The EMBO Journal vol.14 no.14 pp.3425–3433, 1995

The molecular chaperone calnexin is expressed on the surface of immature thymocytes in association with clonotype-independent CD3 complexes

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Immature thymocytes express clonotype-independent CD3 complexes that, when engaged by anti-CD3 antibodies, can signal $CD4^-CD8^-$ thymocytes to differentiate into $CD4^+CD8^+$ cells. Clonotype-independent CD3 complexes consist of CD3 components associated with an unknown 90 kDa surface protein. We now report the surprising finding that this 90 kDa surface protein is the molecular chaperone calnexin, an integral membrane protein previously thought to reside only in the endoplasmic reticulum (ER). We found that calnexin–CD3 complexes escaping to the cell surface utilize interchain associations distinct from those utilized by calnexin–CD3 complexes remaining within the ER. Specifically, we demonstrate that carbohydrate-mediated luminal domain interactions that are necessary for formation of most internal calnexin–CD3 complexes are not required for formation of calnexin–CD3 complexes destined to be expressed on the cell surface, and we provide evidence that cytoplasmic domain interactions between calnexin and CD3ε chains mask calnexin's ER retention signal, permitting calnexin and associated proteins to escape ER retention. Thus, the present study demonstrates that partial T cell antigen receptor complexes can escape the ER of immature thymocytes in association with their molecular chaperone to be expressed at low levels on the cell surface where they may function as a signaling complex to regulate thymocyte maturation.

Key words: calnexin/CD3/endoplasmic reticulum/retention/thymocytes

Introduction

Immature thymocytes up through the $CD4^+CD8^+$ stage of development express on their surface incompletely assembled components of the T cell antigen receptor (TCR) complex that are retained within the endoplasmic reticulum (ER) of mature T cells (Jacobs *et al.*, 1994; Shinkai and Alt, 1994; Wiest *et al.*, 1994). Most notably, immature thymocytes have been found to express heterodimers of CD3γε and CD3δε chains without either clonotypic or surrogate TCR chains and these clonotype-independent CD3 (CIC) complexes are competent to transduce intracellular signals. Indeed, engagement of these surface complexes by anti-CD3 antibody induces $CD4^-CD8^-$ thymocytes to differentiate into $CD4^+CD8^+$ thymocytes (Levelt *et al.*, 1993a,b; Jacobs *et al.*, 1994; Shinkai and Alt, 1994). The mechanism by which CIC complexes escape from the ER without associated clonotypic TCR chains is entirely unknown. However, it is known that surface CIC complexes contain, in addition to CD3 components, an unknown protein of 90 kDa that has been referred to as CD3-associated protein (CD3AP; Wiest *et al.*, 1994), that might play a role in the escape of these complexes from ER retention.

Fully assembled multimeric TCR complexes consisting of CD3 and clonotypic chains are transported efficiently out of the ER; in contrast, individual subunit chains and partially assembled complexes are retained within the ER (Klausner *et al.*, 1990; David *et al.*, 1993). Molecular chaperones are ER-resident proteins that bind transiently to nascent proteins, releasing them so that they can exit from the ER, but bind persistently to malfolded or incompletely assembled proteins, retaining them within the ER (Gething and Sambrook, 1992; Hammond and Helenius, 1994a). Retention within the ER of the molecular chaperones themselves is mediated by receptors that bind to retention sequences on the chaperone molecules and either anchor the chaperone molecules within the ER or retrieve them continuously from a dynamic intermediate compartment between the ER and Golgi (Pfeffer and Rothman, 1987; Pelham, 1988). The sequence motifs responsible for ER retention are known (Munro and Pelham, 1987; Jackson *et al.*, 1990). Retention of luminal ER proteins is mediated by the carboxy-terminal tetrapeptide sequence KDEL (Munro and Pelham, 1987), whereas retention of integral membrane ER proteins is dependent on the carboxy-terminal di-lysine motif KKXX in their cytoplasmic tails (Jackson *et al.*, 1990). While the receptor that binds KDEL sequences on soluble molecular chaperones has been identified (Hardwick *et al.*, 1990; Lewis *et al.*, 1990; Semenza *et al.*, 1990), the receptor that retains integral membrane chaperone molecules remains elusive. Recent evidence indicates that the KKXX di-lysine motif interacts with the coatomer, a polypeptide complex implicated in vesicular trafficking between Golgi cisternae and in retrieval/retention of ER proteins (Cosson and Letourneur, 1994; Letourneur *et al.*, 1994).

The present study was undertaken initially to identify the unknown 90 kDa protein referred to as CD3AP that is associated with CD3 components on the surface of immature thymocytes. Remarkably, we found that CD3AP is calnexin, an integral membrane protein resident within the ER that functions as a molecular chaperone for such immunologically relevant structures as class I and class II major histocompatibility complex-encoded molecules (Anderson and Cresswell, 1994; Jackson *et al.*, 1994;

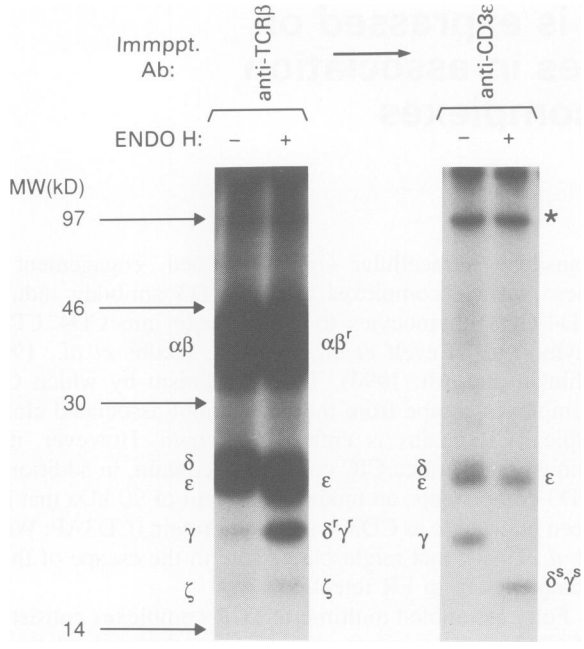

Fig. 1. Immature thymocytes express surface CD3 complexes that are associated with an unknown M_r 90 kDa protein. Immature $CD4^+CD8^+$ thymocytes were ^{125}I-surface-labeled and lysed in 1% digitonin. Clonotypic TCR complexes and clonotype-independent CD3 (CIC) complexes were isolated from the extracts by sequential immunoprecipitation with anti-TCRβ (H57–597) and anti-CD3ε (145–2C11) mAb, respectively. Immunoprecipitated complexes were either mock digested (–) or digested with Endo H (+) and then analyzed by SDS–PAGE under reducing conditions followed by autoradiography. The positions of TCRαβ, CD3γ,δ,ε and ζ chains are indicated. Chains that were resistant to digestion with Endo H ($\alpha\beta^r$ and $\delta^r\gamma^r$) and chains that were sensitive to digestion with Endo H ($\delta^s\gamma^s$) are indicated. *, CD3-associated protein (CD3AP).

