Inhibition of Invariant Chain (Ii)–Calnexin Interaction Results in Enhanced Degradation of Ii but Does Not Prevent the Assembly of αβIi Complexes

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Summary

Calnexin is a resident protein of the endoplasmic reticulum (ER) that associates with nascent protein chains. Among the newly synthesized integral membrane proteins known to bind to calnexin is invariant chain (Ii), and Ii release from calnexin coincides with proper assembly with major histocompatibility complex (MHC) class II heterodimers. Although calnexin association with several membrane glycoproteins depends on interactions involving N-linked glycans, we previously reported that a truncation mutant of mouse Ii (mIi1-107) lacking both N-glycosylation sites was highly effective in associating with MHC class II heterodimers and escorting these dimers through the secretory pathway. This could indicate that calnexin, despite binding to both Ii and class II, is not necessary for the proper interaction of these proteins, or that in contrast to most membrane glycoproteins, the N-linked glycans of Ii are not critical to its interaction with this chaperone. To examine this issue, we have directly explored the binding of calnexin to both Ii truncation mutants lacking the typical sites of *N*-glycosylation or Ii produced in cells treated with tunicamycin to prevent glycan addition. These experiments revealed that either method of eliminating N-linked carbohydrates on Ii also inhibited association with calnexin. A lumenally truncated form of Ii (mIi1-131) that still has N-linked carbohydrates showed a decreased affinity for calnexin compared with intact Ii, however, indicating that calnexin–Ii binding is not determined solely by the sugar moieties. All forms of Ii lacking N-linked sugars and showing defective association with calnexin also had enhanced rates of preendosomal degradation. Despite this effect on degradation rate, tunicamycin treatment did not inhibit the association of class II with glycan-free Ii. These data support the view that calnexin is not an absolute requirement for the proper assembly of class II–Ii nonamers, but rather acts primarily to retain Ii in the ER and to inhibit its degradation. These two properties of calnexin–Ii interaction may help ensure that sufficient intact Ii is available for efficient inactivation of the binding sites of newly synthesized class II molecules, while limiting the ability of excess free Ii to alter the transport properties of the early endocytic pathway.

Invariant chain (Ii)[1] is a type II integral membrane glycoprotein that associates with newly synthesized MHC class II chains shortly after their import into the endoplasmic reticulum (ER). Ii forms noncovalent homotrimers (1), each chain of which associates with a class II αβ heterodimer (2). This nonameric structure is competent for movement out of the ER and through the Golgi complex and *trans*-Golgi network. By either a direct route (3–5) or through a pathway involving the plasma membrane and early endosomes (5–9), the complex moves to more acidic and proteolytic endocytic organelles where Ii is degraded and peptide ligands are acquired by the class II molecules (9–14).

[1]*Abbreviations used in this paper:* DSP, dithiobis[succinylproprionate]; ER, endoplasmic reticulum; Ii, invariant chain; mIi, mouse Ii.

Several different experimental models have been used to explore the events involved in the initial assembly and intracellular trafficking of class II heterodimers and Ii, as well as the fate of subsets of these chains produced in the absence of one or more of the oligomer subunits. Results obtained from studies in microsome-containing in vitro translation systems (15–17), after transient (18–20) and stable (21) transfection of various fibroblast cell lines and using lymphoid cells from mice whose Ii genes have been disrupted by homologous recombination (22–24), all indicate that class II heterodimers can be formed in the absence of Ii. The extent to which heterodimer assembly occurs in the absence of Ii, however, varies greatly among allelic class II products (20, 25). In addition, cells producing class II α and β chains in the absence of Ii show elevated levels of class II

proteins in high molecular weight complexes (26), and similar aggregates are seen in normal lymphoid cells at very short times after protein entry into the ER (27). In normal cells with Ii, these class II chains rapidly leave such aggregates and assemble into typical nonameric complexes with Ii. In the absence of Ii, the class II chains largely remain in aggregates, before degradation. This aggregation-prone behavior of class II is also seen with heterodimers produced in insect cells in the absence of Ii (28) and with class II heterodimers removed from nonameric complexes with Ii using low pH (29). In each of these circumstances, aggregation can be prevented by substituting an antigenic peptide for Ii. This suggests either a special role for binding site occupancy in folding class II so as to avoid nonspecific protein–protein interactions, or direct recognition by the binding site of target structures present on non-MHC protein aggregates.

In both transfected cells (20, 21) and lymphocytes of Ii-deficient mice (22–24), the cohort of class II heterodimers that does form in the absence of Ii shows defective transport from the ER to the Golgi complex. This is characteristically seen as the failure of the α and β chains in these serologically normal heterodimers to acquire endoglycosidase-resistant N-linked glycans, even at late times after synthesis. This transport deficiency can be ascribed to any of several events. One is the aggregation phenomenon, as even $\alpha\beta$ heterodimers are associated with very high molecular complexes of incompletely assembled class II chains under some circumstances. However, Ii produced in the absence of class II also shows delayed exit from the ER (30), yet fails to demonstrate the aggregation typical of class II (26, 27). Another possible mechanism is the association of both Ii and class II chains with the ER-resident protein calnexin (p88, IP90) (31, 32), an interaction that is lost efficiently only upon completion of nonamer assembly.

Calnexin is an integral membrane protein of high abundance in the ER that associates with a large number of newly synthesized membrane-bound and soluble proteins (for reviews see references 33, 34). Release from calnexin coincides with protein folding and oligomer assembly, suggesting that calnexin acts as an ER chaperone. Different protein features have been described to be critical for association with calnexin. Some studies propose that calnexin interaction with newly synthesized proteins is mediated via the transmembrane region (35), whereas other reports suggest a specific role for N-linked glycans (36). Hammond and colleagues (37) have provided evidence that glucose residues on partially trimmed core glycans makes a critical contribution to the interaction between nascent protein chains and calnexin. In these studies protein folding, glucose removal, and release from calnexin coincide. Concordant data on an important role for N-linked glycans in calnexin binding have recently been reported for TCR-α chains (38) and MHC class I molecules (39, 40). In contrast, the CD3ϵ subunit of the TCR complex, which binds strongly to human calnexin in the absence of other receptor polypeptides (41), lacks N-linked glycans. For this latter integral protein, Rajagopalan et al. (41) have suggested that the association with calnexin is regulated by the oligomeric state of the TCR complex. Arunachalam and Cresswell (42) have also recently reported that human class II and Ii chains produced in cells treated with tunicamycin, an inhibitor of core glycan addition, still show association with calnexin.

The notion of calnexin as a chaperone showing prolonged interactions with incompletely folded proteins would be in accord with the ER retention of class II and Ii produced independently of one another, with their association with calnexin shortly after synthesis, and with their efficient release from the ER being related to the loss of this association upon formation of a properly assembled nonamer (31, 32). Such a model of calnexin as a chaperone involved in actively promoting class II–Ii subunit folding and/or association might predict that in the absence of its binding to one or more of these chains, the generation of properly folded oligomers would be inhibited or prevented. Our recent analysis of Ii structure and function revealed, however, that a truncated form of mouse invariant chain (residues 1–107) was quite efficient in assembling with class II and promoting ER to Golgi transport (43). Because this segment of Ii lacked both N-linked glycans as well as most of the lumenal domain of Ii, it was possible that it failed to associate with calnexin, which, if true, would argue against a necessary role of the latter in these aspects of Ii function.

