Cell Surface Expression of Calnexin, a Molecular Chaperone in the Endoplasmic Reticulum*

The folding and assembly of nascent proteins in the endoplasmic reticulum are assisted by the molecular chaperone calnexin, which is itself retained within the endoplasmic reticulum. It was up to now assumed that calnexin was selectively expressed on the surface of immature thymocytes because of a particular characteristic of the protein sorting machinery in these cells. We now report that a small fraction of calnexin is normally expressed on the surface of various cells such as mastocytoma cells, murine splenocytes, fibroblast cells, and human HeLa cells. Surface biotinylation followed by chase culture of living cells revealed that calnexin is continuously delivered to the cell surface and then internalized for lysosomal degradation. These results suggest that there is continuous exocytosis and endocytosis of calnexin, and the amount of calnexin on the plasma membrane results from the balance of the rates of these two events. To study the structural requirement of calnexin for surface expression, we created deletion mutants of calnexin and found that the luminal domain, particularly the glycoprotein binding domain, is necessary. These findings suggest that the surface expression of calnexin depends on the association with glycoproteins and that calnexin may play a certain role as a chaperone on the plasma membrane as well.

A quality control mechanism in the endoplasmic reticulum (ER) ensures that properly folded polypeptides and completely assembled oligomeric complexes are transported to the Golgi and beyond. Calnexin, an ER-resident molecular chaperone, binds transiently to nascent proteins, assisting the folding and assembly while retaining proteins that persist in a mal-folded or incompletely assembled state. The large luminal domain of calnexin contains two sets of repeated structures (1) that recognize mono-glucosylated glycans (2–4). In the ER quality control machinery, calnexin acts as a constituent of the glucosylation/deglucosylation cycle. During this cycle, monoglucosylated glycoproteins bind transiently to calnexin.

ER chaperones are thought to be localized in the ER by their cognate receptor, which constantly retrieves escaped chaperones from a dynamic intermediate compartment between the ER and the Golgi complex. The C-terminal Lys-Asp-Glu-Leu (KDEL) tetrapeptide for luminal chaperones (5) and the C-terminal di-lysine (Lys-Lys-X) motif for membrane-bound chaperones (6) are generally known as the ER retention motif. Calnexin has previously been believed to localize in the ER, because this membrane-bound chaperone has C-terminal charged residues (RKPRRE) that are similar to the ER retention consensus motif and were proven to function as a retention signal (6, 7).

However, immature CD4-CD8 thymocytes were recently found to express calnexin on their surface in association with the CD3 complex (8, 9). In this regard, calnexin associated with Igα/Igβ heterodimer is also expressed on the surface of pro-B cells (10). This escape of calnexin to the cell surface was thought to be implicated in the defective ER retention of immature cells that allows many, but not all, resident ER proteins to reach the cell surface (8). Thus, the surface expression of calnexin/receptor complexes appears to be a common feature of both T and B precursor cells, even though its physiological meaning in early lymphocyte development remains to be determined.

We here report that a small fraction of calnexin is normally expressed on the cell surface regardless of cell type, lineage, or maturation stage of the cell. Calnexin on the cell surface is dynamically turned over by endocytosis. The glycoprotein binding domain is suggested to be prerequisite. Our results suggest that a small fraction of calnexin can escape from the ER and be transported to and expressed on the cell surface, probably by interaction with glycoproteins.

**EXPERIMENTAL PROCEDURES**

**Animals—**C57BL/6 mice were purchased from Japan CLEA Inc. (Tokyo, Japan)

**Cells and Antibodies—**The immature thymocyte cell line KKF was derived from Gross’s leukemia virus-infected BALB/k thymocytes and expresses the pre-T cell receptor complex as described previously (11, 12). P815 is a mastocytoma cell line expressing FcR on the cell surface (13). These cell lines were maintained in RPMI 1640 (Life Technologies, Inc.) supplemented with 5% fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml 2-mercaptoethanol. DAP-3, a mouse fibroblast L cell clone (14), and HeLa cells (ATCC CCL185) were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% FBS, 100 μg/ml l-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml 2-mercaptoethanol.

