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A role for calnexin (IP90) in the assembly of class II MHC molecules

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Major histocompatibility complex (MHC) class II antigens consist of α and β chains that associate intracellularly with the invariant (I) chain. The HLA-DR $\alpha\beta$I complex assembles in the endoplasmic reticulum (ER) into a nonameric structure via progressive addition of three $\alpha\beta$ dimers to a core invariant chain trimer. We have examined intracellular association of $\alpha\beta$I complexes with the resident ER protein calnexin. Calnexin associates rapidly (within 3 min) with newly synthesized α, β and I chains, and remains associated with the assembling $\alpha\beta$I complex until the final $\alpha\beta$ dimer is added, forming the complete nonamer. Dissociation of calnexin parallels egress of $\alpha\beta$I from the ER. These results suggest that calnexin retains and stabilizes both free class II subunits and partially assembled class II-I chain complexes until assembly of the nonamer is complete.

Key words: calnexin/chaperone/HLA-DR/invariant chain/MHC class II

Introduction

Major histocompatibility complex (MHC) class II molecules are heterodimeric cell surface glycoproteins that bind and present peptides derived from internalized antigens to T lymphocytes. The α chain (35 kDa) and the β chain (27 kDa) of class II associate intracellularly with a third subunit, the invariant (I) chain (reviewed in Cresswell, 1992; Neefjes and Ploegh, 1992a). The predominant form of the I chain is p33, with the minor form p35 resulting from an upstream translation initiation site. The I chain promotes the efficient assembly and transport of $\alpha\beta$ heterodimers (Layet and Germain, 1991; Anderson and Miller, 1992), inhibits premature peptide binding to class II (Roche and Cresswell, 1990; Teyton *et al.*, 1990; Newcomb and Cresswell, 1993), and targets or retains class II molecules in endocytic compartments that contain internalized antigen (Bakke and Dobberstein, 1990; Lotteau *et al.*, 1990; Lamb *et al.*, 1991). As a result, class II molecules from I chain deficient mice are poorly expressed at the cell surface and fail to present foreign antigens to T cells (Bikoff *et al.*, 1993; Viville *et al.*, 1993).

Intracellularly, the $\alpha\beta$I complex assembles through a series of distinct intermediates into a nonameric structure. Newly synthesized α and β chains associate in the ER with a core I chain trimer to form a nonamer containing three $\alpha\beta$ dimers and the I chain trimer (Roche *et al.*, 1991; Lamb and Cresswell, 1992). The nonamer is then transported through the Golgi apparatus to an endocytic compartment, where the I chain is proteolytically degraded (Blum and Cresswell, 1988; Neefjes and Ploegh, 1992b). The liberated $\alpha\beta$ dimers can then bind peptide, acquire an SDS-stable conformation, and be transported to the cell surface (Davidson *et al.*, 1991; Germain and Hendrix, 1991).

The assembly of several multimeric proteins in the ER is accompanied by transient non-covalent association with resident ER proteins, such as BiP/GRP78 and GRP94 (reviewed in Gething and Sambrook, 1992; Kelley and Georgopoulos, 1992). BiP, an hsp 70 homologue, associates transiently with free immunoglobulin heavy chains prior to light chain addition (Bole *et al.*, 1986) and more permanently with mutant forms of influenza haemagglutinin (Hurtley *et al.*, 1989) and VSV-G protein (Machamer *et al.*, 1990). BiP also facilitates translocation of polypeptides across the ER membrane (Sanders *et al.*, 1992). GRP94 (gp96, endoplasmin, Erp99), an hsp 90 homologue, associates with unassembled Ig subunits in a complex with BiP (Melnick *et al.*, 1992). Based on comparisons with prokaryotic chaperones, these proteins are thought to prevent aggregation, stabilize and retain partially folded intermediates, and retain misfolded proteins during subunit assembly.

Recently, another resident ER protein, calnexin (IP90, p88), has been shown to associate with unassembled Ig, T cell receptor and MHC class I subunits (Degen and Williams, 1991; Ahluwalia *et al.*, 1992; Degen *et al.*, 1992; Galvin *et al.*, 1992; Hochstenbach *et al.*, 1992). Like the association of BiP with Ig, calnexin associates quantitatively with newly synthesized class I heavy chains and remains associated until the assembly of heavy chain, β_2-microglobulin and peptide is complete (Degen *et al.*, 1992). However, unlike BiP, GRP94 and other soluble heat shock proteins, calnexin is a constitutively expressed transmembrane protein and may function to stabilize intermediates within the lipid bilayer.

In this study, we examine the association of calnexin with class II MHC molecules. We find that calnexin associates rapidly and quantitatively with class II subunits, and remains associated with the assembling class II-I chain complex until the nonameric structure is complete.

Results

Multiple class II alleles coprecipitate with calnexin

In previous experiments in our laboratory, no proteins other than the I chain have been found to associate with class II molecules during the assembly process. These experiments have been carried out in detergents such as $C_{12}E_9$, Triton X-100 (TX-100), and deoxycholate. Since many proteins associate with calnexin in the detergent digitonin, but not in TX-100 (Hochstenbach *et al.*, 1992), we immunoprecipitated class II molecules from unlabelled cells lysed in digitonin and immunoblotted the precipitates with the anti-calnexin antibody AF8. To minimize the immunoglobulin on the blot

