ΔF508 CFTR Pool in the Endoplasmic Reticulum Is Increased by Calnexin Overexpression

Tsukasa Okiyoneda,* Kazutsune Harada,* Motohiro Takeya,† Kaori Yamahira,* Ikuo Wada,‡ Tsuyoshi Shuto,* Mary Ann Suico,* Yasuaki Hashimoto,* and Hiroyuki Kai*§

Departments of *Molecular Medicine and †Pathology, Graduate School of Medical and Pharmaceutical Sciences, Kumamoto University, Kumamoto 862-0973, Japan; and ‡Department of Cell Science, Institute of Biomedical Sciences, Fukushima Medical University School of Medicine, Fukushima 960-1295, Japan

Submitted June 9, 2003; Revised September 9, 2003; Accepted October 3, 2003
Monitoring Editor: Randy Schekman

The most common cystic fibrosis transmembrane conductance regulator (CFTR) mutant in cystic fibrosis patients, ΔF508 CFTR, is retained in the endoplasmic reticulum (ER) and is consequently degraded by the ubiquitin-proteasome pathway known as ER-associated degradation (ERAD). Because the prolonged interaction of ΔF508 CFTR with calnexin, an ER chaperone, results in the ERAD of ΔF508 CFTR, calnexin seems to lead it to the ERAD pathway. However, the role of calnexin in the ERAD is controversial. In this study, we found that calnexin overexpression partially attenuated the ERAD of ΔF508 CFTR. We observed the formation of concentric membranous bodies in the ER upon calnexin overexpression and that the ΔF508 CFTR but not the wild-type CFTR was retained in the concentric membranous bodies. Furthermore, we observed that calnexin overexpression moderately inhibited the formation of aggresomes accumulating the ubiquitinated ΔF508 CFTR. These findings suggest that the overexpression of calnexin may be able to create a pool of ΔF508 CFTR in the ER.

INTRODUCTION

Cystic fibrosis transmembrane conductance regulator (CFTR) is a polytopic integral membrane protein that functions as a cAMP-dependent Cl− channel in the apical membrane of epithelial cells (Anderson et al., 1991; Drumm et al., 1991; Tabcharani et al., 1991; Bear et al., 1992). Mutations in the CFTR gene lead to the absence or malfunction of a regulated Cl− channel in the apical membrane of secretory epithelia, resulting in the clinical symptoms of cystic fibrosis (CF) (Kerem et al., 1989; Riordan et al., 1989; Rommens et al., 1989; Cheng et al., 1990). Therefore, potential CF therapies are aimed at overcoming the functional impairment of various mutant CFTRs, particularly ΔF508 CFTR, in which a phenylalanine at position 508 is deleted from the first nucleotide-binding fold. This mutation is found in ~70% of CF chromosomes and results in a severe form of the disease; >90% of CF patients have at least one ΔF508 allele (Tsui, 1992; Serrara and Collins, 1993). Although ΔF508 CFTR is functionally competent as a cAMP-dependent Cl− channel in model situations where it reaches the plasma membrane (Drumm et al., 1991; Denning et al., 1992; Li et al., 1993), in mammalian cells ΔF508 CFTR fails to reach the plasma membrane.

CFTR biogenesis is inefficient. During insertion into the ER membrane, CFTR interacts with the cytosolic chaperones Hsc70/Hdj-2 and Hsp90, as well as the ER chaperone calnexin (CNX) (Yang et al., 1993; Pind et al., 1994; Loo et al., 1998; Meacham et al., 1999). After ATP-dependent conformational maturation, assisted by cytosolic and ER chaperones, the chaperones release CFTR, and only ~30% of the immature wild-type (wt) CFTR transits to the late secretory pathway, ultimately reaching the plasma membrane (Luksa et al., 1994). However, most of the immature wt CFTR (~70%), and nearly all of immature ΔF508 CFTR, fail to mature and do not transit to the late secretory pathway (Luksa et al., 1994). They are trapped in the ER as core-glycosylated products (~140 kDa) and are ultimately degraded by the ER-associated degradation (ERAD) pathway. In fact, CFTR retained in the ER is ubiquitinated and retrotranslocated to the cytosol where it undergoes proteosomal degradation (Jensen et al., 1995; Knittler et al., 1995; Johnston et al., 1998; Gelman et al., 2002).