An anti-mouse CD3ε mAb (145–2C11) was kindly provided by Dr. J. Bluemone (University of Chicago, Chicago, IL). SPA-860, a polyclonal antibody to the C terminus of canine calnexin, and anti-α-adaptin mAb
and anti-FLAG M2 mAb were purchased from Stressgen Biotechnologies (Victoria, BC, Canada). Transduction Laboratories (Lexington, KY), and Sigma-Aldrich, respectively.

**Cell Surface Biotinylation, Immunoprecipitation, SDS-PAGE Analysis, and Western Blotting—**Cell surface biotinylation, immunoprecipitation, SDS-PAGE, and Western blotting were performed as described previously (12). Cells were solubilized in a lysis buffer (1% digitonin or 1% Nonidet P-40, 50 mM Tris-HCl (pH 7.6), 300 mM NaCl, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 10 mM iodoacetamide).

To determine the quantity of surface calnexin, biotin-labeled proteins were sequentially precipitated twice with streptavidin-agarose beads (Pierce). The supernatant of the beads was collected, and precipitated proteins were eluted with the SDS sample buffer from streptavidin pellets. Volumes of the samples were adjusted so that each sample was derived from equivalent amounts of starting material. In the blocking experiment of glycosylation, KKF cells were incubated with or without tunicamycin (0.5 μg/ml) (Wako Pure Chemical Industries, Osaka, Japan) for 10 h at 37 °C.

**Endocytosis and Exocytosis Experiments—**Endocytosis experiments were performed with chase culture after surface biotinylation. Biotinylated splenocytes were cultured in RPMI 1640 medium containing 10% FBS for 4 h at 0 °C or 37 °C. In some experiments, the cells were incubated in the presence or absence of the inhibitor of lysosomal degradation, methylamine (50 mM) or bafilomycin A1 (0.5 μM) (Sigma).

Before incubation with the 1% Nonidet P-40 lysis buffer, dead cells were removed by pelleting through a cushion of lympholyte M (Cedarlane Laboratories, Hornby, Ontario, Canada). Viabilities were consistently greater than 97%.

For endocytosis assay (8), CT26BL6 splenocytes were pretreated with 2 μg/ml brefeldin A (BFA) (Wako Pure Chemical Industries) for 1 h at 37 °C and then washed twice in Hank's balanced salts solution (HBSS). The cells were resuspended either in HBSS only or in HBSS containing 2 mg/ml Pronase (Sigma) and incubated at 37 °C for 15 min. Pronase treatment was quenched with an equal volume of ice-cold HBSS containing 5% FBS. After three more washes in HBSS containing 5% FBS, any remaining Pronase was inactivated with 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin for 10 min on ice. Pronase-treated cells were resuspended at 107/ml in RPMI 1640 medium containing 10% FBS and cultured for 4 h on ice or at 37 °C in the presence or absence of 2 μg/ml BFA. After culturing, the cells were washed three times with HBSS and surface-labeled with biotin as above. Before solubilization with 1% Nonidet P-40 lysis buffer, dead cells were removed by lympholyte M treatment as above.

**DNA Construction—**The expression vector pMX-ires-GFP was provided by Dr. K. Kitamura (University of Tokyo, Tokyo, Japan) (11). pcDNA3 neo was obtained from Invitrogen Corp. (San Diego, CA). Polymerase chain reaction (PCR) was employed to construct Tac chimeras and FLAG-tagged deletion mutants of calnexin. Template cDNA was prepared from mouse 2B4 T cell hybridomas with a Superscript II kit (Life Technologies, Inc.). The vector containing cDNA for Tac (in-frame with the 10 amino acids downstream from the transmembrane region of Tac) was used as a control to exclude this possibility. α-Adaptin is a subunit of a clathrin-associated protein complex, AP-2, that associates with the cytoplasmic surface of the plasma membrane so that it could not be labeled in live cells by our biotinylation protocol. As expected, the experiments with surface biotinylation using P815 mastocytomas, murine splenocytes, and DAP-3 fibroblast cells and found calnexin on all of these cells as well (Fig. 1C). Since surface biotinylation might also label proteins present inside dead cells whose plasma membrane was not intact, it is possible that contamination of a small number of dead cells could result in a similar observation. In addition to the use of lympholyte M to separate only live cells after biotinylation, we used an Ab recognizing α-adaptin as a control to exclude this possibility. Calnexin was detected only upon surface biotinylation as shown in Fig. 1B.