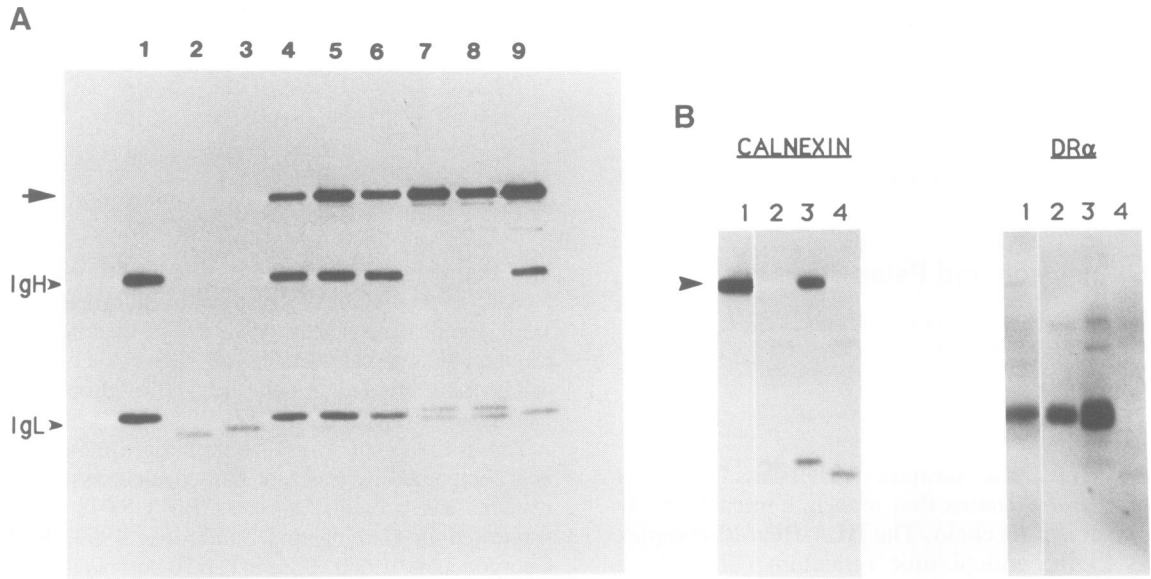

Fig. 1. Coprecipitation of calnexin with HLA-DR3, DR4, DR11 and I-A^k. (**A**) Class II molecules were immunoprecipitated in 1% digitonin from 10^6 cell equivalents from a panel of cell lines, separated by SDS–PAGE, and immunoblotted with the anti-calnexin antibody AF8. T2 cells (class II-negative, lane 1) and Swei cells (DR11, lane 4) were precipitated with DA6.147 (anti-DRα) coupled to Sepharose beads. Swei cells were also precipitated with the control antibody Y3 (lane 2) and the anti-transferrin receptor antibody 1G12 (lane 3). Other class II alleles were precipitated with DA6.147 or 10-2.16 (anti-I-A^k) from the cell lines A2M (DR4, lane 5), T1 (DR7, lane 6), T1.A^k (I-A^k lane 7), T2.A^k (I-A^k, lane 8) and T2.DR3 (DR3, lane 9). (**B**) Left panel. 0.1×10^6 Swei cells (DR11 homozygous) were extracted with digitonin and directly immunoblotted with AF8 as above (lane 1), or 10^6 cells were immunoprecipitated with the antibodies L243 (anti-mature αβ dimers, lane 2), DA6.147 (anti-DRα chain, lane 3) or Y3 (negative control, lane 4), and immunoblotted with AF8. Right panel. The immunoblot in (B) was stripped with SDS and β-mercaptoethanol and reprobed with DA6.147 to demonstrate the presence of DRα chains in both precipitates.

(which is recognized by the secondary antibody) we precipitated with antibodies conjugated to Sepharose beads. Even with this precaution, variable amounts of heavy and light chains are seen in the blot in Figure 1.

There is a 90 kDa AF8-reactive band (see arrowhead) that specifically coprecipitates with anti-class II antibodies from a panel of class II-positive cell lines, but not the class II-negative cell line T2 (Figure 1A, lane 1). In the HLA-DR11 homozygous B-lymphoblastoid cell line Swei, the 90 kDa protein coprecipitates with the anti-class II antibody DA6.147 (lane 4), but not the control antibody Y3 (lane 2) or the anti-transferrin receptor antibody 1G12 (lane 3), even though Swei cells express transferrin receptor. The 90 kDa protein comigrates with immunoprecipitated calnexin and co-precipitates with anti-class I antibodies (data not shown). Class II–calnexin complexes were precipitated with DA6.147 from the hemizygous cell lines A2M (DR4, lane 5) and T1 (DR7, lane 6) and from the transfectant T1.A^k with the anti-I-A^k antibody 10-2.15 (lane 7). The cell line T2 is a class II antigen processing mutant (Riberdy and Cresswell, 1992). To determine if this affects calnexin association, class II molecules were immunoprecipitated from transfectants of T2 (T2.A^k and T2.DR3). Calnexin was also present in these immunoprecipitates (lanes 8 and 9). Therefore, human calnexin associates with human and mouse class II molecules from both wild-type and mutant cell lines.

The association of calnexin and class II is transient

To determine whether calnexin associates with immature and/or mature forms of class II, we immunoprecipitated class II molecules with conformation-specific anti-class II monoclonal antibodies. DA6.147 is an anti-DRα chain antibody that preferentially recognizes free HLA-DR α chains and αβI complexes. The antibody L243, in contrast, recognizes only mature αβ dimers. Using DA6.147 and L243, class II molecules were precipitated from Swei cells and blotted with anti-calnexin as in Figure 1A. Figure 1B shows the coprecipitation of calnexin and class II with DA6.147 (lane 3) but not L243 (lane 2), demonstrating that calnexin associates with immature αβI, but not mature αβ complexes. To show that α chains were present in both precipitations, the blot was stripped and reprobed with DA6.147 (Figure 1B, right panel). Calnexin is the only AF8-reactive band in Swei cell lysates (lane 1), and does not coprecipitate with the negative antibody Y3 (lane 4).

The blot in Figure 1B suggests that the association between calnexin and class II molecules is transient. We therefore compared the rate of assembly and processing of class II αβI complexes as a function of their association with calnexin. The kinetics of assembly and transport of HLA-DR molecules have been well characterized in Swei cells (Machamer and Cresswell, 1982), and are shown in Figure 2A. Swei cells were metabolically labelled for 15 min and chased for up to 8 h. The TX-100 cell lysates were immunoprecipitated with a mixture of DA6.147 (anti-DRα chain) and XD5.A11 (anti-β chain) and separated by two-dimensional electrophoresis. Assembly of the αβI complex occurs within the 15 min pulse-label, and processing of the individual chains by sialylation to more acidic forms is visible within 30 min. Processed I chain (Ip), which contains both N- and O-linked glycans, results in the most dramatic acidic shift. Disappearance of I chain due to proteolytic degradation and release of αβ dimers is apparent at later time points.