To explore this question further, we have directly examined the interactions of normal and mutant mouse Ii chains with calnexin and explored the role of N-linked glycans in this association. Our data reveal that such glycans play an important but not exclusive role in regulating Ii–calnexin interaction, and that Ii–calnexin association is not essential for class II–Ii assembly or transport. Rather, a major function of calnexin seems to be the retention of Ii in the ER and the inhibition of degradation of this retained protein. Both of these features promote effective class II–Ii assembly while protecting the cell from the untoward consequences of free Ii in the endocytic pathway (7, 44, 45).

Materials and Methods

Plasmid Construction. cDNA expression vectors containing inserts coding for wild-type mouse (m) Ii31, mIi19-215, mIi1-107, and mIi19-107 have been previously described (18, 43). A cDNA expression construct containing IP90 (46) was kindly provided by Dr. M. B. Brenner (Harvard Medical School, Boston, MA).

A DNA fragment containing mIi1-131, 5′ translation control elements, and EcoRI, BamHI, and HindIII cloning sites was generated by PCR amplification as previously described (43). pcEXV-3mIi31 was used as template, and the following oligonucleotide primers were used: upstream 5′CCGAATTCAAGCTTACTAGAGGCTAGAGCCATG 3′ and downstream 5′AAGAATTCGGATCCCTATTACCTCGTGAGCAGATGCATCAC3′. The amplified fragment was digested with EcoRI and inserted into the EcoRI cloning site in the cDNA expression vector pcEXV-3 (18). A clone with the insert in the proper transcriptional orientation was selected for use. The wild-type and mutant Ii molecules studied here are shown schematically in Fig. 1.

Transient Expression. A modified DEAE–dextran procedure was used as previously described to transiently transfect COS 7.2

cells (7). After two washes with DME containing 10 mM Hepes, COS cells were incubated with DME/10 mM Hepes containing 400 μg/ml DEAE–dextran, 100 μM chloroquine, and DNA (1 μg of each plasmid). After 4 h at 37°C, the cells were treated with 10% DMSO in PBS for 2 min at room temperature and then incubated in complete medium. 48 h after transfection the cells were used for labeling and immunoprecipitation.

Metabolic Radiolabeling, Immunoprecipitation, and SDS-PAGE Analysis. Transfected COS cells were incubated for 1 h in methionine- and cysteine-free medium. ^{35}S-TRANS label (methionine and cysteine) (500 μCi/ml) was then added and the cells incubated for the indicated time (from 5 to 30 min). Some samples were lysed immediately (pulse) either in 1% NP-40 or in 0.3% CHAPS. Other labeled cell samples were incubated for an additional 15 min or 2 h in medium containing an excess of cold methionine and cysteine (chase), then lysed as indicated. Where indicated, chloroquine (100 μM; Sigma Chemical Co., St. Louis, MO) and/or tunicamycin (10 μg/ml; Sigma Chemical Co.) were added to the culture medium during the prelabeling step and were maintained at these concentrations during pulse-labeling and chase incubations. Brefeldin A (5 μg/ml; Epicentre Technologies, Madison, WI) was added to appropriate samples during the chase only. The precleared lysates were immunoprecipitated using mAbs previously bound to protein G–Sepharose beads, as previously described (43). Treatment of immunoprecipitated proteins with endoglycosidase H was as described (43). For tunicamycin-treated CBA spleen cells, additional preclearing steps were performed using Con A–Sepharose beads. The eluted samples were analyzed by SDS-PAGE in reducing conditions. In the cross-linking experiment, 200 μg/ml of the water-soluble, reducible cross-linking reagent dithiobis[succinimidylproprionate] (DSP; Pierce Chemical Co., Rockford, IL) were added to the lysing buffer.

Results

Relationship between Ii Glycosylation and Calnexin Binding. Hammond et al. (37) have proposed that glucose residues on partially trimmed N-linked glycans play a critical role in regulating calnexin interaction with membrane glycoproteins in the ER, and this hypothesis is well supported by several recent studies (38–40). Nevertheless, some published data argue that such carbohydrate residues are not absolutely required for calnexin binding, including work with human Ii (42). Fig. 2 shows that 4.5 h after synthesis, a greater fraction of the carbohydrates of class II molecules associated with a truncated form of mouse invariant chain lacking the normal N-glycosylation sites (mIi19-107) are resistant to endoglycosidase H digestion than those on either class II expressed in the absence of Ii or expressed in the presence of invariant chain lacking only a segment of the cytoplasmic tail (mIi19-215). These data are consistent with highly efficient export from the ER to the Golgi complex of the class II molecules associated with truncated Ii lacking N-glycosylation sites, a finding that might indicate a loss of calnexin-mediated retention of Ii, as well as the absence of a requirement for Ii–calnexin association during assembly and trafficking of Ii–class II complexes. We therefore undertook to directly examine whether the two N-linked carbohydrates on mouse Ii contributed to the interaction of this molecule with calnexin. Two different approaches were used: drug inhibition of N-glycosylation (tunicamycin) and the study of deletion mutants of the lumenal region of Ii, some of which lacked the residues at which N-glycosylation normally occurs.

Proteins from lysates of pulse-labeled COS cells coexpressing intact mIi31 and human calnexin (IP90) were immunoprecipitated with anti-invariant chain (IN-1; 47) or anti–human calnexin (AF-8; 46) mAbs. AF-8 immunoprecipitates showed a distinct band of coprecipitating mIi31 (Fig. 3 *A*). The AF-8 mAb does not bind to the endogenous simian calnexin present at high concentration in the ER of the COS cells. Therefore, the amount of mIi coprecipitated with human calnexin by this mAb is likely to significantly underestimate the actual level of association of Ii with the total calnexin pool. Similarly, only a modest

