of two polymorphic chains which, in cells normally expressing them, transiently associate with a third, nonpolymorphic molecule, the invariant chain (Ii). To determine differences in the biology of class II molecules synthesized in the presence or absence of Ii, a comparative study was performed of BALB/c 3T3 cells that had been transfected with human class II HLA-DR molecules with or without cotransfection with human Ii. It was observed that in the absence of Ii, at least three high molecular weight proteins coimmunoprecipitate with HLA-DR molecules. These proteins did not coimmunoprecipitate with HLA-DR from cells cotransfected with Ii, nor did they coimmunoprecipitate with class I molecules from any of the transfectants. NH_2-terminal sequence and/or Western blot analysis revealed the identity of two of the proteins as the endoplasmic reticulum (ER) resident stress proteins GRP94 and ERp72. Neither of these proteins was found to have an increased level of synthesis in the Ii^- versus the Ii^+ transfectants, indicating that their synthesis was not induced over constitutive levels. Fluorescence microscopy revealed that in the Ii^- transfectants, the majority of the HLA-DR molecules were present in the ER, whereas in the Ii^+ transfectants, the HLA-DR molecules were found in vesicular structures. We hypothesize that in the absence of Ii, ER resident stress proteins bind to class II molecules and retain them in the ER. This process, in turn, could prevent class II molecules from exiting the ER with endogenous peptides bound in their peptide binding cleft, and therefore could minimize autoimmune responses to endogenously processed self-peptides.

The human class II major histocompatibility DR molecules (HLA-DR) are heterodimeric molecules which, in cells normally expressing them, associate with a third molecule, the invariant chain (Ii)[1], soon after synthesis (1). Although the exact function of Ii is not completely understood, Ii has been described to affect several aspects of class II biology, including biosynthesis and intracellular trafficking of class II molecules (2–6), peptide binding by class II molecules (7–9), and antigen processing and presentation (10, 11). Although Ii is not required for the expression of class II on the cell surface (12, 13), transfection experiments in which BALB/c 3T3 cells were transfected with HLA-DR α and β chains with and without cotransfection with human Ii demonstrated that in the absence of Ii, the majority of the α and β chains remained indefinitely in an immature, endoglycosidase H-sensitive state of lower molecular weight than the mature chains in Ii^+ transfectants (2). Similar results using cell lines transfected with murine class II were subsequently reported by Anderson and Miller (3). These data indicated that in the absence of Ii, the majority of class II molecules might be retained in the endoplasmic reticulum (ER), the intermediate compartment (14), or a pre-*medial*-Golgi compartment. A recent study using a transient transfection system has suggested that this is indeed the case (15). Conversely, Ii has been demonstrated to promote egress from the ER of haplotype mismatched murine class II molecules (16). Although it was proposed that in the absence of Ii, class II molecules might be actively retained in the ER or retrieved from

[1] *Abbreviations used in this paper:* ER, endoplasmic reticulum; ERp72, 72-kD endoplasmic reticulum protein; GRP94, 97-kD glucose-regulated protein; Ii, invariant chain.

a pre-*medial*-Golgi salvage compartment by ER resident proteins (3, 16, 17), no proteins were identified by which such retention or retrieval might be achieved.

During the course of studies on the effect of Ii on the biosynthesis of HLA-DR molecules, we observed that at least three high molecular weight proteins coimmunoprecipitated with class II molecules in cells expressing HLA-DR, but no Ii. We now report that in the absence of Ii, HLA-DR molecules are bound by specific ER resident stress proteins. These proteins, the 97-kD glucose-regulated protein GRP94 (18–20), and the 72-kD ER protein ERp72 (21, 22), are not associated with HLA-DR in cells coexpressing Ii, nor do they associate with class I molecules. We also observed that class II molecules in cells lacking Ii are retained in the ER. We hypothesize that in cells expressing class II molecules in the absence of Ii, these stress proteins are responsible for the retention of class II molecules in the ER.

Materials and Methods

Cell Lines. Generation and culture of the BALB/c 3T3 cell lines transfected with HLA-DRw11 with or without cotransfection with human Ii and culture of the parental BALB/c 3T3 cell line have been previously described (2). The high HLA-DR–expressing human B lymphoblastoid cell line, Swei, has also been previously described (23).

Antibodies and Antisera. The mAb L243, specific for human MHC class II HLA-DR (24), and MK-D6, specific for murine MHC class II I-A^d (25) were obtained from the American Type Culture Collection (Rockville, MD). The mAb 34-5-8, specific for murine MHC class I H-$2D^d$ (26), was a generous gift of Dr. T. Hansen (Washington University School of Medicine, St. Louis, MO). Alkaline phosphatase-conjugated goat anti–rabbit IgG was purchased from Bio-Rad Laboratories (Richmond, CA). Texas red-conjugated goat anti–mouse IgG and fluoroscein-conjugated goat anti–rabbit IgG were purchased from E-Y Laboratories (San Mateo, CA).

Antisera to the NH_2-terminus of GRP94 and COOH-terminus of ERp72 were prepared as follows. The peptides DDEVDVDGTVEEDLGK(C) corresponding to amino acids 1–16 of mature GRP94, and (C)FIDEHATKRSRTKEEL corresponding to amino acids 623–638 of the ERp72 precursor were generous gifts of Cetus, Inc. (Emeryville, CA), and Genetics Institute, Inc. (Cambridge, MA), respectively. The (C) indicates a cysteine residue added to the peptides for the purpose of coupling the peptides to KLH or BSA using the method of Wong, et al. (27). Rabbits were immunized subcutaneously with 0.5 mg of peptide-KLH conjugate in CFA, then boosted with 0.5 mg of peptide-KLH conjugate in IFA at 2-wk intervals until the appropriate antibody titers were obtained as determined by ELISA using the relevant peptide conjugated to BSA.