Schreiber *et al.*, 1994) and the antigen receptors of both B and T lymphocytes (Hochstenbach *et al.*, 1992; David *et al.*, 1993). Thus, the molecular chaperone calnexin that normally retains incompletely assembled CD3 components within the ER is expressed with incompletely assembled CD3 components on the surface of immature thymocytes. We have also characterized the molecular interactions involved in the escape of calnexin–CD3 complexes from the ER, and demonstrate that ER escape involves protein–protein interactions that are distinct from the carbohydrate-mediated luminal domain interactions involved in formation of calnexin–CD3 complexes that are retained within the ER. Thus, the present study provides the first demonstration that a membrane-bound ER molecular chaperone can escape from the ER and be expressed on the cell surface.

Results

Immature thymocytes express on their surface both clonotypic αβTCR complexes and clonotype-independent CD3 (CIC) complexes, each of which contains heterodimers of CD3γε and CD3δε chains (Figure 1). However, the CD3γ and CD3δ glycoproteins in surface CIC complexes are biochemically distinct from those in surface clonotypic TCRαβ complexes in that their *N*-linked sugars are sensitive to digestion with endoglycosidase H (Endo H) (Figure 1), indicating a lack of carbohydrate processing during transit through the Golgi apparatus (Kornfeld and Kornfeld, 1985). In addition, the CD3γ and CD3δ glycoproteins in surface CIC complexes are associated with an unknown protein, referred to as CD3AP (Wiest *et al.*, 1994), of M_r 90–100 kDa which bears no *N*-linked glycans and so is resistant to digestion by Endo H (Figure 1,*) and peptide-*N*-glycosidase F (data not shown).

Table I. Microsequencing of CD3AP purified from BW5147 thymoma cells

Peptide 1	–A–P–V–P–T–G–E–V–Y–F–A–D–S–F–D
Calnexin(42)[a]	–A–P–V–P–T–G–E–V–Y–F–A–D– S–F–D
Peptide 2	–T–H–L–Y–T–L–I–L–N–P–D–N–S–F–E–I
Calnexin(215)	–T–H–L–Y–T–L–I–L–N–P–D–N–S–F–E–I
Peptide 3	–A–A–D–G–A–A
Calnexin(440)	–A–A–D–G–A–A

[a]Amino acid residue in calnexin from which the sequence begins.

CD3AP is a surface form of the molecular chaperone calnexin

To identify CD3AP, we affinity purified CIC complexes, excised and proteolyzed the 90 kDa band and micro-sequenced the proteolytic fragments. All the sequences we obtained were identical to those of the ER-resident molecular chaperone calnexin (David *et al.*, 1993; Schreiber *et al.*, 1994) (Table I). Because CD3 subunits are known to associate with calnexin in the ER (David *et al.*, 1993), we thought it possible that the peptides that we had sequenced were derived from CD3-associated calnexin molecules resident in the ER rather than from surface CD3AP. To determine if surface CD3AP molecules were distinct from or identical with calnexin molecules, we compared the migration of surface-labeled CD3AP molecules in two-dimensional non-equilibrium pH gradient electrophoresis (NEPHGE) gels with that of calnexin molecules identified by sequential blotting of the identical membrane with anti-calnexin antibodies. In Figure 2A (left panel), surface-biotinylated CIC complexes were precipitated by anti-CD3ε mAb, resolved by NEPHGE and visualized by chemiluminescence using enzyme-linked avidin. As expected, biotin surface-labeled CD3γ,δ,ε chains as well as CD3AP were visualized (Figure 2A, left). Surprisingly, reprobing the identical membrane with anti-calnexin antibodies revealed that calnexin molecules comigrated identically with surface CD3AP with respect to both charge and size (Figure 2A, arrows). To further examine the possibility that CD3AP might in fact be calnexin, we assessed the ability of anti-calnexin antibodies to bind surface-labeled CD3AP molecules directly (Figure 2B). Biotin surface-labeled CD3AP molecules that had been precipitated by anti-CD3ε mAb were eluted, resolubilized and exposed to other antibodies with various binding specificities. As can be seen in Figure 2B, biotin surface-labeled CD3AP molecules that had been released from anti-CD3ε precipitates were only recaptured by anti-calnexin antibodies (Figure 2B), demonstrating that CD3AP and calnexin had identical antigenic epitopes. Based on our observations that: (i) CD3AP co-migrated precisely with calnexin in two-dimensional NEPHGE gels; (ii) CD3AP was precipitated by anti-calnexin antibodies; and (iii) partial CD3AP sequences were identical to those