**RESULTS**

**Surface Expression of Calnexin on Various Cells—**Calnexin has not been reported on the surface of cells except for T and B precursor cells (9, 10). To test whether this notion is true, we performed surface biotinylation of splenic T cells and KKF, an immature thymocyte cell line. Cell lysates were immunoprecipitated with anti-CD3 mAb or anti-calnexin Ab. As previously shown (12), calnexin was found to be assembled with the p815 mastocytomas, murine splenocytes, and DAP-3 fibroblast cells and found calnexin on all of these cells as well (Fig. 1A). Unexpectedly, calnexin was expressed on the surface of not only KKF cells but also splenic T cells (Fig. 1A, lanes 3 and 6). The observed band of surface calnexin was detected only upon surface biotinylation as shown in Fig. 1B. To extrapolate from this observation to other types of cells, we performed similar experiments with surface biotinylation using P815 mastocytomas, murine splenocytes, and DAP-3 fibroblast cells and found calnexin on all of these cells as well (Fig. 1C). Since surface biotinylation might also label proteins present inside dead cells whose plasma membrane was not intact, it is possible that contamination of a small number of dead cells could result in a similar observation. In addition to the use of lympholyte M to separate only live cells after biotinylation, we used an Ab recognizing α-adaptin as a control to exclude this possibility. α-Adaptin is a subunit of a clathrin-associated protein complex, AP-2, that associates with the cytoplasmic surface of the plasma membrane so that it could not be labeled in live cells by our biotinylation protocol. As expected, the experiments performed surface biotinylation using P815 mastocytomas, murine splenocytes, and DAP-3 fibroblast cells and found calnexin on all of these cells as well (Fig. 1C). Since surface biotinylation might also label proteins present inside dead cells whose plasma membrane was not intact, it is possible that contamination of a small number of dead cells could result in a similar observation. In addition to the use of lympholyte M to separate only live cells after biotinylation, we used an Ab recognizing α-adaptin as a control to exclude this possibility. α-Adaptin is a subunit of a clathrin-associated protein complex, AP-2, that associates with the cytoplasmic surface of the plasma membrane so that it could not be labeled in live cells by our biotinylation protocol. As expected, the experiments performed similar experiments with surface biotinylation using P815 mastocytomas, murine splenocytes, and DAP-3 fibroblast cells and found calnexin on all of these cells as well (Fig. 1C).

**A Small Fraction of Calnexin Is Expressed on the Cell Surface—**We next determined the fraction of calnexin expressed on the plasma membrane. p815, murine splenocytes, and DAP-3 were surface-biotinylated, and the labeled proteins were sequentially precipitated twice with streptavidin-agarose beads to isolate and remove the biotinylated proteins. Precipitated proteins eluted by the SDS sample buffer from streptavidin beads as well as the residual lysates were subjected to SDS-PAGE analysis with and without brefeldin A. Volumes of the samples were adjusted so that each sample was derived from an equal amount of starting material. Surface calnexin was detected by the first streptavidin precipitation (Fig. 2, lanes 1, 4, and 7), whereas no calnexin was detected in the second precipitates (Fig. 2, lanes 2, 5, and 8), indicating that virtually all of the biotinylated calnexin was in the first streptavidin precipitates. The percentages of calnexin on the plasma membrane calculated from this type of experiment were 1.3% in p815 (Fig. 2, lanes 1 and 3), 8.9% in splenocytes (Fig. 2, lanes 4 and 6), and 5.4% in DAP-3 (Fig. 2, lanes 7 and 9), respectively, of the total calnexin existing in the cell. Therefore, it is conceivable that a small percentage of calnexin is expressed on the cell surface regardless of cell type or maturation stage.