Metabolic Radiolabeling. Cells were metabolically radiolabeled with [^{3}H]leucine and cell lysates prepared as previously described (2). Briefly, 2×10^6 cells were cultured overnight in 75 cm^2 flasks. The flasks were rinsed with PBS, then 3 ml of leucine-free DMEM containing 10% dialyzed FCS, nonessential amino acids, 1 mM L-glutamine, 10 mM Hepes, pH 7.3, and 0.4 mCi/ml [^{3}H]leucine (155 Ci/mmol, New England Nuclear, Boston, MA) was added to each flask and incubated 6 h at 37°C. The flasks were then rinsed with PBS, and frozen at −70°C. After the flasks were thawed, 2 ml of NP-40 cell lysis buffer (PBS, 0.5% NP-40, 200 μg/ml phenyl methylsulfonylfluoride, 50 μg/ml Nα-*p*-tosyl-L-lysine chloromethyl ketone, and 50 μg/ml *N*-tosyl-L-phenylalanine chloromethyl ketone) was added to each flask. After 15 min of agitation on ice, the lysates were centrifuged 1 h at 100,000 *g*, 4°C, and the supernatants collected.

Immunoprecipitations. Immunoprecipitations of metabolically radiolabeled cell lysates were done as previously described (2). For unlabeled cell lysates, cells were harvested, counted, washed three times with PBS, and the cell pellets frozen at −70°C. The pellets were thawed on ice in the presence of NP-40 cell lysis buffer to yield a concentration of 5×10^7 cells/ml. The lysates were centrifuged 1 h at 100,000 *g*, 4°C, and the supernatants collected. Lysates were immunoprecipitated as with metabolically radiolabeled cell lysates, except that no preclearing was done and, since more cell equivalents were used per immunoprecipitation, 25 μl of packed protein G-Sepharose (Pharmacia-LKB, Piscataway, NJ) was used to pellet the increased amount of antibody reacting with the HLA-DR.

For immunoprecipitation of surface class II molecules, 2×10^6 metabolically radiolabeled cells were washed with cold PBS, and resuspended in 300 μl of PBS containing 0.02% sodium azide, 2% fetal bovine serum, and 10 μg L243 anti-DR antibody. The radiolabeled cells were incubated 30 min on ice, washed three times with cold PBS/azide, then mixed with 2×10^7 of nonradiolabeled HLA-DR–expressing human B lymphoblastoid Swei cells to react with any L243 antigen-binding sites not already occupied. After pelleting, the cells were lysed in 1 ml of NP-40 cell lysis buffer. The lysate was microfuged for 15 min at 4°C in a Beckman Microfuge 11 (Beckman Instruments, Inc., Fullerton, CA) set at a speed of 10. The supernatant was transferred to tubes containing 12.5 μl of protein G-Sepharose (Pharmacia-LKB) and rotated for 1 h at 4°C. The protein G-Sepharose was then washed and the class II eluted, as previously described (2).

Gel Electrophoresis and Electroblotting. Immunoprecipitates from metabolically labeled cell lysates were analyzed by SDS-PAGE using 10% gels, fixed, and processed for fluorography as previously described (2). Scanning densitometry was performed using an Ultroscan XL laser densitometer equipped with GelScan XL software (Pharmacia-LKB).

Immunoprecipitates from nonlabeled cell lysates were analyzed on 8% SDS-PAGE gels under the same conditions as above. After electrophoresis, the gel was soaked for 5 min in transfer buffer (10 mM CAPS (3-[cyclohexylamino]-1-propanesulfonic acid), 10% methanol, pH 11.0), then electroblotted for 1 h at 0.5 A, 10°C, onto nitrocellulose (equilibrated in transfer buffer for 15 min before transfer) using a Hoeffer Transphor electroblotter (Hoeffer Scientific Instruments, San Francisco, CA). The blots were then processed for Western analysis or total protein staining as described below.

Western Analysis and Total Protein Staining of Blots. For Western analysis, after transfer, the blots were washed for 5 min in TBS (20 mM Tris, 500 mM NaCl, pH 7.5), blocked for 30 min in 3% gelatin in TBS, then washed for 5 min in TTBS (0.05% Tween 20 in TBS). The blot was then incubated overnight with the primary antibody diluted in 1% gelatin in TTBS. The blot was washed three times for 5 min per wash with TTBS, then incubated for 1 h with alkaline phosphates-conjugated goat anti–rabbit IgG. After three washes with TTBS, the blot was washed once with TBS then incubated with substrate prepared as per the instructions included with the substrate kit (Bio-Rad Laboratories). The reaction was stopped by washing the blot twice with distilled water and the blot allowed to air dry.

For staining of total protein, after transfer, the blot was washed for 30 min to 1 h, at 37°C in 0.3% Tween 20 in PBS. The blot

was then washed three times for 30 min per wash in the same buffer at room temperature. The blot was then rinsed with distilled water and incubated for 30 min at room temperature in colloidal gold staining solution (ISS Gold-Blot; Integrated Separation Systems, Natick, MA). The blot was then rinsed several times with distilled water and air dried.

Immunofluorescence. Cells were grown overnight on coverslips at 5 × 10^4 cells/coverslip in 6-well plates. The cells were then rinsed three times with PBS, then fixed for 30 min in 4% formaldehyde in PBS, washed three times for 10 min per wash with PBS, then quenched for 20 min in PBS containing 0.1 M ammonium chloride, 0.25% gelatin, and 0.01% saponin. The cells were then permeabilized and stained as described by Marks, et al. (28) using L243 (anti-HLA-DR) and rabbit anti-GRP94 antiserum as the primary antibodies, and Texas red conjugated goat anti-mouse IgG and fluorescein-conjugated goat anti-rabbit IgG as the secondary antibodies. After staining, the coverslips were sealed under PBS containing 45% glycerol and 0.1% phenylenediamine and observed on a fluorescence microscope (Axioplan; Zeiss, Oberkochen, Germany) equipped with filters for detection of double-labeling with Texas red and fluorescein. A 63X Planapochromat (Zeiss) objective lense (1.4 numerical aperture) was used to observe and photograph cells.