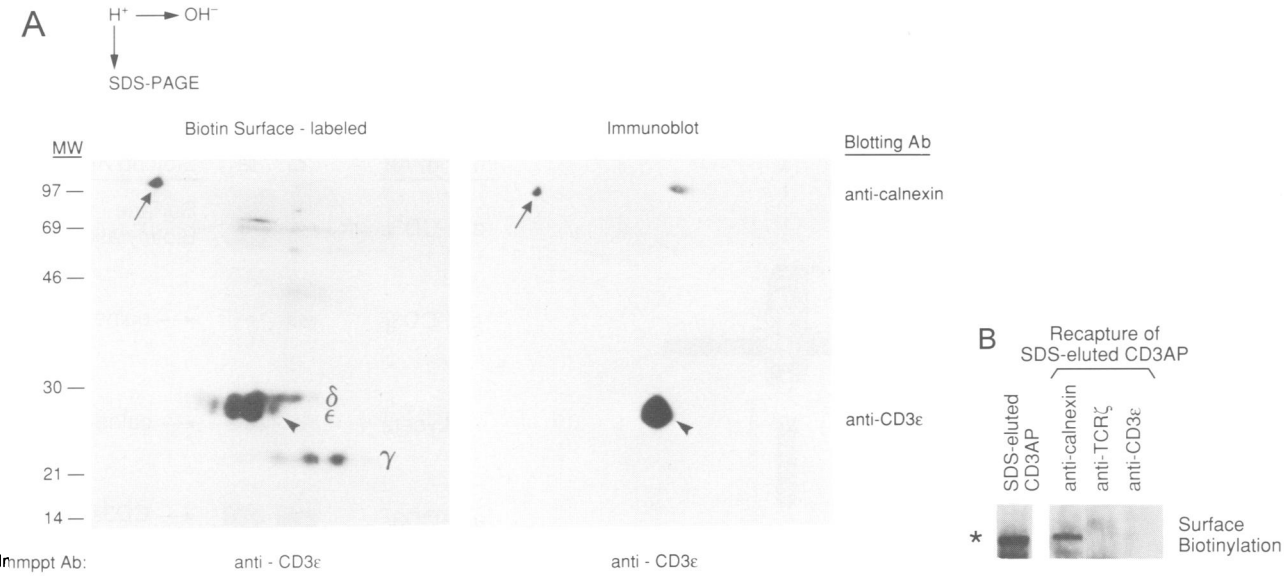

Fig. 2. CD3AP is indistinguishable from the molecular chaperone calnexin. (**A**) Surface-labeled CD3AP co-migrates with calnexin in two-dimensional NEPHGE/SDS–PAGE gels. Immature $CD4^+CD8^+$ thymocytes were surface-labeled with biotin, lysed in digitonin and precleared with anti-TCRβ mAb (H57–597). CIC complexes were then immunoprecipitated with anti-CD3ε mAb (145–2C11), resolved by two-dimensional NEPHGE/SDS–PAGE under reducing conditions and transferred onto an Immobilon PVDF membrane. Surface biotin-labeled proteins were visualized with horseradish peroxidase-conjugated streptavidin (HRP-Av) and chemiluminescence (left panel). Positions of CD3δ,ε,γ and CD3AP (arrow) are indicated. After washing, the membrane was reprobed with anti-calnexin and anti-CD3ε (HMT3.1) antibodies (right panel) followed by [^{125}I]protein A to identify the positions of calnexin (arrow) and CD3ε (arrowhead), respectively. The unlabeled spot is nonspecific. (**B**) Surface-biotinylated CD3AP is specifically recaptured by anti-calnexin antibodies. Biotin-labeled WT BW5147 cells were lysed in digitonin, precleared with anti-TCRβ mAb and immunoprecipitated with anti-CD3ε. Surface-labeled CD3AP was released from the CD3 components by boiling in SDS. After neutralization with NP-40, the SDS eluates were immunoprecipitated with antibodies to calnexin, TCRζ (551) or CD3ε. *, CD3AP.

of calnexin, we conclude that CD3AP is indistinguishable from the molecular chaperone calnexin.

Because calnexin is primarily an ER-resident protein (Hochstenbach *et al.*, 1992; Rajagopalan *et al.*, 1994), we sought to further document that CD3AP is a form of calnexin that is expressed on the surface of immature thymocytes. Since calnexin molecules can be digested by pronase (data not shown), we used pronase to digest the surface of intact cells whose membrane proteins had been biotinylated. We found that extracellular pronase treatment completely digested surface-labeled calnexin molecules on immature thymocytes and BW5147 thymoma cells without affecting the size of their intracellular calnexin pools (Figure 3). These results demonstrate that, unlike internal calnexin molecules, surface biotinylated calnexin molecules are uniquely susceptible to extracellular proteolysis. In addition, these data demonstrate that surface calnexin molecules comprise only a small fraction of the total cellular pool of calnexin.

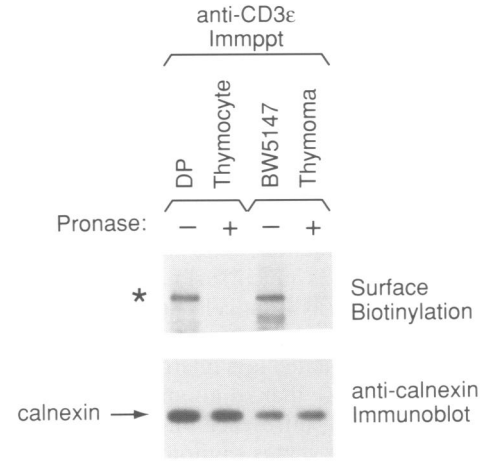

Fig. 3. Surface calnexin is sensitive to digestion of intact cells with pronase. Surface-labeled $CD4^+CD8^+$ (DP) thymocytes and WT thymoma cells were either mock treated for 15 min at 37°C in PBS (–) or proteolyzed in 2 mg/ml pronase (+). After lysis in digitonin and preclearing with anti-TCRβ mAb, CIC complexes were isolated using anti-CD3ε mAb (145–2C11) and resolved by SDS–PAGE under reducing conditions. After transferring the proteins onto an Immobilon membrane, surface biotinylated proteins were visualized using HRP-Av and chemiluminescence. The effect of pronase treatment on the total cellular pool of CD3-associated calnexin was determined by washing the membrane and then blotting with anti-calnexin antibodies followed by [^{125}I]protein A. *, surface-labeled calnexin.

Surface calnexin–CD3 complexes are biochemically distinguishable from those retained intracellularly

Calnexin is an integral membrane chaperone with both luminal and cytoplasmic domains that subserve different molecular functions. The luminal domain of calnexin binds to *N*-linked glycans that have been processed to the monoglucose form (Hammond *et al.*, 1994; Kearse *et al.*, 1994b; Ware *et al.*, 1995), whereas the cytoplasmic domain of calnexin contains a retention motif that is bound by a putative ER retention receptor (Rajagopalan *et al.*, 1994). We therefore reasoned that escape of calnexin–CD3 complexes from the ER was most likely to be a consequence of cytoplasmic domain interactions between calnexin and CD3 that blocked recognition of calnexin's retention motif by an ER retention receptor (Figure 4).