**Delivery of Intracellular Calnexin to the Cell Surface—**The...
fact that calnexin is expressed on the plasma membrane suggests that the molecule is delivered to the cell surface through the secretory pathway. To test this possibility, cells were first treated with Pronase to digest surface proteins present on cells and then placed into culture at 37 °C to allow reestablishment of the surface expression of the proteins (8). After Pronase treatment, calnexin completely disappeared from the cell surface (Fig. 3, lane 1 and 2) and was re-expressed after culturing for 4 h at 37 °C (Fig. 3, lane 3). Reprobing the same membrane with the anti-calnexin Ab detected similar amounts of the total cellular calnexin in each lane, suggesting that equivalent amounts of cells were subjected to the experimental procedures. Comparison of the amounts of biotinylated calnexin in lanes 1 and 3 of Fig. 3 revealed that 30–50% of calnexin of the original level on the cell surface reached the plasma membrane during the 4-h incubation. To confirm that the reappeared calnexin was delivered through the ER-Golgi pathway after new synthesis, the transport to the plasma membrane was blocked by BFA during the reappearance stage. As shown in Fig. 3, lane 4, BFA significantly inhibited about 80% of the reappeared calnexin on the cell surface, whereas the total cellular level of calnexin remained at the same level. These data indicate that the reappeared calnexin on the cell surface was mainly transported through Golgi after new synthesis.

Disappearance of Surface-biotinylated Calnexin—We next examined whether the surface calnexin was stably expressed or continuously endocytosed. Surface-biotinylated cells were cultured for 4 h at 37 or 0 °C. After the culture at 37 °C, most of the surface-biotinylated calnexin was endocytosed (Fig. 4A). However, at 0 °C, the surface level of calnexin remained almost the same (Fig. 4B). The lower panel of Fig. 4A shows that the biotinylated calnexin was released back to the cell surface after 4 h at 37 °C, whereas the surface level of calnexin was not changed at 0 °C (Fig. 4B). These results suggest that the endocytosed calnexin was mainly recycled through the secretory pathway.
ER Retention Motif of Calnexin Effectively Retains the Molecule in the ER—The surface expression of calnexin could result from the relative shortage of the ER retention receptors for the calnexin (70–95%) disappeared from the cell surface (Fig. 4A, lane 3). This was in striking contrast with the T cell receptor α/β chains, which remained mostly on the cell surface during the same period of time (Fig. 4A, lane 6). The disappearance of calnexin was not observed by incubation at 0 °C (Fig. 4, lane 2), demonstrating that calnexin disappeared from the cell surface by dynamic metabolism. The disappearance of the biotinylated surface calnexin by incubation at 37 °C could result from endocytosis and degradation within the cells or secretion into the medium. We could not detect any biotinylated calnexin by immunoprecipitation of the culture supernatant after incubation (data not shown). Then, to confirm that surface calnexin is endocytosed and degraded after internalization, the surface-biotinylated cells were treated by methylamine or bafilomycin, both of which are known to inhibit lysosomal degradation by induce neutralization of the lysosomal acid compartments. Indeed, treatment with either reagent inhibited degradation of biotinylated calnexin (Fig. 4B). 60 and 85% of the disappeared calnexin was recovered onto the cell surface by the treatment of methylamine (lane 9) and bafilomycin (lane 4), respectively. The results indicate that a significant proportion of the surface calnexin was continuously endocytosed and degraded in the lysosomal compartment. These results suggest that continuous exocytosis and endocytosis of calnexin take place and that the amount of calnexin on the plasma membrane results from the balance of the rates of these two events.