The same analysis was performed on pulse–chased Swei cells, solubilized in digitonin and precipitated with AF8. In Figure 2B, the entire two-dimensional gel is included to show that DRα, β and I chains are the predominant labelled proteins that associate with calnexin, even though Swei cells

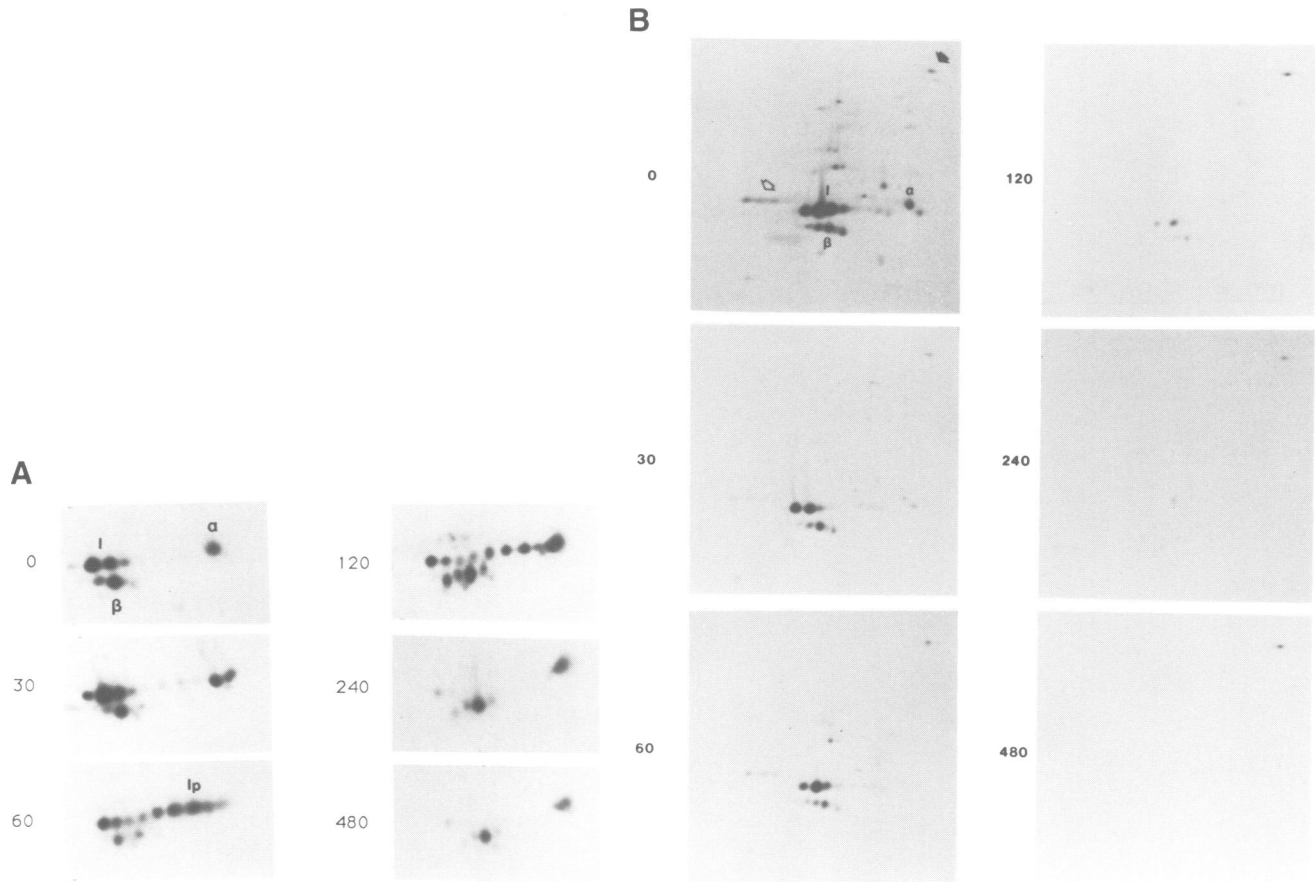

Fig. 2. Assembly of class II and calnexin–class II complexes in Swei cells. (**A**) 15×10^6 Swei cells were pulse-labelled for 15 min and chased for 0–480 min, lysed in 1% TX-100, immunoprecipitated with DA6.147 (anti-DRα chain) and XD5.A11 (anti-β chain), and separated by two-dimensional NEPHGE–SDS–PAGE. (**B**) 15×10^6 Swei cells were pulse–chase labelled as in (A), but lysed in 1% digitonin, immunoprecipitated with AF8 (anti-calnexin), and separated by two-dimensional NEPHGE–SDS–PAGE.

also express the calnexin-binding proteins immunoglobulin and class I MHC. Also visible are the p35 form of the I chain (open arrow) and labelled calnexin (filled arrow). Although labelled calnexin can be immunoprecipitated with AF8, labelled calnexin is not detectably coprecipitated with anti-class II antibodies (data not shown). This is probably because calnexin that associates with class II includes a pool of pre-existing unlabelled molecules, as found for class I–calnexin complexes (Degen and Williams, 1991).

Class II complexes associate with calnexin within the 15 min pulse label, and dissociate with a half-life of 30–60 min. In contrast to the normal processing of α, β and I chains seen in Figure 2A, the calnexin-associated class II remains unprocessed. This is most evident with the I chain, which does not acquire sialic acid modifications. The class II associated with calnexin remains sensitive to endoglycosidase H (data not shown). Since the dissociation of $\alpha\beta$I complexes from calnexin occurs as $\alpha\beta$I complexes are processed, these results suggest that $\alpha\beta$I complexes remain associated with calnexin in the ER and are released prior to transport through the Golgi apparatus.

The intensity of labelled class II molecules that are immunoprecipitated from pulse-labelled cells with anti-class II antibodies is equivalent to the amount of class II molecules coprecipitated with the AF8 antibody (compare the 0 time points in Figure 2A and B). Although the labelled cells in Figure 2A and B were lysed in different detergents (TX-100 and digitonin), the same result is found for Swei cells lysed only in digitonin, and for the cell line T1.DR3 (data not shown). These results suggest that the majority of newly synthesized class II associates with calnexin. Up to 70% of the total pulse labelled DRα chains can be immunodepleted by the AF8 antibody (data not shown). Since the class II–calnexin association is sensitive to detergent conditions, the remaining 30% may have dissociated during solubilization or incubation with the antibody.