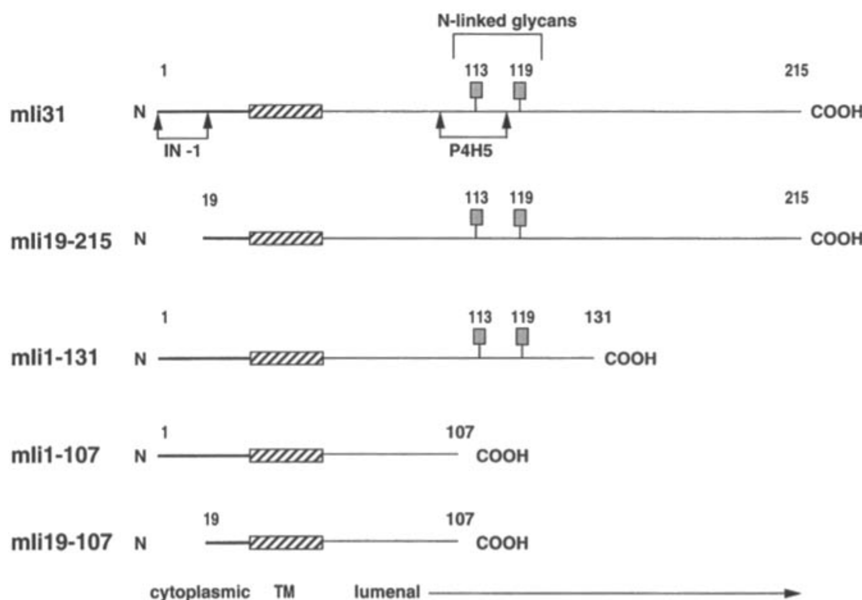

Figure 1. Schematic representation of wild-type mouse Ii31 and truncated forms of Ii used in this study. The striped boxes represent the Ii transmembrane region. Shaded boxes indicate Ii N-linked glycosylation sites. Numbering refers to amino acid positions in the protein. IN-1 and P4H5 mAb binding regions are indicated. *N*, NH_2 terminus of protein; *COOH*, COOH terminus of protein.

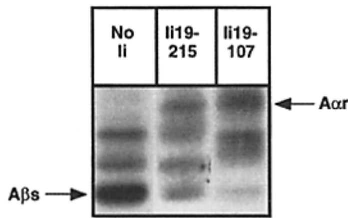

Figure 2. The N-linked glycan-free truncation mutant mIi19-107 assembled with class II shows enhanced ER to Golgi transport. COS cells expressing the class II chains $A\alpha^b$ and $A\beta^b$ alone or together with either mIi19-215 or mIi19-107 were metabolically labeled (30 min), then chased for 4 h. Detergent lysates were immunoprecipitated with Y3P (anti-A^b mAb), treated with endoglycosidase-H (+), then analyzed by SDS-PAGE and autoradiography. *Aβs* indicates the position of Aβ chains with endoglycosidase H–sensitive glycans; *Aαr* indicates the position of Aα chains with endoglycosidase H–resistant glycans.

amount of labeled calnexin is seen in the reciprocal immunoprecipitation with IN-1, due to a large pool of preexisting endogenous calnexin in these transfected cells that does not label under these conditions, and that competes with the newly synthesized, labeled human protein for binding to Ii.

Tunicamycin treatment of transfected COS cells, which inhibited most but not all the addition of core glycans to the overproduced nascent Ii protein chains, resulted in the coprecipitation of little or no nonglycosylated mIi31 with calnexin (Fig. 3 *B*), consistent with several reports on the importance of N-linked oligosaccharide in nascent protein interactions with calnexin (36–40), but in distinction to the data of Arunachalam and Cresswell (42). We then analyzed the association of deletion mutants of Ii with calnexin. As shown in Fig. 3, *C* and *D*, neither mIi1-107 nor mIi1-131 coprecipitated with calnexin.

Given the results obtained with tunicamycin and mIi31, it was surprising to find that mIi1-131, which contains both of the N-glycosylation sites of mIi31 and is extensively glycosylated in COS cells, did not show detectable association with calnexin under these conditions. These results suggest that the sugar moieties alone, while perhaps necessary, are not sufficient for tight binding of Ii to the chaperone. To examine whether the failure to observe calnexin–mIi1-131 interaction under our standard cell lysis conditions reflected a weak but perhaps physiologically significant association between these molecules, pulse-labeled COS cells expressing human calnexin and either mIi1-131 or mIi1-107 were lysed in the presence of the reduction-sensitive cross-linking agent DSP. As shown in Fig. 4, reduction after immunoprecipitation with AF-8 demonstrated that mIi1-131 was associated with calnexin at the time of cell lysis, whereas mIi1-107 was not. Thus, the presence of N-linked carbohydrates on a molecule lacking the complete lumenal portion of Ii (mIi1-131) is sufficient for a less stable interaction with calnexin, whereas the absence of both the sugars and the COOH-terminal segment of Ii (mIi1-107) leads to a lack of any detectable association. These latter results, together with the data shown in Fig. 2 on the effective assembly of class II with mIi19-107, also indicate that Ii interaction with calnexin is not required for the formation of class II–Ii complexes.

Immunoprecipitation experiments using COS cells synthesizing class II α and β, mIi31, and human calnexin in the presence of tunicamycin confirmed the ability of class II to associate with Ii in the absence of detectable calnexin binding (data not shown).

Elimination of Calnexin Association Promotes Ii Degradation in a Preendosomal Compartment of COS Cells. Many proteins that are part of oligomeric assemblies are rapidly degraded in the absence of their partner chains or interaction with resident chaperones. It was thus of interest to examine whether the virtual elimination of Ii–calnexin interactions in cells lacking class II affected the stability of Ii that had not reached it usual site of degradation in endosomes/lysosomes. To accomplish this, we examined the extent of degradation of wild-type Ii and Ii mutants by performing pulse–chase experiments in the presence of chloroquine

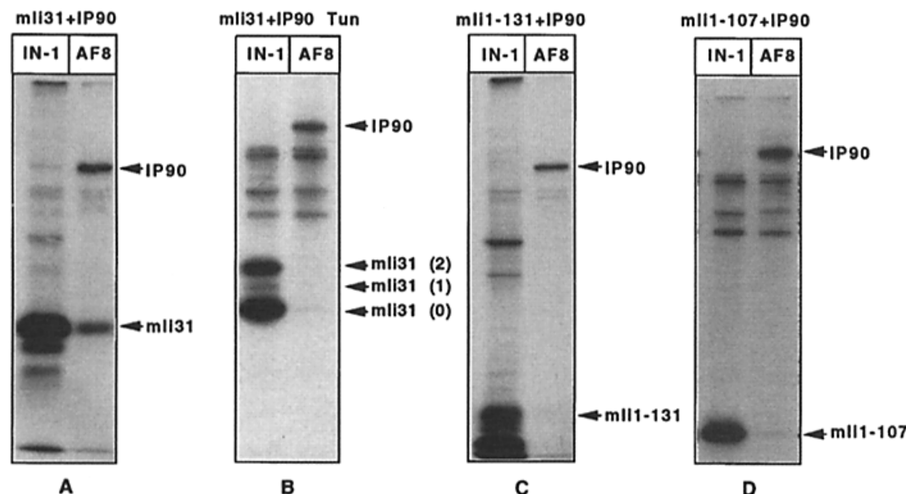

Figure 3. Relationship between Ii glycosylation and calnexin interaction. (*A* and *B*) COS cells expressing IP90 plus mIi31 were metabolically labeled (30 min) in the absence (*A*) or presence (*B*) of tunicamycin. CHAPS lysates were immunoprecipitated with an anti-Ii mAb (IN-1) or an anti-calnexin mAb (AF-8). The eluted boiled samples were analyzed by SDS-PAGE. Arrows on the right of the figure indicate the migration of IP90 and the different glycosylation forms of mIi31 (numbers of carbohydrates shown in parentheses). (*C* and *D*) Pulse-labeled lysates of COS cells expressing either IP90 plus mIi1-131 (*C*) or IP90 plus mIi1-107 (*D*) were immunoprecipitated with an anti-Ii mAb (*IN-1*) or an anti-calnexin mAb (*AF-8*). The boiled eluted samples were analyzed by 10 and 12% SDS-PAGE, respectively. Arrows on the right of the figure indicate the migration of IP90, mIi1-131, and mIi1-107.