To determine if surface and internal calnexin–CD3

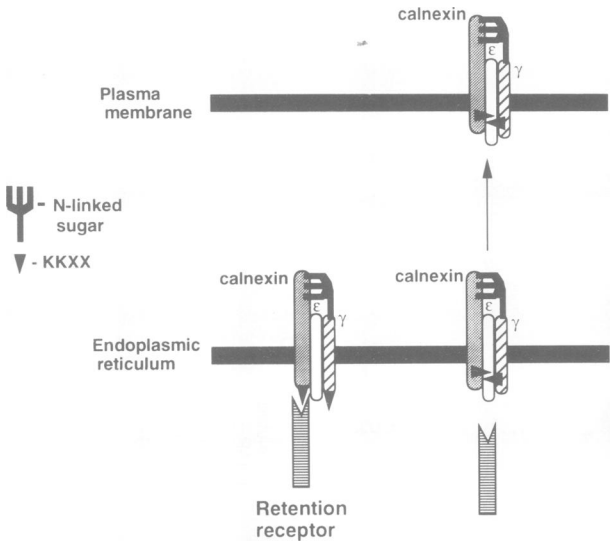

Fig. 4. Model for escape of calnexin–CD3 complexes from the ER. Incompletely assembled CD3 complexes are retained in the ER at least in part by their association with the molecular chaperone calnexin. Calnexin most efficiently binds to CD3 subunits through its luminal domain, which recognizes monoglucosylated *N*-linked glycans (Hammond *et al.*, 1994; Ware *et al.*, 1995). While the luminal domain promotes carbohydrate-mediated interactions, the cytoplasmic tail of calnexin contains an ER retention signal (black triangle), which mediates retention within the ER of both calnexin and associated CD3 subunits. In this model, a number of calnexin–CD3 complexes successfully escape from the ER as a consequence of cytoplasmic domain interactions that mask calnexin's ER retention signal. It might be noted that, whereas the consensus ER retention motif is KKXX, the cytoplasmic tails of calnexin and CD3 contain functional variants of this consensus motif (Letourneur *et al.*, 1992; David *et al.*, 1993).

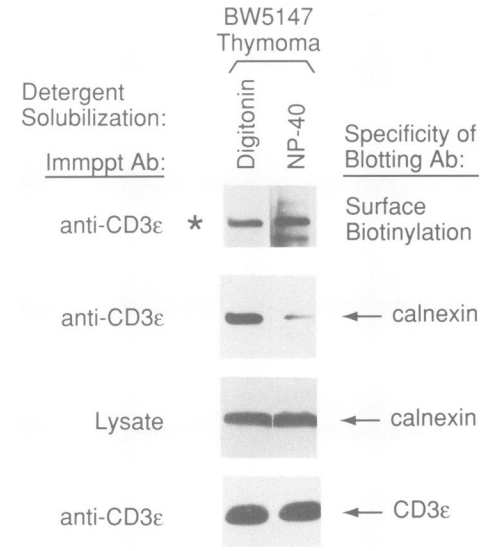

Fig. 5. Calnexin–CD3 complexes expressed on the cell suface are more stable than those retained intracellularly. Biotin-labeled WT BW5147 thymoma cells were lysed in either digitonin or NP-40 and the extracts depleted of TCRβ. CIC complexes isolated with anti-CD3ε mAb (145–2C11), as well as total cell lysates, were resolved by SDS–PAGE under reducing conditions and transferred onto an Immobilon membrane. Surface biotinylated proteins were visualized by HRP-Av and chemiluminescence (top panel). Total cellular pools of calnexin and CD3ε were assessed by washing the membrane and then reblotting with anti-calnexin or anti-CD3ε (HMT3.1) antibodies, respectively, followed by [^{125}I] protein A. *, surface-labeled calnexin.

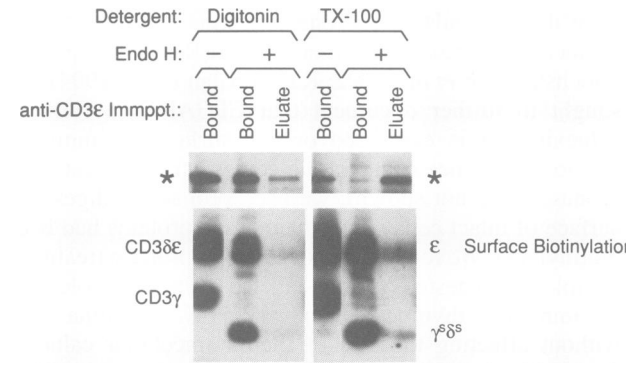

Fig. 6. Surface calnexin–CD3 complexes contain protein–protein interactions that are stable in digitonin but not TX-100. WT thymoma cells were surface biotinylated, lysed in the indicated detergent and precleared with anti-TCRβ mAb. CIC complexes were immunoprecipitated using anti-CD3ε mAb (145–2C11) adsorbed to Sepharose beads and either mock digested (–) or digested with Endo H (+) under nondenaturing conditions. Following digestions, the eluate and bound material were analyzed separately by SDS–PAGE under reducing conditions. Biotinylated proteins were visualized using HRP-Av and chemiluminescence. Positions of CD3 subunits before and after deglycosylation are indicated. γ^s, deglycosylated CD3γ; δ^s, deglycosylated CD3δ; *, surface-labeled calnexin.

complexes contained different intermolecular interactions, we first assessed whether surface and internal calnexin–CD3 complexes were biochemically distinguishable from one another. We determined the relative stabilities of internal and surface calnexin–CD3 complexes upon solubilization in detergents with different potentials to disrupt protein–protein interactions. We found that anti-CD3ε immunoprecipitated as much surface calnexin from cells solubilized in NP-40 as from cells solubilized in digitonin (Figure 5, top row). In contrast, we found that anti-CD3ε immunoprecipitated less internal calnexin from cells solubilized in NP-40 than digitonin (Figure 5, second row). The observed difference in stability of internal complexes in NP-40 versus digitonin did not reflect less efficient protein extraction by NP-40, as both detergents extracted similar amounts of internal calnexin and CD3ε proteins (Figure 5, bottom rows). Thus, NP-40 solubilization dissociated internal calnexin–CD3 complexes but did not disrupt surface calnexin–CD3 complexes, indicating that surface calnexin–CD3 complexes were biochemically distinct from, and more stable than, those retained intracellularly.