Calnexin associates with free α, β and I chains

The pulse–chase analysis of Swei cells in Figure 2B demonstrates that α, β and I chains associate with calnexin, but does not address which of the three chains directly associates with calnexin. To determine whether free α, β and I chains can associate with calnexin, we used a vaccinia virus–T7 polymerase system (Fuerst *et al.*, 1986; Lamb *et al.*, 1991) to transiently express the individual chains in HeLa cells, which express calnexin but are negative for class II and I chain. The results are shown in Figure 3A.

HeLa cells were infected with vaccinia virus encoding T7 RNA polymerase, then transfected with plasmids encoding α, β or I cDNAs with a T7-dependent promoter. Five hours post infection, cells were radiolabelled for 1 h with [^{35}S]methionine and immunoprecipitated with anti-class II (lane 1) or anti-calnexin (lane 2) antibodies. Because calnexin associates with other proteins, bound proteins were removed from the protein G–Sepharose beads with SDS and re-precipitated with conformation-independent anti-class II

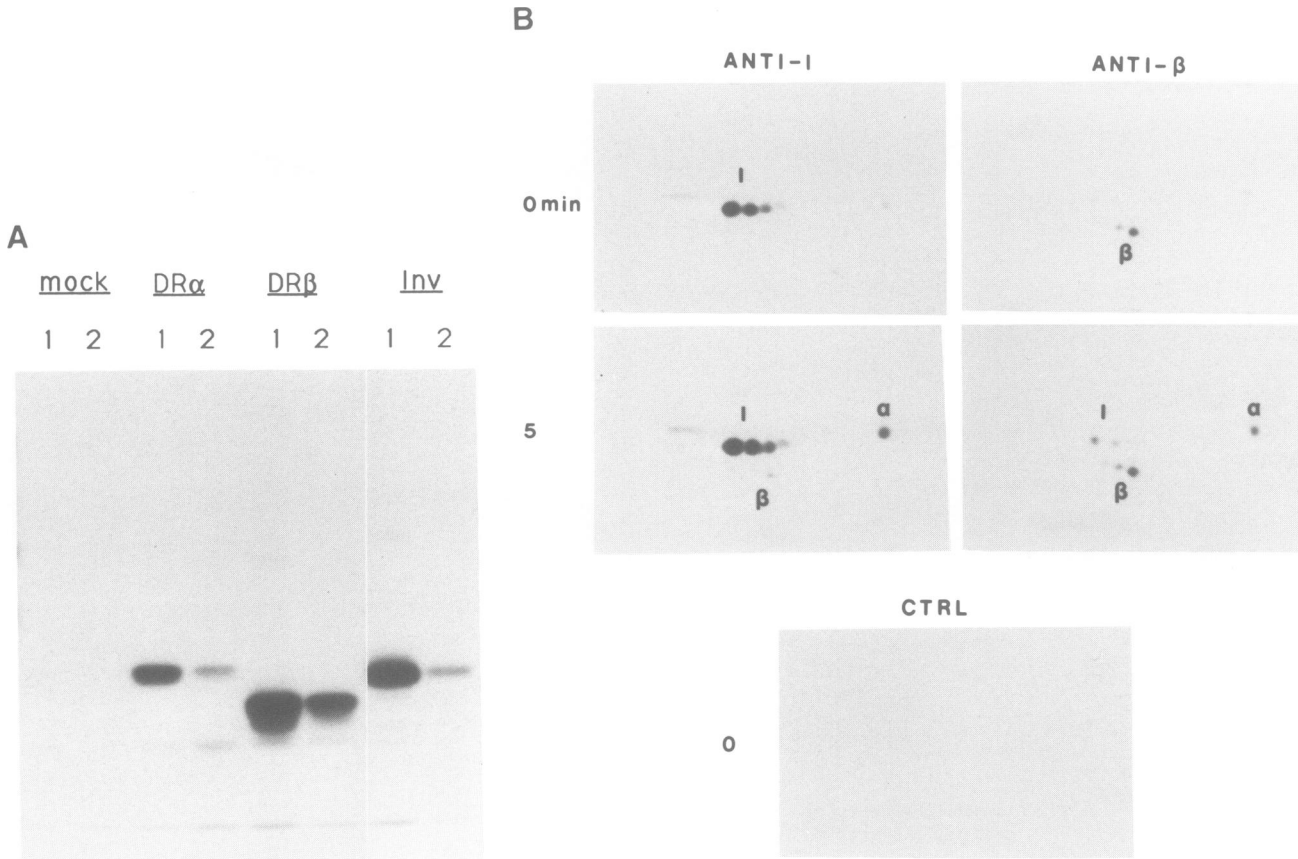

Fig. 3. Association of calnexin with free α, β and I chains and $\alpha\beta$I complexes. (**A**) HLA-DRα, DR11β and the p33 form of the I chain were transiently expressed in vaccinia-infected HeLa cells and metabolically labelled for 1 h. Cell lysates were divided and immunoprecipitated with anti-class II (lane 1) or anti-calnexin (lane 2). The proteins were eluted from the protein G beads with TBS, 2% SDS and 2 mM DTT, diluted with TBS, 1% TX-100 and 10 mM IAA, and reprecipitated with anti-class II or anti-I chain antibodies (DA6.147, XD5.A11 or PIN.1 for α, β or I chains, respectively). Mock-transfected cells were reimmunoprecipitated as above with a mixture of all three anti-class II antibodies (mock lanes 1 and 2). (**B**) Swei cells were metabolically labelled with [^{35}S]methionine for 3 min and either immediately solubilized or chased for 5 min prior to solubilization in TBS/1% digitonin. The extracts were precipitated with anti-calnexin (AF8). Calnexin-associated class II complexes were eluted with TBS/1% TX-100 and reprecipitated with XD5.A11 (anti-β chain), PIN.1 (anti-I chain) or Y3 (control). The complexes were then separated by two-dimensional NEPHGE–SDS–PAGE.

antibodies. This technique is specific. When control antibodies are used for the first precipitation or the reprecipitation no radiolabelled bands are detected (data not shown). In Figure 3, it can be seen that the transiently expressed free α and β chains, and the p33 form of I chain all coprecipitate with anti-calnexin antibodies (lanes 2). The relatively poor association of free chains with calnexin (compare lanes 1 and 2) may be due to overexpression of the chains relative to calnexin, inhibition of host protein synthesis by vaccinia, or weaker affinity of calnexin for individual chains compared with the $\alpha\beta$I complex. We cannot distinguish between these possibilities. The p35 form of the I chain also associates with calnexin in this assay, and free I chain associates with calnexin in the class II-negative cell line T2 (data not shown). These results demonstrate that each of the free chains can associate with calnexin.