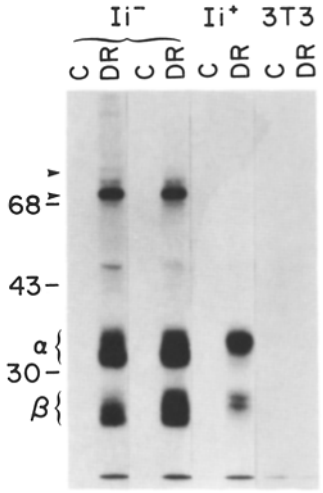

Figure 1. Coimmunoprecipitation of 97- and 74-kD proteins with HLA-DR in Ii^- transfectants metabolically labeled with [^{3}H]leucine. Cell lysates of metabolically radiolabeled cells were immunoprecipitated with the isotype control antibody MK-D6 (*C*) or with the anti-HLA-DR antibody L243 (*DR*), and the immunoprecipitates analyzed by SDS-PAGE at 10^6 cell equivalents per lane on 10% gels. (Ii^-) cells transfected with HLA-DR alone (clones 5E4 and 5A10, respectively); (Ii^+) cells cotransfected with HLA-DR and Ii; (*3T3*) untransfected parental BALB/c 3T3 cell line. Locations of the 97- and 74-kD proteins (►) HLA-DR α chains (α) and β chains (β), and molecular weight markers are shown (*left*).

Results

Association of High Molecular Weight Proteins with HLA-DR in the Absence of Ii. The class II- and Ii-negative BALB/c 3T3 cell line was transfected with HLA-DRw11 with or without cotransfection with human Ii (2). These cells have been demonstrated to be negative for expression of Ii at both the protein and mRNA level (2, 12). Transfected cells were metabolically radiolabeled with [^{3}H]leucine, lysed, the lysate immunoprecipitated with a mAb to HLA-DR, and the immunoprecipitate analyzed by SDS-PAGE. When HLA-DR molecules were immunoprecipitated from metabolically radiolabeled cells transfected with HLA-DR alone, two molecules of ~97 and 74 kD were observed to coimmunoprecipitate (Fig. 1, lanes Ii^-). These proteins did not coimmunoprecipitate with HLA-DR molecules from cells that had been cotransfected with Ii (Fig. 1, lane Ii^+). More of the 74- than the 97-kD protein appeared to be coimmunoprecipitated with HLA-DR from metabolically radiolabeled cells. However, when class II molecules were immunoprecipitated from lysates of transfected cells that had not been metabolically radiolabeled, this was not the case. Approximately equal amounts of the two proteins, as well as a third protein of 72 kD, which was not apparent in immunoprecipitates from metabolically labeled cells, were present in colloidal gold stained blots of SDS-PAGE gels of HLA-DR immunoprecipitates from Ii^- transfectants (Fig. 2 *a*, lanes Ii^-). Thus, it appears that predominantly newly synthesized 74-kD molecules associate with HLA-DR in the absence of Ii, while the 97- and 72-kD molecules which associate with HLA-DR in the absence of Ii must arise from a pool of pre-existing molecules present in the cell before the metabolic labeling of the cells.

Because of high background staining of the lower region of blots stained for total protein with colloidal gold, the DR α chains are not visible in Fig. 2 *a*, which was stained for optimal visualization of the 94-, 74-, and 72-kD proteins. A lighter stain of the same blot is shown in Fig. 2 *c*. As shown in this figure, the immature α chains are present in the Ii^- lanes, but only mature α chains are present in the immunoprecipitate from the Ii^+ transfectant. Because of the use of an 8% gel, the β chains run at the dye front and cannot be seen.

None of the three proteins coimmunoprecipitated with MHC class I molecules from any of the transfectants (Fig. 2 *b*), indicating that the interaction was specific for class II molecules in Ii^- cells.

Identification of the 97- and 72-kD Proteins as GRP94 and ERp72. NH_2-terminal sequence analysis of the 97-kD molecule (data not shown) revealed identity with the first 14 amino acids of the mature form of the ER resident stress protein GRP94 (18, 19), also known as ERp99 or endoplasmin. Identity of the 97-kD molecule was confirmed by Western blot analysis using antisera specific for the NH_2-terminal (Fig. 3 *a*) and COOH-terminal (data not shown) regions of GRP94. When initial yields from NH_2-terminal sequencing of DR chains and GRP94 were compared, a ratio of twelve immature HLA-DR molecules per GRP94 molecule was observed in Ii^- transfectants. This number represents the maximal ratio of class II molecules to GRP94, since the GRP94 molecules are associated with the DR molecules noncovalently. Therefore, it is likely that this ratio is an overestimate due to dissociation of GRP94 molecules from the DR molecules during the immunoprecipitation procedure and washes. Experiments are currently underway to more accurately determine the actual stoichiometry of class II and GRP94 molecules in the complex.

Since no NH_2-terminal sequence could be obtained from the 72-kD protein, various antibodies to ER resident proteins of this size were used in Western blot assays to identify this protein. Antisera specific for the COOH-terminal region (Fig. 3 *b*) or the whole molecule (data not shown) of the ER resident protein ERp72 (21, 22) were found to bind