The increased stability of surface calnexin–CD3 complexes implied that they contained interchain interactions in addition to carbohydrate-mediated luminal domain interactions. To reveal such interactions, we treated anti-CD3 immunoprecipitates with Endo H under nondenaturing conditions to remove the *N*-linked glycans on the CD3 subunits, as calnexin–CD3 complexes would remain intact after Endo H treatment only if they contained interactions that were not carbohydrate mediated (Ware *et al.*, 1995). In fact, for surface complexes solubilized in digitonin, we found that Endo H treatment did not release calnexin into the eluate fraction (Figure 6, left lanes). In contrast, for surface complexes solubilized in the more disruptive detergent TX-100, Endo H treatment did release

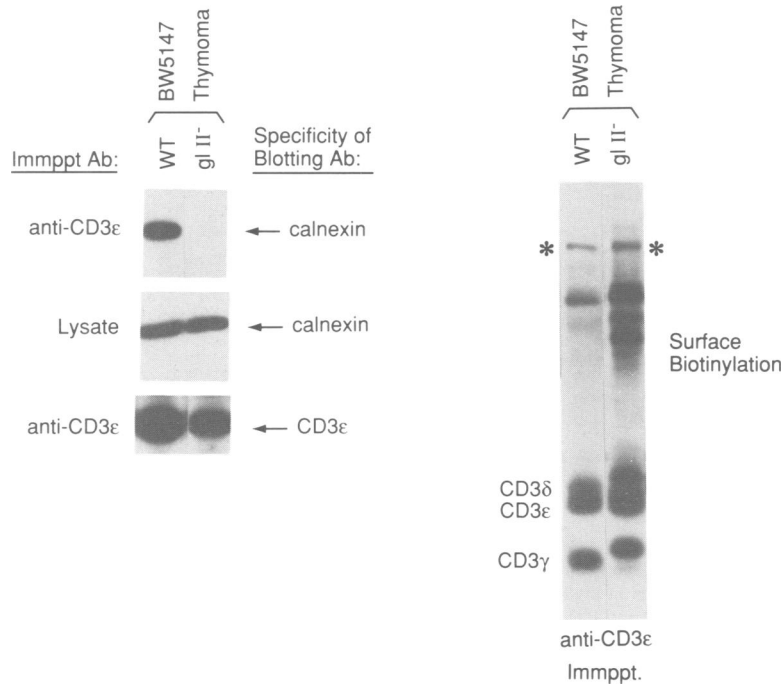

Fig. 7. Carbohydrate-mediated luminal domain interactions are not required for calnexin–CD3 complexes escaping to the cell surface. (Left panel) Equal numbers of WT and glII⁻ BW5147 thymoma cells were lysed in digitonin and the extracts precleared with anti-TCRβ mAb. Anti-CD3ε (145–2C11) immunoprecipitates of the detergent extracts and total cell lysates were resolved by SDS–PAGE and blotted with anti-calnexin and anti-CD3ε (HMT3.1) antibodies as indicated. (Right panel) Equal numbers of WT and glII⁻ BW thymoma cells were surface labeled with biotin, lysed in digitonin and precleared with anti-TCRβ mAb. CIC complexes isolated using anti-CD3ε mAb (145–2C11) were resolved by SDS–PAGE under reducing conditions and blotted onto an Immobilon membrane. Biotin-labeled proteins were visualized using HRP-Av and chemiluminescence. Positions of the CD3 components are indicated. *, surface-labeled calnexin.

calnexin into the eluate fraction (Figure 6, right lanes). These results demonstrate that, in addition to carbohydrate-mediated luminal domain interactions, surface calnexin–CD3 complexes contain additional protein–protein interactions that are stable in digitonin but are disrupted in TX-100.

We next considered that, even though carbohydrate-mediated luminal domain interactions would be expected to promote formation of internal calnexin–CD3 complexes, they might not be necessary for formation of calnexin–CD3 complexes escaping to the cell surface. To determine if internal and surface calnexin–CD3 complexes were affected differently by the absence of carbohydrate-mediated interactions, we analyzed expression of calnexin–CD3 complexes in wild-type (WT) BW5147 thymoma cells and in mutant BW5147 thymoma (glII⁻) cells that lack glucosidase II activity (Reitman *et al.*, 1982) and so cannot trim *N*-linked glycans to the monoglucose form recognized by calnexin's luminal domain (Hammond *et al.*, 1994; Kearse *et al.*, 1994b) (Figure 7). We found that glII⁻ cells contained calnexin and CD3ε in amounts equivalent to WT cells but contained few calnexin–CD3 complexes, as anti-CD3ε immunoprecipitates from glII⁻ cells contained only trace amounts of calnexin (Figure 7, left). In contrast, WT and glII⁻ cells expressed similar amounts of surface calnexin–CD3 complexes (Figure 7, right), demonstrating that formation of calnexin–CD3 complexes escaping ER retention does not depend upon trimming of *N*-linked glycans to the monoglucose form. Taken together, these results indicate that carbohydrate-dependent luminal domain interactions are necessary for formation of intracellularly retained calnexin–CD3 complexes, but are not necessary for assembly of calnexin–CD3 complexes escaping to the cell surface.

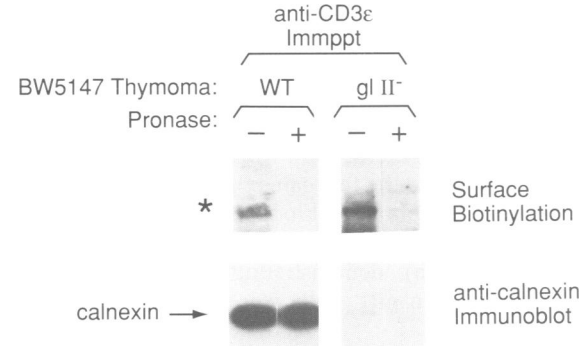

Fig. 8. Most calnexin–CD3 complexes in glII⁻ cells are expressed on the cell surface. Surface-labeled WT and glII⁻ cells were either mock treated (–) or treated with pronase (+), then lysed and immunoprecipitated as in Figure 3. Immunoprecipitates were resolved by SDS–PAGE, transferred to an Immobilon membrane and biotin-labeled proteins visualized using HRP-Av and chemiluminescence (top panels). The effect of extracellular proteolysis on the total cellular pool of CD3-associated calnexin was determined by sequentially blotting the same membrane with anti-calnexin antibodies (bottom panels). *, surface-labeled calnexin.