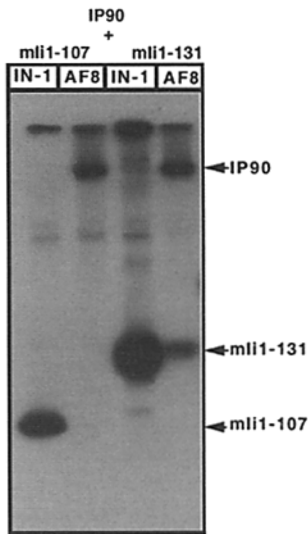

Figure 4. mIi1-131, but not mIi1-107, associates with calnexin. COS cells expressing either IP90 plus mIi1-107 or IP90 plus mIi1-131 were metabolically labeled (30 min) and lysed in CHAPS in the presence of a cross-linking agent, DSP. An anti-Ii mAb (*IN-1*) or an anti-calnexin mAb (*AF-8*) was used in immunoprecipitation. The boiled eluted samples were analyzed under reducing conditions by 12% SDS-PAGE. Arrows on the right of the figure indicate the migration of IP90, mIi1-131, and mIi1-107.

and/or brefeldin A, to minimize any proteolysis of Ii in the endocytic pathway.

Such studies in COS cells expressing either mIi31 or mIi1-107 showed different degrees of stability of these two forms of Ii. The wild-type protein mIi31 was found to be stable during the time frame of our experiments, as shown by the quantitative recovery of pulse-labeled molecules after a 2-h chase (Fig. 5 *A*). mIi1-107 was much more susceptible to preendosomal degradation under the same conditions, with 50% loss of signal during this same time period (Fig. 5 *C*). Additional experiments showed that 75–80% of this form of Ii was degraded by 4 h, whereas mIi31 was largely intact at this time (data not shown). These data indicate a half-life for mIi1-107 of ~2 h under these conditions in transiently transfected COS cells producing large amounts of this protein.

Given that wild-type mIi31 shows clear binding to calnexin whereas the truncation mutant mIi1-107 does not, these results are consistent with the hypothesis that calnexin binding promotes the stability of newly synthesized invariant chains, just as calnexin–TCR-α chain association protects the latter from rapid prelysosomal destruction (38). This model would predict that tunicamycin should affect the rate of degradation of wild-type mIi31 and of mIi1-131, but not the glycan-free mIi1-107. As expected, mIi31 became sensitive to degradation when synthesized in cells cultured in tunicamycin (Fig. 5 *A*). In agreement with the cross-linking data showing calnexin–mIi1-131 association, the fate of mIi1-131 was also dramatically influenced by the presence of tunicamycin, with only 50–60% of the initial pool of labeled molecules remaining after the chase (Fig. 5 *B*), whereas loss of mIi1-107 occurred at the same rate in the presence and absence of tunicamycin (Fig. 5 *C*). These data are in clear accord with the view that calnexin association plays a critical role in protecting Ii from nonendocytic proteolysis.

Effects of Preventing Ii–Calnexin Interaction in Lymphoid Cells Normally Synthesizing Class II and Ii. All the experiments described above were performed using transient overexpression in heterologous fibroblasts. Although our previous experience indicated that many properties of class II and Ii in these cells resembles that in lymphoid cells, some aspects of class II–Ii assembly and transport differ in the fibroblast and physiologic APC models. Therefore, to determine whether our observations concerning a role for calnexin in regulating preendosomal Ii degradation reflect what happens in "professional" APC, we examined the effect of tunicamycin on Ii degradation in freshly isolated lymphoid cells. CBA spleen cells were pulse labeled for 5 min and chased for 15 min in the presence or absence of tunicamycin. Ii immunoprecipitates were then analyzed by SDS-PAGE, autoradiography, and densitometry. In the absence of tunicamycin, mIi31 in these cultured spleen cells showed no significant degradation during this brief chase period. In contrast, only 60% of the initially labeled pool of mIi31 remained after 15 min of chase in cells treated with tunicamycin to prevent glycan addition and interfere with

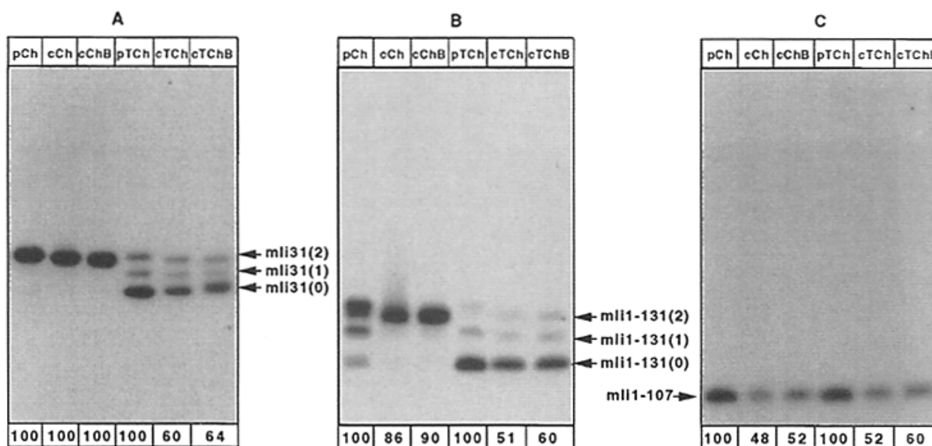

Figure 5. Extensive preendocytic degradation as a result of the inhibition of Ii–calnexin interaction. COS cells expressing mIi31 (*A*), mIi1-131 (*B*), or mIi1-107 (*C*) were pulse labeled (30 min; *p*) and chased (2 h; *c*) in the presence or absence of tunicamycin (*T*). In addition, chloroquine (*Ch*) and/or brefeldin A (*B*) was added to the indicated samples. Lysates were immunoprecipitated with an anti-Ii mAb (IN-1). The boiled eluted samples were analyzed by 12% SDS-PAGE. Arrows on the right of the figure indicate the migration of mIi31, mIi1-131, and mIi1-107, and the labels in parentheses indicate the number of N-linked glycans on each. The numbers below each lane give the chase/pulse intensity ratio as a percentage, as determined by densitometric analysis.