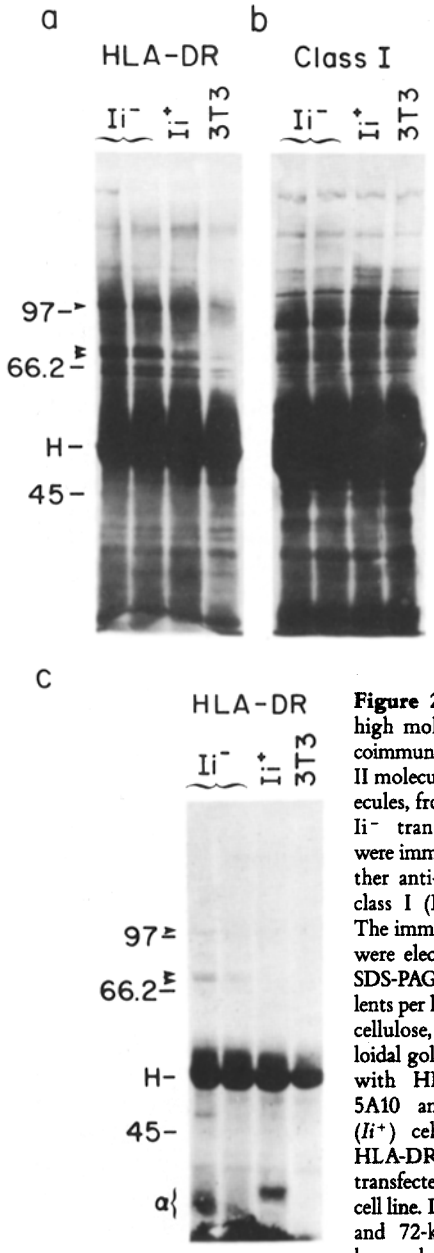

Figure 2. Detection of three high molecular weight proteins coimmunoprecipitating with class II molecules, but not class I molecules, from nonlabeled lysates of Ii^- transfectants. Cell lysates were immunoprecipitated with either anti-HLA-DR (*a*) or anti-class I (H-$2D^d$) (*b*) antibodies. The immunoprecipitated proteins were electrophoresed on an 8% SDS-PAGE gel at 10^7 cell equivalents per lane, transferred to nitrocellulose, and stained with colloidal gold. (Ii^-) cells transfected with HLA-DR alone (clones 5A10 and 5E4, respectively); (Ii^+) cells cotransfected with HLA-DR and Ii; and (*3T3*) untransfected parental BALB/c 3T3 cell line. Locations of the 97-, 74-, and 72-kD proteins (►), the heavy chains of the anti-DR and anti-class I antibodies (*H*), and molecular weight markers are shown (*left*). (*c*) Same blot as in (*a*) except lighter stain, to visualize DRα chains (α).

to the 72-kD protein. These data and the fact that ERp72 also has a blocked NH_2-terminus (M. Green, unpublished observation) demonstrate that the 72-kD protein coimmunoprecipitated with HLA-DR molecules in the absence of Ii is ERp72. Since the NH_2-terminus of ERp72 is blocked, the ratio of class II molecules to ERp72 molecules could not be determined.

Although bands comigrating with GRP94 and ERp72 were occasionally detected in total protein stains of class II immunoprecipitates of transfectants cotransfected with Ii, as well as in control precipitations using the parental BALB/c 3T3 cells, GRP94 and ERp72 were never detected by Western blot analysis of class II immunoprecipitates from these cells. These results indicate that although these proteins comigrate with GRP94 and ERp72 during electrophoresis, they are not GRP94 or ERp72, and are most likely proteins that are nonspecifically precipitated and that have molecular weights similar to these stress proteins.

The identity of the 74-kD protein has yet to be determined. However, Western blot analysis has demonstrated that it is not likely to be a known member of the 70-kD heat shock protein (hsp70) family of proteins, including grp78/BiP (data not shown). Experiments are currently underway to determine the identity of this protein.

Expression of GRP94 and ERp72 Is Not Increased in Transfectants Expressing HLA-DR in the Absence of Ii. Since GRP94 and ERp72 are stress-induced proteins (29–31), it was possible that their expression was induced above constitutive levels in the Ii^- transfectants. In this case, the increased expression could lead to an increase in nonspecific precipitation of these proteins with HLA-DR in the Ii^- transfectants relative to the Ii^+ transfectants. To determine if increased levels of GRP94 and ERp72 were present in Ii^- transfectants, cell lysates from metabolically radiolabeled cells were immunoprecipitated with antisera specific for GRP94 or ERp72. As shown in Fig. 4, no consistent differences in the level of synthesis of the two stress proteins were observed in any of the transfectants relative to the parental BALB/c 3T3 cell line. This indicates that there is no induction of expression of GRP94 or ERp72 in the Ii^+ transfectants over constitutive levels in any of the transfected cell lines. Therefore, the coprecipitation of GRP94 and ERp72 with HLA-DR in the Ii^- transfectants is due to a specific interaction and not to an increase in nonspecific precipitation of GRP94 and ERp72 brought about by excessive levels of these proteins in Ii^- transfectants.

It is interesting to note that the data in Fig. 4 also demonstrate that class II molecules were not observed to coimmunoprecipitate with GRP94 or ERp72 when antisera raised against peptide fragments of these stress proteins were used as the precipitating antibodies.

The Majority of HLA-DR Molecules Are Retained in the ER in Ii^- Transfectants. Although the level of HLA-DR expressed on the surface of Ii^- versus Ii^+ transfectants is approximately equivalent (2), scanning densitometry of the DR α and β chain bands in Fig. 1 indicated that three times more total HLA-DR molecules were present in the Ii^- than in Ii^+ transfectants. Since both types of transfectants have approximately equal surface expression of HLA-DR, the observation that the Ii^- transfectants contain three times as much total class II as the Ii^+ transfectants implies that two-thirds of the class II molecules must remain intracellular in the Ii^- transfectants. These observations are in agreement with the previous demonstration that ~60% of the α and β chains in Ii^- cells remain nonterminally glycosylated (2), suggesting that these molecules do not transit the Golgi apparatus in Ii^- transfectants, and therefore probably do not reach the surface of the cell. If this is the case, then it would be predicted that only the mature, terminally glycosylated α and β chains would be found on the cell surface of the Ii^- transfectants. As shown in Fig. 5, when surface HLA-DR molecules are