In the absence of carbohydrate-mediated luminal domain interactions, calnexin–CD3 complexes were most likely to form in glII⁻ cells as a result of cytoplasmic

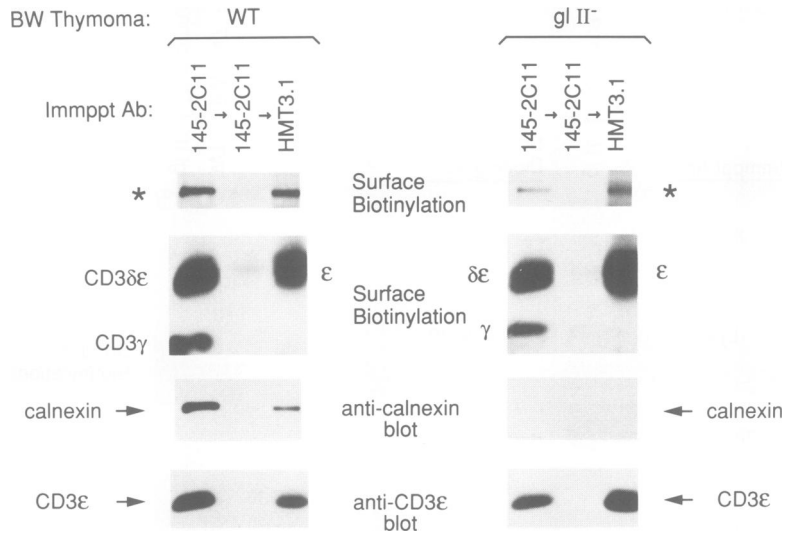

Fig. 9. CIC complexes consisting only of CD3ε and calnexin molecules are expressed on the surfaces of both WT and glII⁻ cells. WT and glII⁻ thymoma cells were biotin surface-labeled, lysed in digitonin and precleared with anti-TCRβ mAb. CIC complexes containing CD3γε and δε heterodimers were isolated using 145–2C11, an anti-CD3ε mAb that recognizes CD3ε only when assembled with CD3γ or δ. Subsequent immunoprecipitation of these extracts with HMT3.1, an anti-CD3ε mAb that recognizes CD3ε chains independently of other CD3 components, was then used to isolate CIC containing only CD3ε. Immune complexes were resolved by SDS–PAGE under reducing conditions and transferred to an Immobilon membrane. Biotin-labeled proteins were visualized using HRP-Av and chemiluminescence; total intracellular pools of CD3 and calnexin were determined by sequentially blotting the same membrane with anti-calnexin and anti-CD3ε (HMT3.1) antibodies. Positions of surface-biotinylated components are indicated. Calnexin and CD3ε molecules detected by immunoblotting are indicated by arrows. *, surface-labeled calnexin.

domain interactions that, with some frequency, would result in ER escape. Consequently, even though glII⁻ cells contained relatively few calnexin–CD3 complexes, most of the calnexin–CD3 complexes that they did contain might be expressed on the cell surface. To address this possibility, surface biotinylated calnexin–CD3 complexes were digested with extracellular pronase and the effect on total cellular calnexin–CD3 complexes was assessed by immunoblotting. Extracellular pronase treatment digested surface biotinylated calnexin molecules from both WT and glII⁻ cells (Figure 8, top). Most importantly, pronase treatment destroyed the few calnexin–CD3 complexes that were detectable by immunoblotting in glII⁻ cells, whereas it had no detectable effect on such complexes in WT cells (Figure 8, bottom), demonstrating that most calnexin–CD3 complexes in glII⁻ cells were in fact expressed on the cell surface.

Interaction of calnexin with isolated CD3ε chains

Finally, we reasoned that release of calnexin from the ER of immature thymocytes was most likely to involve cytoplasmic domain interactions between calnexin and CD3ε chains because: (i) CD3ε is not a glycoprotein and so has no *N*-linked glycans to interact with calnexin's luminal domain, and (ii) CD3ε chains do not contain a cytoplasmic retention signal that might prevent ER release even when calnexin's retention sequence is masked (Letourneur and Klausner, 1992). Thus, calnexin–CD3ε complexes lacking associated CD3γ,δ chains should be competent to escape to the cell surface. To test this prediction, we utilized two different anti-CD3ε mAbs: 145–2C11, which binds CD3ε chains only if they are assembled with CD3γ or δ chains (Bonifacino *et al.*, 1989); and HMT3.1, which binds isolated CD3ε chains (Born *et al.*, 1987). Lysates from surface biotinylated WT and glII⁻ thymoma cells were first immunodepleted with 145–2C11 (to precipitate all CD3γε- and CD3δε-containing complexes), and then with HMT3.1 (to precipitate all remaining CD3ε proteins) (Figure 9). Consistent with the specificities of the precipitating mAbs, 145–2C11 precipitates contained surface biotinylated CD3γ,δ and ε chains, whereas HMT3.1 precipitates contained surface biotinylated CD3ε, but not CD3γ and δ, chains (Figure 9). Most importantly, both 145–2C11 and HMT3.1 precipitates contained surface biotinylated calnexin molecules (Figure 9), documenting that WT and glII⁻ cells did express surface complexes consisting only of calnexin and CD3ε chains in addition to surface calnexin–CD3γε,δε complexes. Furthermore, this experiment demonstrates that complexes consisting only of calnexin and CD3ε chains were expressed disproportionately on the surface of both WT and glII⁻ cells relative to their internal pools. That is, glII⁻ cells expressed more surface calnexin–CD3ε complexes than surface calnexin–CD3γε,δε complexes; and WT cells, which contained fewer internal calnexin–CD3ε complexes than calnexin–CD3γε,δε complexes, expressed equivalent numbers of calnexin–CD3ε and calnexin–CD3γε,δε complexes on their cell surface (Figure 9). Thus, these results support the concept that calnexin–CD3ε interactions promote, with some frequency, calnexin's escape from the ER of immature thymocytes.

Discussion

The present study reports the unexpected observation that calnexin is expressed on the surface of immature thymocytes in association with CD3 components. We found that calnexin–CD3 complexes expressed on the cell surface differ from calnexin–CD3 complexes retained within the ER in that surface complexes are susceptible

to extracellular proteolysis, exhibit differential detergent stability and can be formed without carbohydrate-mediated interactions involving calnexin's luminal domain. Escape of calnexin–CD3 complexes to the cell surface appears to be a consequence of cytoplasmic domain interactions between calnexin and CD3ε chains that interfere with recognition of calnexin by ER retention receptors. These findings are especially curious because calnexin is thought to be the molecular chaperone that ordinarily retains incompletely assembled CD3 components within the ER. We think it is precisely because calnexin binds to unassembled CD3 components in the ER that masking of calnexin's ER retention signal results in escape of some calnexin–CD3 complexes from the ER to the cell surface.