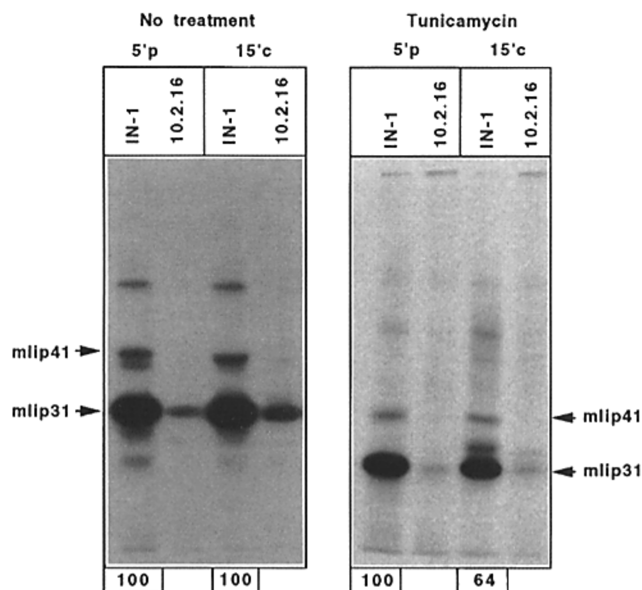

Figure 6. The inhibition of Ii–calnexin interaction using tunicamycin promotes rapid Ii degradation but does not prevent assembly of the α/β Ii complexes in normal cells. Spleen cells from CBA mice were pulse labeled for 5 min and then chased for 15 min in the absence (*left*) or presence (*right*) of tunicamycin. Tunicamycin-treated lysates were precleared with Con A–Sepharose beads. An anti-Ii mAb (*IN-1*) or an anti-MHC class I mAb (*10.2.16*) was used in immunoprecipitations. Immunoprecipitates were separated by 12% SDS-PAGE. The numbers below each lane give the chase/pulse intensity ratios as a percentage, as determined by densitometric analysis. mIip41 refers to the form of mIi containing the alternatively spliced exon 6b.

calnexin association (Fig. 6), suggesting an even more rapid loss of Ii in the putative absence of calnexin binding than was seen in the COS system.

We also examined the association of Ii with class II under these conditions. MHC class II–associated Ii and total Ii were quantified by densitometry from 10.2.16 and IN-1 immunoprecipitates, respectively. The ratio of Ii in the 10.2.16 versus IN-1 precipitates, which indicates the proportion of the total Ii pool that is complexed with αβ heterodimers, decreased from 3.8 to 2 in the presence of tunicamycin. Total protein synthesis, including class II, was, however also decreased nearly 50% in the treated cells, so correcting for the smaller class II pool subject to 10.2.16 precipitation, these data indicate that a treatment expected to eliminate Ii–calnexin association has little effect on the initial extent of Ii–class II assembly. These data are consistent with the results shown in Fig. 2 concerning effective assembly of class II with the truncation-mutant mIi19-107 and experiments using tunicamycin-treated COS cells coexpressing class II, intact Ii, and human calnexin (data not shown).

Discussion

Ii and class II are among the large number of integral membrane proteins known to associate with the ER resident protein calnexin shortly after synthesis (31, 32). These associations are not efficiently disrupted until the complete nonameric complex of three class II αβ heterodimers on a trimeric core of Ii is formed, after which rapid exit of this complex from the ER can be detected. These data suggest that calnexin might play an important role in facilitating the assembly of the class II–Ii oligomer, in addition to retaining the component chains in the ER until proper coassembly is complete. These studies did not reveal, however, the fate of either class II or Ii in the absence of such calnexin binding. Studies in other model systems have implicated calnexin as a chaperone involved in the folding pathway for both soluble (48–50) and membrane-bound (37) proteins, whereas other investigations have provided evidence for calnexin acting primarily as a retention protein, regulating release of otherwise properly folded but incompletely assembled subunits of oligomeric proteins from the ER (41, 51). Studies of both TCR-α chains (38) and MHC class I heavy chains (39, 40), have also indicated that at least some component chains of oligomeric protein assemblies are rapidly degraded in the absence of such calnexin binding.

Our previous studies of Ii mutants lacking portions of the lumenal domain raised a question about the need for calnexin binding in the function of Ii. Two truncated forms of Ii encompassing residues 1–107 or 19–107 lacked sites for addition of the N-linked glycans shown for some other proteins to be essential for association with calnexin, but nevertheless were very effective in performing the secretory pathway functions of Ii, which include assembly with class II α and β chains, enhanced formation of properly assembled αβ heterodimers, prevention of aggregation of class II chains with each other and/or other ER proteins, and efficient transport of class II dimers through the Golgi complex (43). We thus chose to examine the role of N-linked glycans in the interaction of calnexin with the invariant chain, and upon finding differential association of this chaperone with distinct forms of Ii, to assess the impact of a lack of calnexin–Ii binding on Ii assembly and assembly with class II.

Two different approaches were used: either treatment of cells with tunicamycin, an inhibitor of core oligosaccharide addition, or expression of a truncated form of Ii lacking the sites for addition of N-linked carbohydrate. Most or all measurable calnexin interaction with Ii was lost in the absence of such N-linked glycans, in agreement with the work of several investigators in other model systems (37–40) but in contrast to recent studies using human Ii (42). The differences between our experiments and this prior investigation cannot be readily ascribed to the use of distinct detergents for cell solubilization, as we have observed the same results reported here with either CHAPS or digitonin (Romagnoli, P., unpublished observations). Despite the high level of conservation of Ii structure during evolution, species differences remain a possible explanation for these apparently discrepant findings, especially given the existence of alternative forms of Ii produced by human (52) but not mouse cells. These forms have unique ER retention properties (53) that might supplant those of calnexin. This in turn could lead to a distinct physiology of interaction be-

tween Ii and this chaperone. Alternatively, the association of human calnexin with mouse Ii might be weaker than for the human–human combination.

Our experiments have also revealed that the strength of interaction of calnexin with Ii is not determined solely by these oligosaccharides. Detection of calnexin binding to the mIi1-131 truncation mutant by immunoprecipitation required chemical cross-linking, despite the addition of N-linked glycans to this form of Ii. Thus, protein structural features, in this case controlled by the COOH-terminal lumenal region of the invariant chain, either affect calnexin–carbohydrate interaction or, independently from the sugars, promote association with calnexin. Such an ability of protein structure to directly regulate calnexin binding is in excellent agreement with observations that some secreted or membrane proteins lacking N-linked glycans nevertheless interact strongly with calnexin (41), with other data on a role for glucose-containing N-linked glycans in initiating but not maintaining calnexin–protein interactions (39, 40), and evidence that for some secretory proteins, such as thyroglobulin undergoing disulfide maturation, the redox state of the protein seems to regulate calnexin interaction (50). Human and mouse Ii may differ in the degree to which this protein structure–dependent binding to (human) calnexin can take place in the absence of a glycan-driven initiating event, perhaps explaining the differences between this study and that of Arunachalam and Cresswell (42).