While the results of the transient expression system demonstrate that calnexin can associate with free α, β and I chains, it does not address what happens in the normal assembly process. To examine the initial events of calnexin–class II assembly, Swei cells were metabolically labelled for 3 min and chased for 5 min. Class II–calnexin complexes were then immunoprecipitated with anti-calnexin. To elute intact class II complexes from calnexin, the immunoprecipitates were incubated with 1% TX-100, which dissociates class II from calnexin. Although elution with TX-100 is not as efficient as elution with SDS, $\alpha\beta$I complexes remain intact in TX-100. The eluted proteins were then reprecipitated with anti-β (XD5.A11), anti-I (PIN.1) or control (Y3) antibodies. Therefore, any α chain or I chain that coprecipitates with anti-β chain must have been associated with β chain–calnexin complexes. Likewise, any α or β chains that coprecipitate with anti-I chain must have been associated with I chain–calnexin complexes. This technique allows us to examine what combinations of α, β or I-containing complexes associate with calnexin. Since each individual chain can bind calnexin, these experiments do not address which chain mediates the association of $\alpha\beta$I with calnexin.

After a 3 min metabolic label, the most intense spots reprecipitated with the anti-β antibody XD5.A11 are β chains, although very faint α and I chain spots are visible (Figure 3B, upper right panel). Therefore, the majority of β chains that associate with calnexin within 3 min of translation exist as β–calnexin complexes, and not associated with α or I chains. It is unlikely that the newly synthesized β chains are associating with unlabelled α or I chains, because labelled β chains are not coprecipitated with the anti-I chain antibody (upper left panel). After 5 min of chase, the anti-β antibody coprecipitates with more intense α and I chain spots (lower right panel), demonstrating the formation of $\alpha\beta$I–calnexin

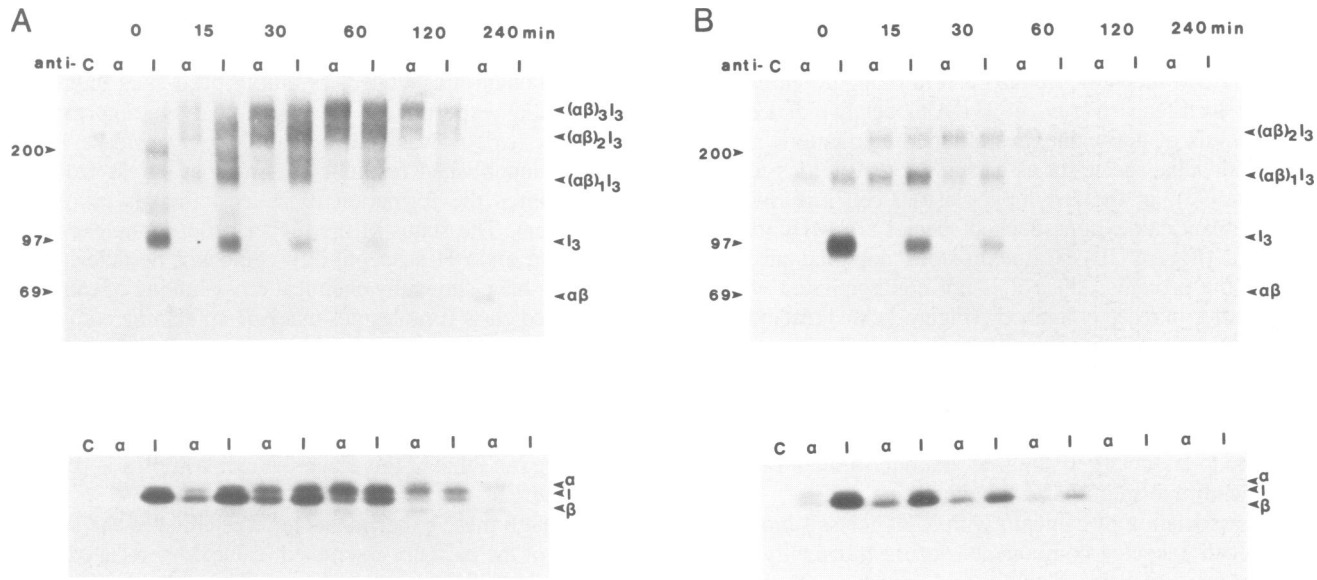

Fig. 4. Association of calnexin with class II assembly intermediates. (**A**) 6×10^6 Swei cells were pulse-labelled with [^{35}S]methionine for 15 min and chased for 0–240 min. Cells were extracted in BS/1% $C_{12}E_9$ with 200 μM DSP. After quenching the cross-linker with glycine, class II was immunoprecipitated with Y3 (negative control), DA6.147 or PIN.1 coupled to Sepharose beads. The precipitates were divided and separated by non-reducing 5–10% gradient SDS–PAGE (upper panel) or reducing 10.5% SDS–PAGE (lower panel). (**b**) Swei cells were pulse–chase labelled as in (A), lysed in BS/1% digitonin without DSP, and immunoprecipitated with AF8 (anti-calnexin). The calnexin-associated proteins were eluted with BS/1% $C_{12}E_9$ and then cross-linked with 200 μM DSP. Class II was immunoprecipitated as above and separated by non-reducing gradient SDS–PAGE (5–10% acrylamide, upper panel) or reducing SDS–PAGE (10.5% acrylamide, lower panel).

complexes. As with the XD5.A11, reprecipitation of calnexin complexes with PIN.1 (anti-I) yields a predominant I chain spot at the pulse time point (I–calnexin, upper left panel), with coprecipitated α and β spots evident after 5 min of chase ($\alpha\beta$I–calnexin, bottom left panel). Similar results are seen when calnexin complexes are reprecipitated with the anti-α chain antibody DA6.147, with α chain visible at the pulse time point, and α, β and I chains visible at the chase point (data not shown). Therefore, calnexin associates rapidly (possibly cotranslationally) with newly synthesized free α, β and I chains in Swei cells. These α–calnexin, β–calnexin and I–calnexin complexes combine within minutes to form $\alpha\beta$I–calnexin complexes.