Immature thymocytes have been shown recently to express on their surface CIC complexes consisting of CD3 components that are associated with an unknown 90 kDa membrane protein referred to as CD3AP (Wiest *et al.*, 1994). The present study identifies CD3AP as being indistinguishable from calnexin, the ER-resident molecular chaperone that associates with partially assembled TCR components within the ER (David *et al.*, 1993). Indeed, calnexin, CD3γ and CD3δ proteins contain ER retention motifs in their cytoplasmic tails that are thought to be bound by ER retention receptors, preventing their escape from the ER (Letourneur and Klausner, 1992; Rajagopalan *et al.*, 1994). Consequently, calnexin–CD3 complexes can only escape from the ER to the cell surface when their cytoplasmic tails are not bound by ER retention receptors. It is possible that modifications such as phosphorylation of calnexin's cytoplasmic tail might occur in immature thymocytes and interfere with recognition of calnexin's retention sequence by ER retention receptors. It is also possible that immature thymocytes have insufficient numbers of ER retention receptors to retain all the calnexin–CD3 complexes that are present within their ER. However, the present study provides evidence that in immature thymocytes polypeptide interactions between calnexin and CD3 chains can induce ER escape, presumably by masking calnexin's ER retention signals. Specifically, the present study demonstrates that surface calnexin–CD3 complexes rely on different interchain interactions from those present in internal calnexin–CD3 complexes, which are predominantly dependent upon carbohydrate-mediated luminal domain interactions. Thus, we found that: (i) carbohydrate-mediated luminal domain interactions were unnecessary for formation of calnexin–CD3 complexes that escaped to the cell surface; (ii) surface calnexin–CD3 complexes were more stable than internal calnexin–CD3 complexes, indicating that surface complexes contained protein–protein interactions in addition to those present in internal complexes; and (iii) complexes consisting only of calnexin and CD3ε chains existed even though CD3ε contains no *N*-linked glycans to mediate interactions with calnexin's luminal domain.

Why were surface calnexin–CD3 complexes detected on immature thymocytes and not mature T cells? In contrast to mature T cells, immature thymocytes cannot assemble CD3 components efficiently into complete TCRαβ complexes (Kearse *et al.*, 1994a). Consequently, immature thymocytes contain large numbers of unassembled CD3 components within their ER, including unassembled CD3ε proteins (Wiest *et al.*, 1993). Thus, we think that greater numbers of calnexin–CD3 complexes form in the ER of immature thymocytes than in the ER of mature T cells, leading to greater surface expression of CIC complexes on immature thymocytes. Indeed, an inverse relationship between expression of surface CIC complexes and expression of surface TCRαβ complexes has previously been noted (Wiest *et al.*, 1994).

While the present study is the first to demonstrate surface expression of an integral membrane ER protein, the concept that cytoplasmic tail interactions can mask ER retention signals and permit escape from the ER to the cell surface is not novel. Indeed, it is precisely the masking of ER retention signals by assembly of individual TCR components into a multisubunit receptor complex that is thought to permit assembled TCR complexes to exit the ER (Letourneur and Klausner, 1992). Consequently, it seems quite reasonable to consider that ER retention signals on molecular chaperones might also be masked by cytoplasmic domain interactions with other proteins in the ER. We think that it is the interaction of calnexin with CD3ε proteins in particular that masks calnexin's retention signals because: (i) CD3ε chains do not themselves possess an ER retention signal within their cytoplasmic tails (Letourneur and Klausner, 1992); and, most importantly, (ii) we found that complexes consisting only of calnexin and CD3ε chains were over-represented on the cell surface relative to their internal pools. Nevertheless, we think that CD3ε chains are relatively inefficient at masking calnexin's retention sequence. Indeed, previous experiments localizing calnexin–CD3ε complexes by immunofluorescence in transfected fibroblasts failed to detect surface expression except upon removal of calnexin's ER retention sequence (Rajagopalan *et al.*, 1994).

Despite its relative inefficiency, escape of calnexin–CD3 complexes from the ER of immature thymocytes may have important biological consequences for T cell development in that it results in expression of surface complexes that are potentially able to signal the further differentiation of immature $CD4^-CD8^-$ thymocytes into $CD4^+CD8^+$ cells (Levelt *et al.*, 1993a,b; Jacobs *et al.*, 1994; Shinkai and Alt, 1994). Since the cytoplasmic domains of all CD3 subunits contain tyrosine-based activation motifs (Chan *et al.*, 1994), any intracellular signals transduced by CIC complexes can be ascribed to the CD3 components associated with calnexin and need not be attributed to calnexin itself, although we have not ruled out the possibility that surface calnexin molecules may transduce signals as well. It is also possible that calnexin molecules might subserve a non-signaling function on the surface of immature thymocytes, such as ligand binding. Calnexin has been described as a Ca^{2+}-dependent animal lectin (Bergeron *et al.*, 1994) displaying a tropism for glycoproteins bearing monoglucosylated carbohydrates (Hammond *et al.*, 1994; Hammond and Helenius, 1994b). Since potential calnexin substrates are expressed on the surface of immature thymocytes (Kishi *et al.*, 1991; Figure 1), calnexin might function to promote cell–cell contact between thymocytes or between immature thymocytes and the thymic stroma. Indeed development of thymocytes and the thymic stroma has been shown to be a co-dependent process (van Ewijk *et al.*, 1994) involving cell–cell interactions that could conceivably be promoted by calnexin.

Interestingly, a surface complex is present on immature B cells that may be analogous to surface calnexin–CD3 complexes on immature thymocytes and may function similarly to transduce differentiation signals (Karasuyama *et al.*, 1994).

In addition to expressing CIC complexes, immature thymocytes can also express pre-Tα complexes on their cell surfaces, consisting of CD3 components in association with a disulfide-linked heterodimer of TCRβ and pre-Tα, a recently described invariant 33 kDa glycoprotein important in early thymocyte development (Groettrup *et al.*, 1993; Saint-Ruf *et al.*, 1994). Since pre-Tα complexes contain clonotypic TCRβ chains, they are physically distinct from CIC complexes and appear later in ontogeny. However, CIC complexes may represent an early precursor of pre-Tα complexes whose escape from ER retention may require similar mechanisms to those described here for CIC complexes.

In conclusion, we have demonstrated that the molecular chaperone calnexin is expressed on the surface of immature thymocytes and on the surface of immortalized lines of immature thymocytes. While we have found surface calnexin to be associated with CD3 components, surface calnexin may also be expressed in association with other molecules as well. Thus, the present study provides a novel molecular basis for surface expression of partial receptor complexes on immature thymocytes that may transduce intracellular signals important for thymocyte development.