Fig. 2. A small fraction of total calnexin on the cell surface. Surface-biotinylated cells of P815 (lanes 1–3), spleen cells (SpI) (lanes 4–6), and DAP-3 (lanes 7–9) were lysed with 1% Nonidet P-40 and then sequentially precipitated twice with streptavidin-agarose beads. Streptavidin pellets were eluted in SDS sample buffer, and the eluates were diluted with lysis buffer to adjust the volumes to their residual supernatants. Equal aliquots derived from the samples were subjected to SDS-PAGE and immunoblot analysis with anti-calnexin Ab.

Fig. 3. Delivery of intracellular calnexin to the cell surface. Murine splenocytes were either mock-treated (−) (lane 1) or Pronase-treated (+, 2 mg/ml Pronase) (lanes 2–4), followed by culture for 4 h at 0 °C or at 37 °C in the presence (lane 4) or absence (lanes 1 and 3) of 2 μg/ml BFA. After incubation, the cells were surface-biotinylated, lysed in 1% Nonidet P-40 lysis buffer, and immunoprecipitated (IP) with anti-calnexin Ab. The lower panel shows the same membrane reblotted with anti-calnexin Ab. This result represents three independent experiments.

Fig. 4. Disappearance of surface-biotinylated calnexin. A, surface-biotinylated murine splenocytes were divided equally into three lots. One of them was immediately lysed in 1% Nonidet P-40, and the others were cultured for 4 h at 0 or 37 °C, after which the cells were lysed in the same lysis buffer. The lysates were immunoprecipitated (IP) with anti-calnexin (CNX) Ab or 2C11 (anti-CD3e Ab). The lower panel shows the same membrane reblotted with anti-calnexin Ab. B, an endocytosis experiment was performed similar to A in the presence of inhibitors of lysosomal degradation, methylamine (50 mM) or bafilomycin A1 (0.5 μM). The lower panel shows the same membrane reblotted with anti-calnexin Ab. This result represents three independent experiments.
FIG. 5. ER retention motif of calnexin effectively retains the molecules in the ER. A, schematic representations of Tac (human interleukin-2 receptor α chain) and Tac chimeric molecules. Tac-E19 has the leader peptide (LP) and the luminal (LM) and transmembrane (TM) domain of Tac fused to the cytoplasmic tail (CP) of adenovirus gene product E19. TTC was constructed by ligating the whole cytoplasmic domain of calnexin to the C terminus of the cytoplasmic domain. TCC expresses the transmembrane and cytoplasmic domain of calnexin with the luminal region of Tac. B, HeLa cells were surface-biotinylated and lysed in 1% Nonidet P-40 lysis buffer. The lysates were immunoprecipitated (IP) with normal rabbit serum (NRS) and anti-calnexin Ab (CNX), respectively. C, expression of Tac and Tac chimeras on the surface of transfected cells. The constructs depicted in A in the retroviral vector pMX-IRES-GFP were transiently transfected into HeLa cells. 48 h after transfection, cells were analyzed by flow cytometry. The GFP-positive populations of the respective transfected cells (40.5% for mock, 30.4% for Tac, 28.0% for Tac-E19, 37.2% for TTC, and 26.6% for TCC) were stained with phycoerythrin-anti-Tac mAb, and their histograms were shown. Intracellular staining of each transfecnt with phycoerythrin-anti-Tac mAb is shown in the histograms in the right panel. It was confirmed that GFP levels were correlated with the expression of Tac proteins. D, aliquots of transfected HeLa cells used in C were lysed in 1% Nonidet P-40 lysis buffer and immunoprecipitated (IP) and blotted with anti-C-terminal calnexin Ab (CNX-C). Densitometric analysis showed that the expression of transfected Tac-chimeras was 3-fold higher than the endogenous calnexin for both TTC and TCC. Since the percentage of Tac chimera-positive cells in the total population was 37.2% for TTC and 26.6% for TCC, the Tac chimeras were expressed at 8 and 11 times higher level than the endogenous calnexin for TTC and TCC, respectively.