The lectin-like chaperone calnexin participates in the ER retention and ERAD of ΔF508 CFTR (Pind et al., 1994). Calnexin is a type I transmembrane protein localized in the ER that associates selectively with incompletely folded glycoproteins containing monoglucosylated N-linked oligosaccharides (Wada et al., 1991). Calnexin transiently interacts with newly synthesized CFTR and dissociates after it attains a native conformation (Pind et al., 1994). If CFTR cannot attain a native conformation, as is the case for ΔF508 CFTR, calnexin fails to dissociate from the misfolded CFTR (Pind et al., 1994). The prolonged interaction of ΔF508 CFTR with calnexin results in
the ERAD of ΔF508 CFTR (Pind et al., 1994; Ward et al., 1995). Therefore, calnexin seems to lead it to the ERAD pathway. However, the role of calnexin in the ERAD remains controversial. Previous reports indicated that a diminished rate of physical dissociation of substrates from calnexin leads to ERAD in mammalian cells (Cabrall et al., 2000, 2001), suggesting that calnexin plays a role in substrate recognition. In contrast, interactions between calnexin and glycoproteins enhanced the degradation, indicating that calnexin prevents ERAD (Chen et al., 1998; Wilson et al., 2000).

In this study, we tried to clarify the role of calnexin in the ERAD of ΔF508 CFTR by using calnexin overexpression system. Our results showed that calnexin overexpression increased the ER retention of ΔF508 CFTR but partially attenuated the ERAD. Moreover, calnexin overexpression induced the formation of concen-tric membranous (CM) bodies of the ER where calnexin and ΔF508 CFTR accumulated. ΔF508 CFTR in CM bodies was not ubiquitinated, and calnexin overexpression inhibited formation of aggresomes and high molecular weight (HMW) complexes induced by a proteasomal inhibitor.

MATERIALS AND METHODS

Materials

The following antibodies were used in this study: mouse monoclonal anti-CFTR (C-terminus specific) (clone 24–1; Genzyme/Techen, Cambridge, MA), rabbit polyclonal anti-calnexin (C-terminus specific) (anti-CN; Sigma-Aldrich); Strepsyn Biotechnologies, Victoria, BC, Canada), rabbit polyclonal anti-ubiquitin (anti-Ub; FL-76; Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal anti-ubiquitinated proteins (anti-Ub-Protein; clone FK2; Affiniti Bioreagents, Golden, CO), mouse monoclonal anti-20S proteasome subunit α7 (anti-20S; clone HC8; Affiniti Bioreagents), mouse monoclonal anti-vimentin (clone V9; Santa Cruz Biotechnology), mouse monoclonal anti-Hsp70 (clone C21; Sigma-Aldrich; Stressgen Biotechnologies), rat monoclonal anti-Hsc70 (clone 1B5; SPA-815; Stressgen Biotechnologies), rabbit polyclonal anti-calreticulin (anti-CRT; SPA-600; Stressgen Biotechnologies), mouse monoclonal anti-KDEL receptor (anti-KDELr; clone 10C3; Stressgen Biotechnologies), rabbit polyclonal anti-protein disulphide isomerase (PDI) (SPA-890; Stressgen Biotechnologies), mouse monoclonal anti-β-COP (clone maD; Sigma-Aldrich, St. Louis, MO), mouse monoclonal anti-KDEL receptor (anti-KDELr; clone KR-10; Stressgen Biotechnologies), mouse monoclonal anti-Hsc70 (clone 1B5; SPA-815; Stressgen Biotechnologies), rabbit polyclonal anti-calnexin (anti-CN; Sigma-Aldrich), mouse monoclonal anti-Lamp-1 (clone UH1; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), and mouse monoclonal anti-Flag M2 (Sigma-Aldrich) antibodies.

M6C-132 and moesinazole were purchased from Calbiochem (San Diego, CA), and brefeldin A and cycloheximide were purchased from Sigma-Aldrich.

Cell Lines

Baby Hamster kidney (BHK) cells stably expressing green fluorescent protein (GFP-CFTR; BHK-GRFP) and GFP-ΔF508 CFTR (ΔF508-BHK); and hamster ovary (CHO) cells stably expressing CFTR (CFTR-CHO) and ΔF508 CFTR (ΔF508-CHO) were grown and maintained as described previously (Lukacs et al., 1994; Loo et al., 1998). CFTR- and ΔF508-CHO cells were maintained in a minimal essential medium (MEM) containing 7% fetal bovine serum, antibiotics, and 200 μM methotrexate. CFTR- and ΔF508-BHK cells were maintained in DMEM/F-12 medium containing 10% fetal bovine serum, antibiotics, and 500 μM methotrexate. CFBE41o −ΔGFP (ΔF508-ΔGFP) cells were grown in MEM with 10% fetal bovine serum and antibiotics on glass-bottomed culture dishes with human fibronectin. To increase CFTR expression, cells were incubated with 1 μM CFTR- and ΔF508-BHK cells) or 2 μM sodium butyrate (CFTR-, ΔF508-CHO, 161HBE140 +, and CFBE41o −ΔGFP cells) for 20–24 h before analysis.