Ii is a very proteolytically sensitive protein (54–56), a property that is central to its function as a type of propiece or "disposable chaperone" for class II molecules. Cathepsin-mediated proteolytic cleavages in the endocytic pathway (57) sequentially remove (7) structurally discrete regions of Ii (57a). This process eventually exposes a functional class II–binding site (7, 43, 58) able to interact with denatured and/or degraded protein antigens in endocytic locations. Recent in vitro studies have demonstrated that the CLIP segment of Ii (59) that is critical to Ii–class II interactions (43, 60, 61) is especially sensitive to proteases when Ii is not bound to class II (57a, 62). In contrast to these in vitro results, Ii has been shown to be quite stable in the ER of living cells (1, 30). This makes physiologic sense, as it is essential that newly synthesized Ii in the ER remain intact in the CLIP region if it is to properly associate with class II and carry out its various preendocytic functions. The present study shows that several distinct maneuvers (tunicamycin treatment, truncation) that share the property of inhibiting Ii–calnexin association result in enhanced secretory pathway degradation of Ii. The rate of degradation was extremely fast in normal spleen cells ($t_{1/2}$ of ~15 min) compared with COS cells ($t_{1/2}$ of 2 h). In the latter cells, very high level synthesis of this protein may saturate the proteolytic machinery, decreasing the effective degradation rate. These data imply that Ii is inherently susceptible to proteolytic attack when unbound to either calnexin or class II. A primary function of Ii association with calnexin may therefore be to protect the large pool of free Ii from such destruction, so that it can serve to efficiently interact with the entire cohort of newly synthesized class II.

Could the increased rate of degradation be an artifact arising from protein misfolding due to either absence of N-linked glycans on Ii produced in the presence of tunicamycin, or misfolding due to the removal of the bulk of the COOH-terminal segment of Ii in the case of mIi1-107? The similar extent of degradation of both full-length and shorter forms of Ii, together with the recent structural data (57a) supporting our model that the lumenal regin of Ii has two discrete domains (43), argue against this possibility. It is also inconsistent with our previous (43) and current demonstration that mIi1-107 or mIi19-107 rapidly and efficiently associate with coexpressed class II and effectively promote its egress from the ER compartment, functions that should not occur if these forms of Ii were substantially misfolded. Finally, experiments reported here using both transfected COS cells and normal spleen cells also show that initial assembly of intact Ii with class II is not substantially affected in cells producing Ii in the presence of tunicamycin, which in the COS model could be directly shown to prevent calnexin association. It thus seems more likely that calnexin association protects intrinsically sensitive sites of properly conformed, non-class II–associated Ii from protease attack than that this molecule helps fold Ii into a protease-resistant structure.

We and others have also shown that the production of high levels of Ii, especially in the absence of class II coexpression, leads to disturbances in the structure and function of early endosomal compartments (7, 44, 45). Cells expressing substantial amounts of free, full-length Ii show characteristic enlarged early endosomes and a delay in endocytic trafficking from these early compartments to late endosomes/lysosomes (7, 63). It is difficult to produce stably transfected cells with high free-Ii levels, apparently due to these endocytic disturbances (unpublished observations). Although milder alterations in endosomal function may be a physiologically relevant effect of Ii that helps coordinate the flow of antigen and class II–Ii complexes in APC (7, 63), excess movement of Ii from the secretory pathway to the endocytic pathway is clearly undesirable. Calnexin association would be expected to retain Ii in the ER, limiting these deleterious effects of high levels of Ii expression. In accord with this possibility, the data in Fig. 2 and our prior work (43) indicate that mIi1-107 or mIi19-107, which are not tightly associated with calnexin, leave the ER more rapidly than mIi31, which is bound to this chaperone. Thus, by stabilizing associated Ii and simultaneously retaining it in the ER, calnexin allows the attainment of the high molar ratios of Ii to class II necessary to ensure that Ii and not other ER ligands interact with newly synthesized class II binding sites while preventing this accumulation of free Ii from adversely affecting cell survival.

Although roles for calnexin can thus be found in the prevention of Ii degradation and in Ii retention, we were unable to discern a necessary contribution of calnexin to effective assembly of Ii with MHC class II molecules. This was true of mIi1-107 or mIi19-107 in COS cells, intact Ii in tunicamycin-treated COS transfectants, and mIi31 in normal spleen cells. Analysis of Ii and associated $A\alpha^k A\beta^k$

behavior at later times in the spleen cells was complicated by the very rapid loss of Ii under tunicamycin treatment conditions, so that less labeled Ii was available for continued assembly or coexport with class II. Taken together, these data seem most consistent with calnexin playing a facilitating, but not essential, role in the contribution of Ii to control of class II assembly and transport in the secretory pathway. The lack of an absolute role may arise from redundancy in the function of various ER chaperones. Alternatively, calnexin might play a quantitative role that optimizes the function of proteins produced at a given biosynthetic rate, reducing the overall metabolic load on a cell while allowing vital functions requiring certain concentrations of exported proteins to be maintained. This would be consistent with the present observations that calnexin helps maximize the availability of newly synthesized Ii for assembly with class II while not being essential for this interaction. A similar contribution to the optimal use of components of other oligomeric protein assemblies would explain the consistent finding of calnexin association with components of the T and B cell receptors, or monomers of viral glycoproteins.

We thank Mickey Marks, Paul Roche, and Caetano Reis e Sousa for their careful reading and helpful suggestions during the preparation of this manuscript, and members of the Lymphocyte Biology Section for critiques and discussions during the course of this study.

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Received for publication 22 May 1995 and in revised form 8 August 1995.