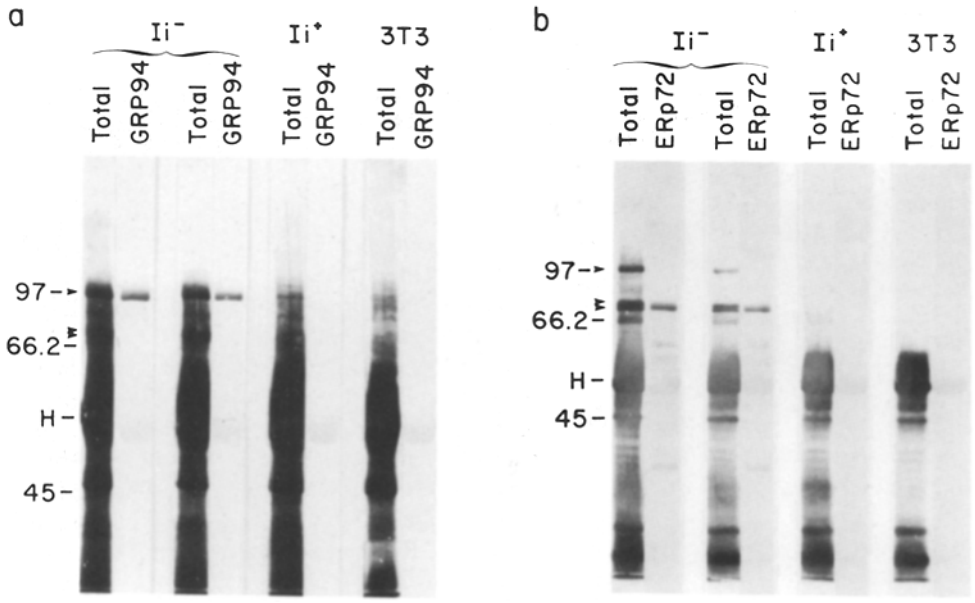

Figure 3. Identification of 97- and 72-kD proteins as GRP94 and ERp72, respectively. Cell lysates were immunoprecipitated with anti-HLA-DR antibody, and the immunoprecipitates electrophoresed on 8% SDS-PAGE gels at 5×10^7 cell equivalents per lane for all lanes except for total protein in (*b*), which was at 10^7 cell equivalents per lane. After electrophoresis, the gels were transferred to nitrocellulose and either stained for total protein (*Total*) or probed with antiserum specific for GRP94 (*a*) or ERp72 (*b*). Legends are as described in Fig. 2.

immunoprecipitated from metabolically radiolabeled cells, only mature α and β chains are found in the immunoprecipitates. Since in Ii⁻ transfectants, approximately one-third of the total class II molecules are expressed on the surface of the cells, approximately one-third of the class II molecules are processed to maturely glycosylated molecules, and only these mature molecules are found on the cell surface, it can be concluded that 60–70% of the class II molecules in Ii⁻ transfectants remain in a pre-*medial*-Golgi compartment and virtually all, if not all, of the remaining fraction of class II molecules which do become terminally glycosylated are ex-

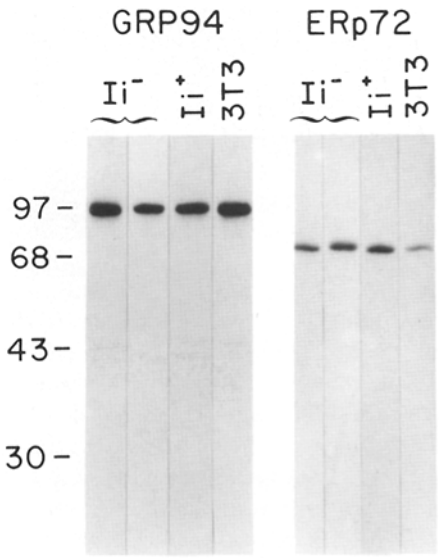

Figure 4. GRP94 and ERp72 expression is not increased in Ii⁻ transfectants. Metabolically radiolabeled cell lysates were immunoprecipitated with antisera specific for GRP94 or ERp72, then the immunoprecipitates analyzed by SDS-PAGE using 10% gels. Legends are as described in Fig. 1.

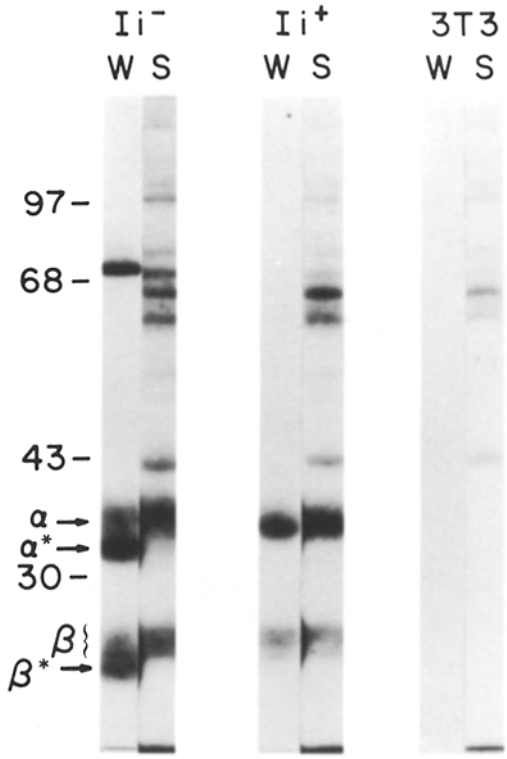

Figure 5. Only mature DRα and β chains are expressed on the surface of Ii⁻ transfectants. Metabolically radiolabeled cells were either lysed and immunoprecipitated as whole cell lysates (*W*) or the surface DR immunoprecipitated (*S*) by incubating intact cells with L243, washing, then lysing in the presence of an excess of an HLA-DR–expressing human B lymphoblastoid cell line to block binding of any labeled internal DR molecules from the labeled cell lines. The lysates were then mixed with protein G-Sepharose and precipitated as described in Materials and Methods. (*Ii⁻*) Cells transfected with HLA-DR alone; (*Ii⁺*) cells cotransfected with HLA-DR and Ii; (*3T3*) untransfected parental BALB/c 3T3 cell line. The positions of mature DRα and β chains (α and β, respectively), the immature DRα and β chains (α^* and β^*, respectively), and molecular weight markers are shown (*left*).