Fig. 6. The glycoprotein binding domain is important for effective expression of calnexin on the cell surface. A, tunicamycin prevented the delivery of calnexin (CNX) to the cell surface. Before surface biotinylation, KKF cells were incubated with (+) or without (−) 0.5 μg/ml tunicamycin for 10 h at 37 °C. The cell lysate of biotinylated KKF was immunoprecipitated (IP) with anti-calnexin Ab (CNX) (upper panel). The lower panel shows the same membrane reblotted with anti-calnexin Ab. This result represents two independent experiments. B, schematic structures of FLAG-tagged deletion mutants of calnexin. All constructs contain FLAG epitope tag at two bases after the signal sequence cleavage site. F-WT is the full-length calnexin construct, and F-ΔN1 and F-ΔN2 represent calnexin mutants lacking the glycoprotein binding domain; F-ΔN1 lacks the region encoding amino acids 3–391, and F-ΔN2 lacks amino acids 254–456. F-ΔC lacks the C-terminal RKPRRE-ER retention motif of calnexin. LP, leader peptide; LM, luminal domain; TM, transmembrane domain; CP, cytoplasmic tail. C, expression of the FLAG-tagged epitope on the surface of transfected cells. HeLa cells were transiently mock-transfected (right panel, dotted line) and transfected with F-WT, F-ΔN1, F-ΔN2 and F-ΔC, respectively (right panel, heavy line). Cell surface staining was performed using anti-FLAG mAb followed by fluorescein isothiocyanate-conjugated secondary Ab. The data are presented by dot plots (left panel). Intracellular staining of each transfectant with B. Blp.
proteins is a characteristic of immature lymphocytes and that the surface expression of calnexin seems to be regulated by protein glycosylation. Therefore, we postulated that the glycoprotein binding domain of calnexin might regulate the cell surface expression. To test this possibility, we constructed the FLAG-tagged full-length calnexin (F-WT), the FLAG-tagged calnexin with the N-terminal deletion including the glycoprotein binding sites (F-ΔN1 and F-ΔN2), and the FLAG-tagged calnexin with the C-terminal deletion including the di-lysine like motif (F-ΔC) (Fig. 6B). These constructs were transiently transfected into HeLa cells, and the surface and intracellular expressions of calnexin were analyzed. As compared with F-WT and F-ΔC, F-ΔN1 and F-ΔN2 were expressed on the cell surface at a strikingly lower level (Fig. 6, C and D) despite the fact that the total cellular levels of chimeric proteins were similar (Fig. 6C, right panels). In this experiment, it remains the possibility that F-ΔN1 and F-ΔN2 were unfolded states. To clarify this point, we investigated whether overexpression of FLAG-tagged constructs could induce the unfolded protein response (19, 20), which results in the up-regulation of genes encoding ER-resident chaperones such as calnexin, calreticulin, and Bip (GRP78). Since the endogenous calnexin could not be distinguished from the transfected calnexin in the transfecteds, we examined unfolded protein response by analyzing the expression of Bip. The expression levels of Bip mRNA in all transfecants were increased by the treatment with tunicamycin, which is often used as a positive inducer of ER stress (Fig. 6E). In contrast, the level of Bip expression was not changed in transfecants expressing FLAG-tagged calnexins (Fig. 6E, lanes 1, 3, 5, and 7). These results suggest that the expression of FLAG-tagged constructs did not alter the quality control systems in the ER. Thus, the glycoprotein binding domain is suggested to be important for the expression of calnexin on the cell surface.

**DISCUSSION**

Recent reports have shown that several soluble ER resident proteins are found on the cell surface. These are calreticulin (CRT), protein disulfide isomerase (PDI), 78-kDa glucose-regulated protein (GRP78), and 94-kDa glucose regulated protein (GRP94) (21–24). These ER luminal proteins commonly have KDEL-containing proteins strictly in the ER. In this report, we demonstrated that this type of ER retention is not efficient enough to keep the protein level of calnexin. COPI, cytosolic coat complex, is known to directly interact with the ER retention motif (25, 26). COPI coats are known to play a significant role in the retrograde transport of ER resident proteins, and it functions in many different traffic pathways including early to late endosomes (27, 28). Therefore, an intriguing possibility would be that the endocyted calnexin is delivered to the lysosomal compartment by association with COPI; further experiments will be required to elucidate the mechanism of endocyted calnexin and for lysosomal targeting.