Dissociation of calnexin from class II parallels assembly of the complete nonamer

Figures 2B and 3B demonstrate that calnexin rapidly associates with α, β and I chains within minutes of translation, but dissociation of the $\alpha\beta$I complex is not completed until 120 min after translation. Since class II molecules assemble in the ER through a defined series of intermediates (Lamb and Cresswell, 1992), this suggested that calnexin remains associated with the $\alpha\beta$I complex until assembly is complete. Class II assembly intermediates can be detected with the thiol-cleavable, homobifunctional chemical cross-linker DSP. Swei cells were pulse–chase radiolabelled and lysed in the detergent $C_{12}E_9$ containing DSP (Lamb and Cresswell, 1992). The extracts were immunoprecipitated with anti-DRα or I chain antibodies and separated on 5–10% SDS non-reducing gradient gels (Figure 4A, top panel) or 10.5% SDS reducing gels (Figure 4A, bottom panel). After a 15 min pulse, the I chain trimer (90 kDa) is evident, as well as several $\alpha\beta$-containing bands. Of these, the 150 kDa $\alpha\beta$-containing band increases in intensity during the 15–30 min chase (lower arrowhead). As the chase progresses, two bands of ~200 kDa (middle arrowhead) and 260 kDa (upper arrowhead) become more prominent as the I chain trimer and the 150 kDa band become fainter. The α-containing bands include β chain, since they are removed by preclearing with an anti-β antibody (Lamb and Cresswell, 1992). The incremental addition of $\alpha\beta$-containing 60 kDa proteins to the complex shows that $\alpha\beta$ dimers progressively associate with the core I chain trimer until the $(\alpha\beta)_3I_3$ nonamer is assembled. At later chase points, free $\alpha\beta$ dimers (62 kDa) are released from the complex as the I chain is degraded. Reduction of the cross-linker shows that the only radiolabelled proteins in the complex are α, β and I chains (Figure 4A, bottom panel).

To examine the multimeric structure of calnexin-associated class II molecules, Swei extracts were first immunoprecipitated with AF8 and the class II molecules dissociated from calnexin with the detergent $C_{12}E_9$. DSP was then added, and the lysates were treated as in Figure 4A. The results are shown in Figure 4B. During the chase, the calnexin-associated complexes progress from free I chain trimers (90 kDa) to multimers consisting of one or two $\alpha\beta$ dimers associated with the core I chain trimer (150 and 200 kDa bands). In contrast to Figure 4A, we do not see the uppermost 260 kDa band [the complete $(\alpha\beta)_3I_3$ nonamer], and we do not see free $\alpha\beta$ dimers. These results demonstrate that calnexin remains associated with the $\alpha\beta$I complex through the assembly process until the final $\alpha\beta$ dimer is added to form the $\alpha\beta$I nonamer.

Discussion

Calnexin was defined as a ubiquitous ER phosphoprotein associated with members of the canine signal sequence receptor complex, SSRα and SSRβ, as well as a novel 25 kDa glycoprotein of the same complex, gp25L (Wada *et al.*, 1991). It has also been identified as an 88 kDa component (p88) associated with MHC class I molecules

(Degen and Williams, 1991; Ahluwalia *et al.*, 1992; Galvin *et al.*, 1992), and as a 90 kDa protein (IP90) associated with human T cell receptor subunits and immunoglobulin heavy chains (Hochstenbach *et al.*, 1992). Although two-dimensional gel analysis of anti-calnexin immunoprecipitations reveals >50 individual species of associated proteins (Hochstenbach *et al.*, 1992), in the B-lymphoblastoid cell line Swei the predominant calnexin-associated species are MHC class II subunits (Figure 2B). Calnexin does not bind all transmembrane proteins, since it is not coprecipitated with an anti-transferrin receptor antibody (Figure 1), and metabolically labelled transferrin receptor does not coprecipitate with the anti-calnexin antibody (data not shown). The CD5 monomer and the CD28 homodimer also do not coprecipitate with calnexin (Hochstenbach *et al.*, 1992). Therefore, calnexin appears to associate with a broad but limited range of transmembrane multimeric glycoproteins.

The association of calnexin with MHC class I molecules and T cell receptor components occurs transiently in the early stages of their assembly and transport (Degen and Williams, 1991; Hostenbach *et al.*, 1991; David *et al.*, 1993). In our hands, while calnexin associates with several mouse and human class II alleles it clearly associates with individual DRα, DRβ and I chain subunits expressed transiently in HeLa cells. In Swei cells, calnexin also associates with free newly synthesized α, β and I chains prior to $\alpha\beta$I complex formation (Figure 3). An apparent exception to the finding that proteins are transiently associated with calnexin may be the signal sequence receptor complex. In the experiments reported by Wada *et al.* (1991) association was maintained through extensive purification procedures, and calnexin was co-immunoprecipitated with SSRα from cells extracted with a conventional non-ionic detergent, TX-100, rather than digitonin. The signal sequence receptor complex was identified by its ability to be cross-linked to nascent polypeptides in the ER (Gorlich *et al.*, 1992), and is therefore believed to be in close proximity to the site of protein translocation. The rapid association of calnexin with individual class II subunits (<3 min) suggests that calnexin may function with the signal sequence receptor complex to bind the polypeptides cotranslationally.

While calnexin initially associates with free class II subunits, it remains associated during the assembly of the $\alpha\beta$I complex. Examination of Figure 3B suggests that the α subunit binds to calnexin-associated I chain molecules before the β subunit. Thus, the intensity of the α subunit spot at 5 min in the anti-I chain immune precipitate is much higher than that of the β-subunit. This is consistent with the early findings of Kvist *et al.* (1982), who found that α subunit association with I chain preceded that of the β subunits. Examination of class II assembly intermediates by pulse–chase labelling and chemical cross-linking previously showed progressive addition of three $\alpha\beta$ dimers to the core I trimer (Lamb and Cresswell, 1992), with no free detectable $\alpha\beta$ dimers present during the assembly process. Combining the data, it seems likely that $\alpha\beta$ dimerization occurs rapidly on calnexin-associated I chain trimers after the initial binding of an α chain. This would be consistent with published data indicating that the I chain itself performs a chaperone-like function in the assembly of $\alpha\beta$ dimers (Layet and Germain, 1991; Anderson and Miller, 1992). The chaperone function of the I chain is not obligatory for $\alpha\beta$ dimerization, however, since some $\alpha\beta$ dimers are expressed on the cell surface in the absence of I chain (Miller and Germain, 1986; Sekaly *et al.*, 1986). The role of calnexin, and other chaperones, in this I chain-independent assembly process is unknown.