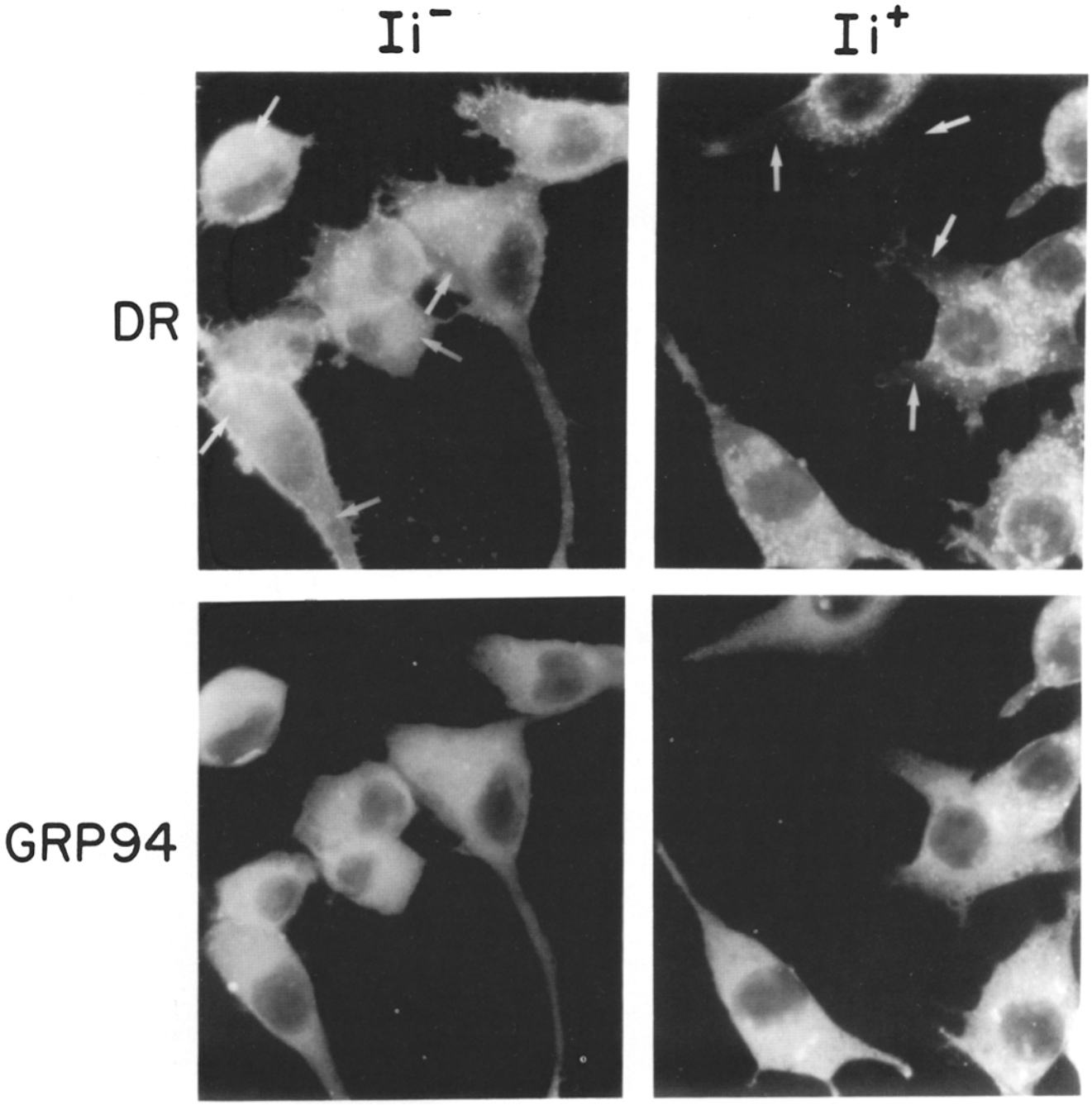

Figure 6. Localization of HLA-DR in ER in Ii⁻ vs. Ii⁺ transfectants. Permeabilized cells were stained for HLA-DR and GRP94 then examined by fluorescence microscopy. Cells representative of their respective transfectants are shown. Columns: (*Ii⁻*) cells transfected with HLA-DR alone; (*Ii⁺*) cells cotransfected with HLA-DR and Ii. Rows: (*DR*) staining with anti-HLA-DR antibody L243 (Texas red); (*GRP94*) staining with anti-GRP94 antiserum (fluorescein). (*Arrows*) Regions of cells containing few class II⁺ punctate bodies for contrast of staining of these regions in Ii⁻ vs. Ii⁺ transfectants.

pressed on the cell surface. This is in contrast to class II molecules in cells coexpressing Ii, in which virtually all of the class II molecules exit the ER and are processed within 4–6 h after synthesis (2).

These observations, in conjunction with the observation that HLA-DR molecules associate with ER resident proteins in the absence of Ii, led to the hypothesis that HLA-DR molecules must be accumulating intracellularly, most likely in the ER, intermediate compartment, or *cis*-Golgi compartment. Such a retention of class II molecules in the ER and Golgi has been reported for HLA-DR molecules in transient expression experiments using Ii⁻ cells (15). To test whether

HLA-DR molecules might be retained in the ER or Golgi apparatus in Ii^- transfectants, cells were double-stained for HLA-DR and the ER resident protein GRP94, then examined by fluorescence microscopy. As shown in Fig. 6, little or no difference in distribution of GRP94 molecules was observed in Ii^- versus Ii^+ transfectants. In both cell types, a diffuse, reticular pattern of staining extending almost to the periphery of the cells was observed, indicating that the ER extended throughout the majority of the cell in BALB/c 3T3 fibroblasts.

However, when distribution of HLA-DR molecules was compared in Ii^- versus Ii^+ transfectants, distinctly different patterns of distribution of class II molecules were observed in the respective transfectants. In the transfectants coexpressing Ii, class II molecules were distributed throughout the cells as punctate, vesicular bodies with little or no staining of the areas around or void of these vesicles (see *arrows*, Fig. 6, column *Ii*$^+$, row *DR*). Such staining is consistent with the presence of class II molecules in a post-Golgi, possibly endosomal compartment. Since staining of the ER with anti-GRP94 demonstrated the presence of ER throughout most of the cell, the lack of staining in the areas around the structures containing the class II molecules indicates that little of the class II molecules remain in the ER.