References

1. Marks, M.S., J.S. Blum, and P. Cresswell. 1990. Invariant chain trimers are sequestered in the rough endoplasmic reticulum in the absence of association with HLA class II antigens. *J. Cell Biol.* 111:839–855.
2. Roche, P.A., M.S. Marks, and P. Cresswell. 1991. Formation of a nine-subunit complex by HLA class II glycoproteins and the invariant chain. *Nature (Lond.).* 354:392–394.
3. Neefjes, J.J., V. Stollorz, P.J. Peters, H.J. Gueze, and H.L. Ploegh. 1990. The biosynthetic pathway of MHC class II but not class I molecules intersects the endocytic route. *Cell.* 61: 171–183.
4. Peters, P.J., J.J. Neefjes, V. Oorschot, H.L. Ploegh, and H.J. Geuze. 1991. Segregation of MHC class II molecules from MHC class I molecules in the Golgi complex for transport to lysosomal compartments. *Nature (Lond.).* 349:669–676.
5. Benaroch, P., M. Yilla, G. Raposo, K. Ito, K. Miwa, H.J. Geuze, and H.L. Ploegh. 1995. How MHC class II molecules reach the endocytic pathway. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:37–49.
6. Cresswell, P. 1985. Intracellular class II HLA antigens are accessible to transferrin-neuraminidase conjugates internalized by receptor-mediated endocytosis. *Proc. Natl. Acad. Sci. USA.* 82:8188–8192.
7. Romagnoli, P., C. Layet, J. Yewdell, O. Bakke, and R.N. Germain. 1993. Relationship between invariant chain expression and MHC Class II transport into early and late endocytic compartments. *J. Exp. Med.* 177:583–596.
8. Roche, P.A., C.L. Teletski, E. Stang, O. Bakke, and E.O. Long. 1993. Cell surface HLA-DR-invariant chain complexes are targeted to endosomes by rapid internalization. *Proc. Natl. Acad. Sci. USA.* 90:8581–8585.
9. Castellino, F., and R.N. Germain. 1994. Extensive trafficking of newly synthesized MHC class II-invariant chain complexes in the endocytic pathway and appearance of peptide-loaded class II in multiple compartments. *Immunity.* 2:73–88.
10. Amigorena, S,. J.R. Drake, P. Webster, and I. Mellman. 1994. Transient accumulation of new class II MHC molecules in a novel endocytic compartment in B lymphocytes. *Nature (Lond.).* 369:113–120.
11. Qiu, Y., X. Xu, A. Wandinger-Ness, D.P. Dalke, and S.K. Pierce. 1994. Separation of subcellular compartments containing distinct functional forms of MHC class II. *J. Cell Biol.* 125:595–605.
12. Tulp, A., D. Verwoerd, B. Dobberstein, H.L. Ploegh, and J. Pieters. 1994. Isolation and characterization of the intracellular MHC class II compartment. *Nature (Lond.).* 369:120–126.
13. West, M.A., J.M. Lucocq, and C. Watts. 1994. Antigen processing and class II MHC peptide-loading in human B-lymphoblastoid cells. *Nature (Lond.).* 369:147–151.
14. Rudensky, A.Y., M. Maric, S. Eastman, L. Shoemaker, P.C. DeRoos, and J.S. Blum. 1994. Intracellular assembly and transport of endogenous peptide-MHC class II complexes. *Immunity.* 1:585–594.
15. Kvist, S., K. Wiman, L. Claesson, P.A. Peterson, and B. Dobberstein. 1982. Membrane insertion and oligomeric assembly of HLA-DR histocompatibility antigens. *Cell.* 29:61–69.
16. Bijlmakers, M.J., P. Benaroch, and H.L. Ploegh. 1994. Assembly of HLA DR1 molecules translated in vitro: binding of peptide in the endoplasmic reticulum precludes association with invariant chain. *EMBO (Eur. Mol. Biol. Organ.) J.* 13: 2699–2707.
17. Hedley, M.L., R.G. Urban, and J.L. Strominger. 1994. Assembly and peptide binding of major histocompatibility complex class II heterodimers in an in vitro translation system. *Proc. Natl. Acad. Sci. USA.* 91:10479–10483.
18. Miller, J., and R.N. Germain. 1986. Efficient cell surface expression of class II MHC molecules in the absence of associated invariant chain. *J. Exp. Med.* 164:1478–1489.

19. Sekaly, R.P., C. Tonnelle, M. Strubin, B. Mach, and E.O. Long. 1986. Cell surface expression of class II histocompatibility antigens occurs in the absence of the invariant chain. *J. Exp. Med.* 164:1490–1504.
20. Layet, C., and R.N. Germain. 1991. Invariant chain promotes egress of poorly expressed, haplotype-mismatched class II major histocompatibility complex $A\alpha A\beta$ dimers from the endoplasmic reticulum/cis-Golgi compartment. *Proc. Natl. Acad. Sci. USA.* 88:2346–2350.
21. Anderson, M.S., and J. Miller. 1992. Invariant chain can function as a chaperone protein for class II major histocompatibility complex molecules. *Proc. Natl. Acad. Sci. USA.* 89: 2282–2286.
22. Viville, S., J. Neefjes, V. Lotteau, A. Dierich, M. Lemeur, H. Ploegh, C. Benoist, and D. Mathis. 1993. Mice lacking the MHC class II-associated invariant chain. *Cell.* 72:635–648.
23. Bikoff, E.K., L.-Y. Huang, V. Episkopou, J. Van Meerwijk, R.N. Germain, and E.J. Robertson. 1993. Defective major histocompatibility complex class II assembly, transport, peptide acquisition, and $CD4^+$ T cell selection in mice lacking invariant chain expression. *J. Exp. Med.* 177:1699–1712.
24. Elliott, E.A., J.R. Drake, S. Amigorena, J. Elsemore, P. Webster, I. Mellman, and R.A. Flavell. 1994. The invariant chain is required for intracellular transport and function of major histocompatibility complex class II molecules. *J. Exp. Med.* 179:681.
25. Bikoff, E.K., R.N. Germain, and E.J. Robertson. 1995. Allelic differences affecting invariant chain dependency of MHC class II subunit assembly. *Immunity.* 2:301–310.
26. Bonnerot, C., M.S. Marks, P. Cosson, E.J. Robertson, E.K. Bikoff, R.N. Germain, and J.S. Bonifacino. 1994. Association with BiP and aggregation of class II molecules synthesized in the absence of invariant chain. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:934–944.
27. Marks, M.S., R.N. Germain, and J.S. Bonifacino. 1995. Transient aggregation of major histocompability complex class II chains during assembly in normal spleen cells. *J. Biol. Chem.* 270:10475–10481.
28. Stern, L.J., and D.C. Wiley. 1992. The human class II MHC protein HLA-DR1 assembles as empty $\alpha\beta$ heterodimers in the absence of antigenic peptide. *Cell.* 68:465–477.
29. Germain, R.N., and A.G. Rinker, Jr. 1993. Peptide binding inhibits protein aggregation of invariant-chain free class II dimers and promotes seletive cell surface expression of occupied molecules. *Nature (Lond.).* 363:725–728.
30. Simonis, S., J. Miller, and S.E. Cullen. 1989. The role of the Ia-invariant chain complex in the posttranscriptional processing and transport of Ia and invariant chain glycoproteins. *J. Immunol.* 143:3619–3625.
31. Schreiber, K.L., M.P. Bell, C.J. Huntoon, S. Rajagopalan, M.B. Brenner, and D.J. McKean. 1994. Class II histocompatibility molecules associate with calnexin during assembly in the endocytoplasmic reticulum. *Int. Immunol.* 6:101–111.
32. Anderson, K.S., and P. Cresswell. 1994. A role for calnexin (IP90) in the assembly of class II MHC molecules. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:675–682.
33. Bergeron, J.J., M.B. Brenner, D.Y. Thomas, and D.B. Williams. 1994. Calnexin: a membrane-bound chaperone of the endoplasmic reticulum. *Trends Biochem. Sci.* 19:124–128.
34. Hammond, C., and A. Helenius. 1993. A chaperone with a sweet tooth. *Curr. Biol.* 3:884–886.
35. Margolese, L., G.L. Waneck, C.K. Suzuki, E. Degen, R.A. Flavell, and D.B. Williams. 1993. Identification of the region on the class I histocompatibility molecule that interacts with the molecular chaperone, p88 (calnexin, IP90). *J. Biol. Chem.* 268:17959–17966.
36. Ou, W.J., P.H. Cameron, D.Y. Thomas, and J.J. Bergeron. 1993. Association of folding intermediates of glycoproteins with calnexin during protein maturation. *Nature (Lond.).* 364: 771–776.
37. Hammond, C., I. Braakman, and A. Helenius. 1994. Role of N-linked oligosaccharide recognition, glucose trimming, and calnexin in glycoprotein folding and quality control. *Proc. Natl. Acad. Sci. USA.* 91:913–917.
38. Kearse, K.P., D.B. Williams, and A. Singer. 1994. Persistence of glucose residues on core oligosaccharides prevents association of $TCR\alpha$ and $TCR\beta$ proteins with calnexin and results specifically in acclerated degradation of nascent $TCR\alpha$ proteins within the endoplasmic reticulum. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:3678–3686.
39. Ware, F.E., A. Vassilakos, P.A. Peterson, M.R. Jackson, M.A. Lehrman, and D.B. Williams. 1995. The molecular chaperone calnexin binds Glc1Man9GlcNAc2 oligosaccharide as an initial step in recognizing unfolded glycoproteins. *J. Biol. Chem.* 270:4697–4704.
40. Zhang, Q., M. Tector, and R.D. Salter. 1995. Calnexin recognizes carbohydrate and protein determinants of class I major histocompatibility complex molecules. *J. Biol. Chem.* 270: 3944–3948.
41. Rajagopalan, S., Y. Xu, and M.B. Brenner. 1994. Retention of unassembled components of integral membrane proteins by calnexin. *Science (Wash. DC).* 263:387–390.
42. Arunachalam, B., and P. Cresswell. 1995. Molecular requirements for the interaction of class II major histocompatibility complex molecules and invariant chain with calnexin. *J. Biol. Chem.* 270:2784–2790.
43. Romagnoli, P., and R.N. Germain. 1994. The CLIP region of invariant chain plays a critical role in regulating MHC class II folding, transport, and peptide occupancy. *J. Exp. Med.* 180:1107–1113.
44. Pieters, J., O. Bakke, and B. Dobberstein. 1993. The MHC class II-associated invariant chain contains two endosomal targeting signals within its cytoplasmic tail. *J. Cell Sci.* 106: 831–846.
45. Arunachalam, B., C.A. Lamb, and P. Cresswell. 1994. Transport properties of free and MHC class II-associated oligomers containing different isoforms of human invariant chain. *Int. Immunol.* 6:439–451.
46. David, V., F. Hochstenbach, S. Rajagopalan, and M.B. Brenner. 1993. Interaction with newly synthesized and retained proteins in the endoplasmic reticulum suggests a chaperone function for human integral membrane protein IP90 (calnexin). *J. Biol. Chem.* 268:9585–9592.
47. Koch, N., S. Koch, and G.J. Hämmerling. 1982. Ia invariant chain detected on lymphocyte surfaces by monoclonal antibody. *Nature (Lond.).* 299:644–645.
48. Le, A., J.L. Steiner, G.A. Ferrell, J.C. Shaker, and R.N. Sifers. 1994. Association between calnexin and a secretion-incompetent variant of human alpha 1-antitrypsin. *J. Biol. Chem.* 269:7514–7519.
49. Wada, I., W.J. Ou, M.C. Liu, and G. Scheele. 1994. Chaperone function of calnexin for the folding intermediate of gp80, the major secretory protein in MDCK cells. Regulation by redox state and ATP. *J. Biol. Chem.* 269:7464–7472.
50. Kim, P.S., and P. Arvan. 1995. Calnexin and BiP act as sequential molecular chaperones during thyroglobulin folding