In initial experiments we found that the intermediate complex $(\alpha\beta)_1I_3$ was sensitive to endoglycosidase H (C. Lamb, unpublished results), arguing that egress from the ER requires the formation of at least $(\alpha\beta)_2I_3$ or $(\alpha\beta)_3I_3$ (nonamer). The status of the higher order complexes with regard to endo H susceptibility could not be determined. Here we have shown by chemical cross-linking of calnexin-associated class II molecules that $\alpha\beta$I-containing complexes of the same mobility as $(\alpha\beta)_1I_3$ and $(\alpha\beta)_2I_3$ are associated with calnexin while the nonamer is not. It seems probable that calnexin dissociates from the $\alpha\beta$I complex when the final $\alpha\beta$ dimer is added to form the complete nonameric structure, and that at this stage the $\alpha\beta$I complex is released from the ER.

Although these experiments have established the stoichiometry of the partially assembled, calnexin-associated, class II–I chain complexes, they do not address the stoichiometry of the class II–I chain–calnexin complex itself. Since calnexin associates with free I chains (Figure 3B), and remains associated as I chain trimerizes (Figure 4B and data not shown), it is likely that multiple calnexin molecules are associated with the trimer. Calnexin may dissociate from each individual α, β and I chain subunit as the α and β subunits associate with the I chain trimer. The I chain subunits within the trimer that are not yet associated with $\alpha\beta$ dimers presumably would remain associated with calnexin. Thus calnexin may act as a 'scaffold' on which assembly occurs. Addition of the final $\alpha\beta$ dimer to the complex would then be accompanied by dissociation of the final calnexin molecule.

The kinetics of calnexin–class II association and dissociation are consistent with calnexin functioning to retain assembling class II molecules. Similar results strongly suggest that calnexin functions to retain class I molecules in the ER until assembly is complete. Class I MHC molecules consist of a trimolecular complex, containing a transmembrane heavy chain, a soluble light chain (β_2-microglobulin) and peptide, which assemble in the ER. Calnexin associates rapidly and quantitatively with class I heavy chains and remains associated when β_2-microglobulin binds. Dissociation of the class I–calnexin complex correlates with transport of class I out of the ER, and is delayed in mutants which fail to generate β_2-microglobulin or peptides (Degen *et al.*, 1992). Similar experiments with assembling T cell receptors argue that individual β-chains remain associated with calnexin for long periods until assembly is completed (David *et al.*, 1993). The C-terminal residues of human calnexin (IP90) are proposed to encode an ER retention signal (David *et al.*, 1993) which may mediate the retention of associated proteins.

There is also an ER localization signal on the cytoplasmic extension of the p35 form of the I chain that confers ER retention of p35, and retention of the more abundant p33 form due to formation of mixed trimers with p35 (Lamb and Cresswell, 1992; Arunachalam *et al.*, 1994). As a result, the I chain remains in the ER in the absence of class II association (Marks *et al.*, 1990). Association of $\alpha\beta$ dimers is thought to mask the I chain retention signal, resulting in transport of the $\alpha\beta$I complex. However, several lines of evidence suggest that the p35 extension is not the only mechanism governing ER retention of class II molecules.

First, p35 is not expressed in murine cells, and is only a minor component of the total invariant chain in human cells. Second, p33, when expressed alone, can transport to endosomes, but the majority remains in the ER (Bakke and Dobberstein, 1990). Finally, $\alpha\beta$ dimers, in the absence of I chain, may remain in the ER (Layet and Germain, 1991; Anderson and Miller, 1992; Schaiff *et al.*, 1992). Because calnexin binds to each of the individual subunits, it is a likely candidate for retention of these molecules in the absence of p35, and may cooperate with p35 to retain higher order multimers until assembly is complete.

The non-covalent association of calnexin with assembling multimeric proteins is similar to the association of chaperones with their substrates. Given the intensity and proportion of radiolabelled class II subunits coprecipitated by anti-calnexin antibodies, and the association of higher order multimers with calnexin, it is unlikely that the association with calnexin represents a dead-end pathway for misfolded proteins. Rather, calnexin may function like the prokaryotic chaperones of the hsp60 and hsp70 families by stabilizing partially folded intermediates, thereby preventing aggregation of unassembled subunits. Unlike prokaryotic and eukaryotic chaperones, calnexin is a transmembrane protein, and may have a specialized function in the assembly of transmembrane proteins. Deletion constructs of class I MHC molecules suggest that calnexin binds the transmembrane region of class I heavy chains (D.Williams, personal communication). Recent experiments have shown that free TCRα chains are secreted into the lumen of the ER in the absence of TCRβ (Shin *et al.*, 1993). Although the TCRα transmembrane regions, unlike class II subunits, contain several charged amino acids, association of free transmembrane regions with integral membrane proteins such as calnexin may have a general function in the assembly of oligomers of membrane proteins. The efficient assembly of class II molecules has been found to depend upon the presence of the correct transmembrane regions of the individual α- and β-subunits (Cosson and Bonafacino, 1992).

Two soluble resident ER proteins, GRP94 and ERp72, have been shown to associate with class II subunits in the absence of I chain (Schaiff *et al.*, 1992). In wild-type cells, I chain is present in excess over α and β chains (Machamer and Cresswell, 1982), so the association is not found. GRP94 and ERp72 may bind solely to class II molecules that misfold in the absence of I chain (Peterson and Miller, 1990), or bind transiently to α and β chains prior to I chain addition. Since the association of calnexin and free chains is very rapid, binding of GRP94 or ERp72 to free chains prior to calnexin addition is unlikely. Rather, GRP94 and/or ERp72 may form a ternary (or tetrameric) complex with calnexin and free α or β chains, as BiP forms a ternary complex with GRP94 and immunoglobulin heavy chains (Melnick *et al.*, 1992). Upon addition of I chain, the soluble chaperones would dissociate. Such a relay system of cooperation has been described for the prokaryotic chaperones DnaK and GroEL (Langer *et al.*, 1992). In this manner, soluble and transmembrane ER chaperones may stabilize different segments of the polypeptide chain prior to assembly.