It is known that a small fraction of residual proteins of intracellular organelles other than the ER is also expressed on the plasma membrane. These include a lysosomal residual protein, lamp-1 (29–31), and a resident of the trans-Golgi network (TGN), TGN38 (32–34). These proteins contain the tyrosine-based sorting signal in their cytoplasmic tails, which interacts with clathrin-adaptor complexes. The intracellular localization of proteins mediated by these tyrosine signals is known to be saturable (18). In contrast, the present study showed that this is not the case for the di-lysine motif. The overexpression of exogenous di-lysine motif-containing proteins resulted in the increased surface expression of neither exogenous proteins themselves nor endogenous proteins with a di-lysine motif (Fig. 5). Since di-lysine signals interact with COPI, components of another class of coated vesicles, the results suggest that COPI is not easily saturated and probably exists much more abundantly than the AP complex in cells. Another attributable difference between these two sorting systems is their sites of function in the cell. In the case of clathrin-coated vesicles, their site of sorting is between trans-Golgi network and the plasma membrane, which resides near the end of the secretory pathway. By contrast, COPI is mostly concentrated on the cis-Golgi membrane, and this location is at the starting site of the secretory pathway, apart from the plasma membrane.

A major question then is how a fraction of calnexin escapes from the ER to reach the plasma membrane. Our data using several deletion mutant calnexins suggest that the glycoprotein binding site in the luminal domain of calnexin appears important for its exit from the ER. The possibility that the

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Footnote:

*Y. Okazaki, H. Ohno, K. Takase, T. Ochiai, and T. Saito, unpublished observation.*
failure of such deletion mutant calnexins to be expressed on the cell surface might be related to the unfolded protein structure of these mutants is unlikely because the expression of the mutants did not induce any unfolded protein response. Therefore, the simplest explanation would be that calnexin can escape from the ER through a specific binding to glycoproteins as a chaperone, and this association may cover the retention signal. The association of the CD3 complex in immature thymocytes as well as the Igα/Igβ heterodimer in pro-B cells may hinder the di-lysine motif of calnexin from the binding with COPI. In this study, however, we could not detect any specific molecule(s) associated with calnexin at a semi-stoichiometric amount on the surface of various cells under the condition that detected the association of calnexin with the CD3 complex on the immature thymocyte cell line. It is possible that calnexin associates with a small amount, but yet a variety, of glycoproteins in various type of cells. It provides a striking contrast to the situation in immature lymphocytes in which the surface calnexin largely associates with pre-T or pro-B receptor complexes and may emphasize the biological importance of the specific interaction of calnexin-immune receptor complexes.

It was recently reported that the expression of calnexin is up-regulated when cells contact a substrate that induces cell adhesion (35). Moreover, calreticulin, a luminal homologue of calnexin largely associates with pre-T or pro-B receptor complexes and this association may cover the retention signal from the ER through a specific binding to glycoproteins as a chaperone, and this association may cover the retention signal for integrin-initiated signaling. The simplest explanation would be that calnexin can escape from the ER through a specific binding to glycoproteins as a chaperone, and this association may cover the retention signal. Moreover, calreticulin, a luminal homologue of calnexin, is known to be essential for integrin-mediated cell adhesion (35). Moreover, calreticulin, a luminal homologue of calnexin, is known to be essential for integrin-mediated cell adhesion (35).

In conclusion, the present study demonstrates that a variety of cells previously thought to keep calnexin exclusively within the ER express calnexin at a low level on their surface and that the luminal glycoprotein binding region seems to be responsible for the expression. Further experiments will be required to elucidate its physiological implications.

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