When the distribution of class II molecules in Ii^- transfectants was studied, little punctate staining was observed relative to cells coexpressing Ii. The vesicles that were present were in a pattern that appeared to be distinct from that observed in the cells coexpressing Ii. Although the vesicles in the Ii^+ transfectants were largely concentrated around the nucleus of the cell, in the Ii^- transfectants the class II-containing vesicles appeared more randomly distributed throughout the cell, or were found closer to the periphery of the cell. These observations suggest that the class II-containing vesicles in the Ii^- cells are distinct from those in Ii^+ cells. However, the present data do not conclusively demonstrate this. Further fluorescence or electron microscopic studies using double-staining for class II molecules and endosomes or lysosomes will be required to determine whether or not the class II-containing vesicles in Ii^- and Ii^+ cells are the same or different. In addition to having fewer class II-containing vesicles, diffuse staining of class II molecules was observed throughout the cell in the Ii^- transfectants (see *arrows*, Fig. 6, column *Ii*$^-$, row *DR*), in a pattern resembling that of GRP94. These patterns are consistent with the majority of the class II molecules remaining in the ER in the Ii^- cells. These results suggest that in cells expressing class II molecules in the absence of Ii, the majority of the class II molecules reside in the ER.

Discussion

It has been proposed that in the absence of Ii, MHC class II molecules might be retained in the ER or retrieved from a pre-*medial*-Golgi salvage compartment by ER resident proteins (3, 16, 17). Such a recycling pathway has been reported for class I molecules in which unassembled murine class I molecules recycled between the ER and *cis*-Golgi (32). However, no proteins responsible for retrieving the class I molecules from the Golgi apparatus to the ER were found. The data in this report for the first time provide evidence that the ER resident proteins GRP94 and ERp72 bind to class II molecules not associated with Ii in the ER, and are likely retaining these class II molecules in the ER and/or retrieving them from a *cis*- or pre-Golgi salvage compartment.

GRP94 and ERp72 are stress-induced proteins (29–31) that are retained in the ER (22) via COOH-terminal KDEL (M. Green, unpublished observation) and KEEL (33) sequences, respectively. Because GRP94 and ERp72 associate with HLA-DR in the absence of Ii, it is likely that these proteins are preventing HLA-DR molecules not associated with Ii from exiting the ER or are retrieving them to the ER from a pre-*medial*-Golgi compartment. Although it is clear that the majority of HLA-DR molecules in Ii^- transfectants do not transit the Golgi apparatus as evidenced by their lack of terminally glycosylated carbohydrates (2, 3), the exact location of their retention is not known. It has been demonstrated that ER retention is achieved by retrieval of ER retained proteins from post-ER compartments (34, 35), and that the receptor for the KDEL ER retention signal is located in or near the Golgi compartment (36, 37). Such a scenario would indicate that class II molecules are in fact exiting the ER in Ii^- cells, and that their retention in the ER is due to retrieval of the class II–stress protein complex from a post-ER, pre-*medial*-Golgi compartment. Such a system has been proposed for class I molecules in which it was hypothesized that retrograde transport was the mechanism by which unassembled class I molecules are recycled to the ER (32).

The data presented in this manuscript strongly suggest that GRP94 and ERp72 are responsible for preventing transit of HLA-DR molecules through the Golgi apparatus in the absence of Ii. The data in this report does not allow delineation of the mechanism by which GRP94 and ERp72 recognize class II molecules in the Ii^- cells. Because it has been demonstrated that class II molecules synthesized in the absence of Ii have an altered conformation (11), GRP94 and ERp72 could be recognizing misfolded class II molecules leading to their retention in the ER. In this case, the stress proteins would be unable to associate with class II molecules that have achieved a proper conformation because of their synthesis in the presence of Ii, and would therefore be unable to retain the class II molecules in the ER. This mechanism would imply that once a proper conformation has been achieved by the class II molecules, GRP94 and ERp72 would be unable to bind to class II molecules even in the absence of Ii.

An alternative scenario would be that GRP94 and ERp72 are capable of binding to properly folded class II molecules, but Ii prevents or displaces the binding of GRP94 and ERp72 to class II molecules. In this scenario, two nonexclusive mechanisms could be occurring. In one, Ii binds to nascent class II molecules and blocks binding of GRP94 or ERp72 to the class II molecules. In the second mechanism, stress proteins bind to the nascent class II molecules but are rapidly displaced by Ii. In this case, the stress proteins would be transiently

associated with class II molecules in cells coexpressing Ii. However, since the stress proteins would be rapidly displaced by the available Ii, very little GRP94 or ERp72 would be associated with class II molecules and could be below the level of detection of the systems used in this study. With either mechanism, as long as Ii is associated with the class II molecules, the complex would be able to be transported out of the ER. Unlike the mechanism discussed above where the stress proteins recognize misfolded class II molecules, this alternative model would imply that GRP94 and ERp72 would be capable of binding class II molecules not associated with Ii, whether or not the class II molecules had already achieved a proper conformation.

GRP94 and ERp72 are stable, long-lived proteins (22), which could account for the observation that little or none of these proteins were observed in immunoprecipitates of metabolically radiolabeled cells. The reason that these proteins have not previously been found associated with class II molecules in the absence of Ii may be that previous studies analyzed immunoprecipitates from lysates of metabolically labeled cells (3, 16, 17). In these experiments, the association of GRP94 and ERp72 with class II molecules would have gone undetected. The observation that under normal labeling conditions the GRP94 and ERp72 molecules which become associated with class II molecules in Ii^- cells are not labeled makes the study of the kinetics of their association impossible to perform using standard pulse-chase experiments. Neither could such an experiment be used to determine if these stress proteins might transiently associate with newly synthesized class II molecules before binding of Ii in Ii^+ cells. Using a standard pulse, none of the stress proteins that become associated with class II molecules would be detected because of a lack of incorporated label. Using longer labeling times would make it impossible to determine the age of the associated class II molecules.