Materials and methods

Cell lines

The HeLa cell line, the B-LCL lines Swei (DR11) and A2M (DR4) and the TxB hybrid lines T1 (DR7) and T2 (class II$^-$) were maintained in Iscove's modified DMEM (Gibco, Grand Island, NY) with 5% calf serum (Hyclone, Logan, UT) and 20 μg/ml gentamicin. Stable genomic HLA-DR3 or I-A^k transfectants of T2 have been described (Riberdy and Cresswell, 1992).

Antibodies

The anti-calnexin monoclonal antibody AF8 was the generous gift of Dr M.Brenner (Hochstenbach *et al.*, 1992). The monoclonal antibodies Y3 (anti-H-2K^b, Jones and Janeway, 1981), 10-2.16 (anti-I-A^k, Oi *et al.*, 1978), DA6.147 (anti-HLA DRα chain, Guy *et al.*, 1982), L243 (anti-HLA DR $\alpha\beta$ dimer, Lampson and Levy, 1980), XD5.A11 (anti-class II β chain, Radka *et al.*, 1994), PIN.1 (anti-I chain, Lamb and Cresswell, 1992) and 1G12 [anti-transferrin receptor (TfR), Davis and Cresswell, 1990] have been described.

Metabolic labelling and immunoprecipitations

For pulse-radiolabelling of cells, 30×10^6 cells were radiolabelled for 15 min with 1.5 mCi [^{35}S]methionine Trans-label (ICN Biochemical, Costa Mesa, CA), and labelling was stopped with cold PBS. For the chase labelling, the pulsed cells were diluted with a 15-fold excess of cold methionine and incubated for up to 8 h at 37°C. Cells were extracted on ice at $2-5 \times 10^6$ cells/ml with 10 mM Tris, 150 mM NaCl, pH 7.4 (TBS), containing 1% TX-100 (Sigma, St Louis, MO), 0.5 mM PMSF and 0.1 mM TLCK. Post-nuclear supernatants were precleared for 1 h with normal rabbit serum and protein G–Sepharose (Pharmacia, Piscataway, NJ) and then precipitated with specific antibody (2 μl ascites/ml extract) and protein G–Sepharose. Pellets were washed three times with TBS/0.1% TX-100. To coprecipitate class II–calnexin complexes, cells were lysed as above, but with TBS–1% digitonin (Wako, Richmond, VA) instead of TX-100. After immunoprecipitation, the pellets were washed with TBS–0.1% digitonin.

Elution and reprecipitation of immunoprecipitates

Proteins were eluted from immunoprecipitates by denaturation by adding 100 μl TBS, 2% SDS and 2 mM dithiothreitol and boiling for 3 min. The supernatants were diluted with 1 ml TBS, 1% TX-100 and 10 mM iodoacetamide, incubated for 30 min at 25°C, and then reprecipitated with antibody and protein G–Sepharose as described above. The precipitates were washed with TBS–0.1% TX-100 prior to electrophoresis. For elution of class II molecules from calnexin immunoprecipitates under native conditions, 0.5 ml TBS containing 1% TX-100 or 1% $C_{12}E_9$ were added. After 15 min at 25°C, the beads were pelleted and re-eluted with 0.5 ml TBS with 1% TX-100 or 1% $C_{12}E_9$. The supernatants were pooled and precipitated as above.

Two-dimensional PAGE

Two-dimensional gel analysis with NEPHGE (3/10 ampholytes; Bio-Rad, Richmond, VA) in the first dimension and 10.5% SDS–PAGE in the second dimension was performed as described (O'Farrell *et al.*, 1977).

Transient cDNA expression in HeLa cells

Transient expression of cDNAs in HeLa cells was performed essentially as described in Lamb *et al.* (1991). HeLa cells were infected with a recombinant vaccinia virus encoding T7 RNA polymerase, vTF7-3, and transfected with HLA-DR α, β and I cDNAs driven by the T7 promoter (Fuerst *et al.*, 1986). This process results in high levels of expression of transfected genes. Subconfluent HeLa cells in 25 cm^3 flasks were infected with vTF7-3 at a multiplicity of infection of 20 for 30 min in serum-free medium. The medium was removed, and 10 μg of the cDNAs were added with 10 μl of lipofectin (BRL) in serum-free medium. After 2.5 h the medium was removed, the cells were trypsinized, incubated for 1 h in methionine-free medium, and then labelled for 1–2 h with [^{35}S]methionine as above.

Immunoblotting

Cells (10^6) were lysed in 1% digitonin and immunoprecipitated with antibody coupled to Sepharose beads. The proteins were eluted from the beads with non-reducing Laemmli sample buffer, reduced with β-mercaptoethanol, separated by 10.5% SDS–PAGE, then electroblotted for 1.5 mA h. For unprecipitated cell lysates, 10^5 cell equivalents were lysed in digitonin and loaded on the gel. The blots were probed by chemiluminescence (ECL; Amersham, Arlington Heights, IL) using a 1:16 dilution of AF8 supernatant or 1:4 dilution of DA6.147 supernatant and a 1:4000 dilution of HRP-conjugated anti-Ig secondary antibody (Pel-Freez Biologicals, Brown Deer, WI).

DSP cross-linking

For cross-linking in cell extracts, 6×10^6 cells were extracted at 2×10^6 cells/ml in 10 mM bicine, 150 mM NaCl, pH 8.2 (BS) and 1% polyoxy-

ethylene 9 laurylether ($C_{12}E_9$) (Sigma), containing 200 μM dithiobis (succinidmidyl) propionate (DSP; Sigma) for 30 min on ice. The cross-linking was stopped with 10 mM glycine. PMSF and TLCK were added and samples were immunoprecipitated with Y3, DA6.147 or PIN.1 conjugated to CNBr-activated Biogel A50m beads (Bio-Rad). Pellets were washed three times with BS/0.1% $C_{12}E_9$. For cross-linking of calnexin-associated proteins, 6×10^7 cells were extracted at 2×10^6 cells/ml in BS/1% digitonin without DSP, immunoprecipitated with AF8, and eluted with BS/1% $C_{12}E_9$. DSP (200 μM) was added to the supernatants for 30 min on ice, quenched with 10 mM glycine and immunoprecipitated with antibodies conjugated to Biogel beads as above.

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