in the endoplasmic reticulum. *J. Cell Biol.* 128:29–38.

51. Degen, E., M.F. Cohendoyle, and D.B. Williams. 1992. Efficient dissociation of the p88 chaperone from major histocompatibility complex class-I molecules requires both β_2-microglobulin and peptide. *J. Exp. Med.* 175:1653–1661.
52. Strubin, M., E.O. Long, and B. Mach. 1986. Two forms of the Ia antigen-associated invariant chain result from alternative initiations at two in-phase AUGs. *Cell.* 47:619–625.
53. Schutze, M.P., P.A. Peterson, and M.R. Jackson. 1994. An N-terminal double-arginine motif maintains type II membrane proteins in the endoplasmic reticulum. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:1696–1705.
54. Nowell, J., and V. Quaranta. 1985. Chloroquine affects biosynthesis of Ia molecules by inhibiting dissociation of invariant (γ) chains from α–β dimers in B cells. *J. Exp. Med.* 162: 1371–1376.
55. Blum, J.S., and P. Cresswell. 1988. Role for intracellular proteases in the processing and transport of class II HLA antigens. *Proc. Natl. Acad. Sci. USA.* 85:3975–3979.
56. Nguyen, Q.V., W. Knapp, and R.E. Humphreys. 1989. Inhibition by leupeptin and antipain of the intracellular proteolysis of Ii. *Hum. Immunol.* 24:153–163.
57. Maric, M.A., M.D. Taylor, and J.S. Blum. 1994. Endosomal aspartic proteinases are required for invariant-chain processing. *Proc. Natl. Acad. Sci. USA.* 91:2171–2175.

57a. Park, S.-J., S. Sadegh-Nasseri, and D.C. Wiley. 1995. Invariant chain made in *E. coli* has an exposed N-terminal segment that blocks antigen binding to HLA-DR1 and a trimeric segment that binds empty HLA-DR1. *Proc. Natl. Acad. Sci. USA.* In press.

58. Roche, P.A., and P. Cresswell. 1991. Proteolysis of the class II-associated invariant chain generates a peptide binding site in intracellular HLA-DR molecules. *Proc. Natl. Acad. Sci. USA.* 88:3150–3154.
59. Riberdy, J.M., J.R. Newcomb, M.J. Surman, J.A. Barbosa, and P. Cresswell. 1992. HLA-DR molecules from an antigen-processing mutant cell line are associated with invariant chain peptides. *Nature (Lond.).* 360:474–477.
60. Freisewinkel, I.M., K. Schenck, and N. Koch. 1993. The segment of invariant chain that is critical for association with major histocompatibility complex class II molecules contains the sequence of a peptide eluted from class II polypeptides. *Proc. Natl. Acad. Sci. USA.* 90:9703–9706.
61. Bijlmakers, M.J., P. Benaroch, and H.L. Ploegh. 1994. Mapping functional regions in the lumenal domain of the class II-associated invariant chain. *J. Exp. Med.* 180:623–629.
62. Avva, R.R., and P. Cresswell. 1994. In vivo and in vitro formation and dissociation of HLA-DR complexes with invariant chain-derived peptides. *Immunity.* 1:763–774.
63. Gorvel, J.P., J.M. Escola, E. Stang, and O. Bakke. 1995. Invariant chain induces a delayed transport from early to late endosomes. *J. Biol. Chem.* 270:2741–2746.