A similar complex has been reported in which murine class I molecules are transiently associated with an 88-kD protein in the ER (38). As with the class II/GRP94/ERp72 complexes, the 88-kD protein was not detected by standard labeling procedures, but required 24 h of metabolic labeling before the 88-kD protein could be detected in anti-class I immunoprecipitates. The 88-kD protein was found to be associated with class I molecules only in the ER and dissociated from the class I molecules before their transport to the Golgi apparatus. Unlike the complex reported here between class II molecules and GRP94 and ERp72, detection of the class I–88-kD molecule complex required chemical crosslinking, indicating that the association of class II molecules with GRP94 and ERp72 is likely to be of higher avidity than the complex formed between the class I and 88-kD molecules. It should also be noted that the 88-kD protein was determined not to be GRP94. The similarity of the systems suggests a general mechanism by which MHC molecules might be transiently retained in the ER by ER resident proteins until proper subunit assembly or until a proper conformation is achieved. At this point, the ER resident proteins dissociate from the MHC molecules, allowing the MHC molecules to proceed to the Golgi apparatus. In the case of the Ii^- transfectants, completion of the complex or a proper conformation of the class II molecules is not achieved. The GRP94 and ERp72 molecules do not dissociate from the class II molecules, and retain the class II molecules in the ER.

It is interesting that although the class II/GRP94/ERp72 complex is stable enough to be detected without chemical crosslinking when immunoprecipitated with anti–HLA-DR antibody, the class II molecules do not coimmunoprecipitate when antisera to GRP94 or ERp72 peptides are used as the immunoprecipitating antibodies. The possibility exists that the antisera are directed against regions of the GRP94 or ERp72 molecules that are not accessible to the antibodies when these stress proteins are complexed to class II molecules. Alternatively, binding of GRP94 or ERp72 to class II molecules could induce conformational changes in the stress proteins destroying the antigenic determinant(s) recognized by the antipeptide antisera. The fact that class II molecules do not coimmunoprecipitate with GRP94 or ERp72 antipeptide antisera does not invalidate the specificity of the coimmunoprecipitation observed with anti-DR antibody. As demonstrated in Fig. 2, GRP94 and ERp72 coimmunoprecipitate with class II molecules only in the absence of Ii. If this were a nonspecific interaction between class II molecules or one of the reagents used in the immunoprecipitation, then GRP94 and ERp72 would be expected to be coimmunoprecipitated with class II molecules in the Ii^+ transfectants, or, if the interaction was with one of the reagents used in the immunoprecipitations, in the immunoprecipitations using lysates from the untransfected parental cells. These data indicate that the formation of the class II/GRP94/ERp72 complex is specific, inasmuch as the absence of Ii is a requirement for stable complex formation.

The demonstration that the stimulation of B cells with LPS, which induces an increase in the expression of class II molecules, also increases expression of GRP94 and ERp72 3–10-fold (21) while the expression of other ER proteins remains constant, suggests that class II molecules and GRP94 and ERp72 may be coregulated. Such coregulation would further support the hypothesis that these two ER proteins play an important role in class II biology in cells normally expressing class II molecules, and are not an artifact of the system used in this study.

The question arises as to why any surface expression of HLA-DR is observed in the Ii^- transfectants. Normally, class II molecules are expressed in cells that also express Ii. In this physiological situation, very few class II molecules not associated with Ii would be present in the ER. Therefore only few class II molecules would be available to be bound by GRP94 and ERp72. However, in the Ii^- transfectants, none of the class II molecules are associated with Ii. This high concentration of class II in the absence of Ii could overload the system and allow the escape of some of the HLA-DR molecules from a salvage compartment, permitting their expression on the cell surface. Although the ratio is possibly artificially high, the observed 12:1 ratio of class II molecules to GRP94 molecules is consistent with the hypothesis that

the system is being overloaded, allowing some escape of class II molecules through the Golgi apparatus, and ultimately, to the cell surface.

Because Ii blocks binding of peptides to class II molecules (7–9), class II molecules which have not bound Ii could potentially bind endogenously synthesized peptides in the ER. T cell recognition of these peptides bound to class II molecules could potentially initiate an autoimmune response. It is possible that in cells normally expressing both class II molecules and Ii, GRP94 and ERp72 act to scavenge class II molecules not associated with Ii and prevent their egress from the ER or retrieve them back to the ER from a *cis*- or pre-Golgi salvage compartment. In this context, GRP94 or ERp72 may help maintain the separation of the endogenous and exogenous pathways of antigen presentation, and prevent autoimmune responses by blocking the exit of non-Ii-associated class II molecules from the ER or Golgi apparatus.

It is interesting to note that class II-containing vesicles were present in transfectants expressing class II in the absence of Ii. Using transfected HeLa cells, Lotteau, et al. (6) observed that class II-containing vesicles were present only in transfectants coexpressing Ii, and proposed that Ii was necessary for targeting class II molecules to endosomal compartments. The presence of vesicular structures containing class II molecules in the absence of Ii can possibly be explained by increased recycling of class II molecules from the surface of the cell to endocytic vesicles in BALB/c 3T3 cells relative to HeLa cells. Although Ii might be necessary for delivery of nascent class II molecules to endocytic vesicles, class II molecules that have reached the cell surface via the default pathway could enter the endocytic pathway by endocytosis. In this report, it was observed that there were fewer vesicles containing class II molecules and the vesicles were closer to the cell periphery in Ii^- transfectants versus Ii^+ transfectants. These observations are consistent with the idea that in the cells expressing class II molecules in the absence of Ii, class II molecules are transported to the surface via the default pathway and enter the observed vesicles by endocytosis after reaching the cell surface. Alternatively, these vesicles could be completely distinct from those in the endocytic pathway. Further experiments are necessary to distinguish between these possibilities and, if these vesicles are part of the endocytic pathway, to determine if their origin is distinct from the endocytic vesicles observed in the presence of Ii.

In addition to being relevant to the study of the biology of class II molecules, the observations in this report provide a model with which to study the specificity of binding of specific stress proteins to different sets of proteins in the ER. As discussed above, the interaction of GRP94 and ERp72 with class II molecules exhibits a degree of specificity as demonstrated in part by the fact that, to date, no other molecules have been found to associate with these two stress proteins. In addition, conventional stress proteins, such as BiP, which have previously been shown to bind to proteins in the ER (reviewed in references 39 and 40), were not found to bind to class II molecules in the absence of Ii. By studying the differences in the structures of the proteins recognized by these two sets of stress proteins, the structures that signal the stress proteins to bind to their specific ligands can be determined.

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