Materials and methods

Cells and reagents

Immature $CD4^+CD8^+$ thymocytes were isolated from adult C57BL/6 mice by panning on plates coated with anti-CD8 (Nakayama *et al.*, 1989). BW5147 cells (Hyman and Stallings, 1974) and the $glII^-$ variant, PHAR2.7 (Reitman *et al.*, 1982), were maintained in RPMI-1640 supplemented with 10% fetal calf serum (FCS). The following mAbs were used: (i) anti-TCRβ (H57–597) (Kubo *et al.*, 1989); (ii) anti-CD3ε (145–2C11) (Leo *et al.*, 1987; and (iii) anti-CD3ε (HMT3.1) (Born *et al.*, 1987). Polyclonal rabbit antibodies specific for either the C-terminal tail or a fusion protein containing the luminal domain of mouse calnexin (5′ 1100 bp fragment) were generated as described (Schreiber *et al.*, 1994). Rabbit polyclonal antibodies reactive with the C-terminus of TCRζ (551) were generated by immunization with C-terminal peptide coupled to chicken serum albumin as described (Cenciarelli *et al.*, 1992).

Surface labeling and pronase digestion

Lactoperoxidase-catalyzed surface labeling with ^{125}I was performed as described (Samelson *et al.*, 1985). Labeling of the cell surface with sulfo-NHS-biotin (Pierce Chemical, Rockford, IL) was performed as follows. Cells were washed three times in HBSS, resuspended at either 200×10^6/ml for $CD4^+CD8^+$ thymocytes or 20×10^6/ml for BW5147 thymoma cells, and labeled with 1 mg/ml sulfo-NHS-biotin in HBSS for 30 min on ice. Labeling was terminated with 25 mM lysine/HBSS and the cells were washed a total of three times in the same buffer. Viabilities were determined and were consistently >95%. Cells to be proteolytically digested were incubated for 15 min at 37°C in 2 mg/ml pronase in phosphate-buffered saline (PBS) at 2×10^6/ml and 20×10^6/ml for BW5147 thymoma and $CD4^+CD8^+$ thymocytes, respectively. Proteolysis was quenched with HBSS containing 5% FCS and 100 μg/ml DNase then, after three more washes in HBSS containing 5% FCS, any remaining pronase was inactivated by treating the cell suspension with 0.5 mM PMSF/0.1 mM TLCK for 10 min on ice.

Immunoprecipitation, microsequencing and Endo H digestion

Cells were lysed in buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1.8 mg/ml iodoacetamide, aprotinin 40 μg/ml and leupeptin 20 μg/ml) containing either 1% digitonin (Wako, Kyoto, Japan), 1% NP-40 (Calbiochem, La Jolla, CA) or 1% Triton X-100 (RPI, Mount Prospect, IL) for 20 min on ice. Extracts were clarified by microcentrifugation for 10 min at 4°C and then immunoprecipitated for 2 h with antibodies preadsorbed to protein A–Sepharose (Pharmacia, Uppsala, Sweden) (Wiest *et al.*, 1993). Preparative immunopreciptiation of CIC complexes for microsequencing was performed as follows. Briefly, 5×10^9 BW5147 thymoma cells were lysed in digitonin, and the clarified extracts depleted of CIC complexes using anti-CD3ε mAb (145–2C11) covalently coupled to Sepharose at 10 mg/ml of resin. Immune complexes were eluted with 0.2 M glycine pH 2.5, concentrated by TCA precipitation and resolved by SDS–PAGE under reducing conditions. Resolved proteins were transferred to nitrocellulose (Millipore, Bedford, MA) and visualized by staining with 0.2% (w/v) Ponceau S in 1% (v/v) acetic acid, following which the 100 kDa protein band was excised. The excised band was proteolysed *in situ* using lysyl endopeptidase C (Boehringer Manheim, Indianapolis, IN) as described (Egerton *et al.*, 1992). Peptides isolated by reversed phase high performance liquid chromatography using an Applied Biosystems model 130 (Applied Biosystems, Foster City CA) were subjected to automated Edman degradation using an Applied Biosystems model 473A protein sequenator. For elution/recapture assays, immune complexes were disrupted by boiling in 1% SDS. SDS eluates were then quenched with 10 volumes of 1% NP-40 lysis buffer and immunoprecipitated as above. Endo H digestion of immune complexes was performed under both denaturing and non-denaturing conditions. Endo H digestions under denaturing conditions were performed as follows: immune complexes adsorbed to protein A–Sepharose were boiled in 10 μl 1% SDS, quenched with 10 volumes of Endo H buffer (75 mM sodium phosphate pH 6.1, 75 mM EDTA, 0.1% NP-40), and incubated for 12 h at 37°C in the presence or absence of 5000 units of Endo H (New England Biolabs, Beverly, MA). (Note: 10 NEB units are equal to 1 IUB milliunit.) Endo H digestions under non-denaturing conditions were performed as follows: immune complexes adsorbed to protein A–Sepharose were incubated for 12 h at 37°C in digestion buffer (0.1 M sodium phosphate pH 6.1 with 0.15 M NaCl) containing the indicated detergent at 0.1% and 5000 units Endo H (New England Biolabs).

Electrophoresis and immunoblotting

Both one- and two-dimensional NEPHGE/SDS–PAGE were performed as described (Wiest *et al.*, 1994). Gels were transferred to Immobilon PVDF membranes (Millipore Corp., Bedford, MA) in a TE-52 transphor apparatus (Hoeffer Scientific Instruments, San Francisco, CA) for 4 h in buffer containing 50 mM Tris, 384 mM glycine, 20% methanol and 0.1% SDS. For biotinylated samples, the membranes were blocked for 1 h at room temperature in PBS containing 5% milk protein and 0.4% Tween-20 (Fisher Scientific, Pittsburgh, PA), washed three times for 5 min each in PBS containing 0.4% Tween-20 (PBS-T) and probed for 1 h with horseradish peroxidase-conjugated streptavidin (Southern Biotechnology Associates, Inc., Birmingham, AL) at 80 ng/ml in PBS-T. After washing the membranes three times for a total of 30 min, they were developed for 1 min using the Renaissance system (Dupont/NEN, Boston, MA), and exposed to Reflection film (Dupont/NEN). Membranes to be re-probed with antibodies were washed in PBS-T, blocked in 5% milk protein/PBS and blotted with either rabbit anti-calnexin C-terminal antiserum at 1:500 or 25% supernatant from the HMT3.1 hybridoma (anti-CD3ε), both in 5% milk/PBS. Blots were then washed three times for 5 min each following which bound antibody was visualized by probing with [^{125}I]protein A at 1 μCi/ml and autoradiography (Wiest *et al.*, 1993).

Acknowledgements

We thank Drs Pierre Henkart, Juan Bonifacino and Yousuke Takahama for critically reading the manuscript, and Dr Oddmund Bakke for helpful discussions.

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Received on March 30, 1995; revised on May 3, 1995