Recombinant Adenovirus

Recombinant adenovirus expressing human calnexin (Ad-CN) or LacZ (Ad-LacZ) based on adenovirus 5 (Ad5) was produced as described previously (Okiyone et al., 2002).

SDS-PAGE and Western Blotting

Subconfluent (90–100%) CFTR- and ΔF508-CHO cells grown on six-well plates were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed at 4°C in 100 μl of radioimmuno precipitation assay (RIPA) buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mg/ml sodium deoxycholate, and 1% NP-40) containing 1% protease inhibitor cocktail (Sigma–Aldrich) and centrifuged at 15,000 × g for 10 min at 4°C. The supernatant was prepared for SDS-PAGE using 6 or 7.5% polyacrylamide gels. Protein was electrophoretically transferred into membranes and maintained in 0.1% skim milk or 0.1% Tween 20 in PBS for 1 h at RT, and incubation with the primary antibodies specified in the figure legends was for 1 h at RT. The membranes were then washed three times in 0.05% Tween 20 in PBS and incubated with the appropriate secondary antibody for 1 h at RT. After washing, membranes were incubated with ECL detection reagents (Amersham Biosciences, Piscataway, NJ) and analyzed by luminescent image analyzer (LAS-1000; Fujifilm, Tokyo, Japan).

Pulse-Chase Analysis and Immunoprecipitation

Pulse-chase analysis and immunoprecipitation were carried out as described previously (Lukacs et al., 1994). Subconfluent CFTR- and ΔF508-CHO cells infected with or without Ad-CNx were incubated for 30 min in methionine- and cysteine-free MEM 48 h after infection, and then pulse-labeled for 20 min with 100 μCi/ml [35S]methionine and [35S]cysteine (3,700 Ci/mmol; Amersham Biosciences). For chasing, the cells were washed three times with PBS and the labeling medium was replaced by complete MEM containing 1 mM cycloheximide. Radiolabeled CFTR was isolated by immunoprecipitation. The cells and then lysed with ice-cold PBS three times at 4°C in 1 ml of RIPA buffer containing 1% protease inhibitor cocktail. Samples were centrifuged at 15,000 × g for 10 min at 4°C, and the supernatant was incubated for 2 h at 4°C with monoclonal anti-CFTR antibody immobilized in protein G Sepharose 4 Fast Flow (Amersham Biosciences). Immune complexes were precipitated, followed by four washes with 1 ml of RIPA buffer. Immunoprecipitated proteins were eluted for 15 min at 37°C with 2× concentrated loading buffer and analyzed on 6% SDS-PAGE gels. The gels were dried and analyzed with a BAS imaging plate scanner (BAS-2000, Fujifilm). The radioactivity associated with CFTR was quantified using Image Gauge software (version 3.4, Fujifilm).

Transfection

Transfection was performed using TransIT-L1 transfection reagents (Mirus, Madison, WI). Subconfluent cells grown on glass-bottomed culture dishes were transfected with 2 μg of plasmid DNA per dish.

Small Interfering RNA (siRNA) Preparation and Transfection

Specific siRNA was designed as described previously (Elbashir et al., 2001). We used the 21-nucleotide (nt) sense strand (5′-GACUGAUCAGCUCCUG-CU4dTdT, coding region 384–402 relative to the start codon) and the 21-nt antisense strand (5′-AGCAAGUGCUCCAGUCGd4dTdT, coding region 402 relative to the start codon). siRNA duplex was prepared as described previously (Elbashir et al., 2001). Transient transfection with siRNA was performed by using TransIT-TKO (Mirus) as described by the manufacturer. siRNA duplex was used at a concentration of 10 nM.

Immunocytochemical Analysis and Confocal Laser Scanning Microscopy

Subconfluent cells grown on glass-bottomed culture dishes were washed twice with PBS, fixed in 3.7% paraformaldehyde in PBS for 20 min at RT, and permeabilized with 0.1% Triton-X in PBS for 20 min at RT. For immunostaining with anti-Golgi 58k protein or Lamp-1 antibodies, cells were permeabilized with 0.5% Triton-X in PBS or immunofluorescence buffer (150 mM NaCl, 5 mM Na2EDTA, 15 mM Tris-Cl, 0.1% gelatin, 0.01% saponin, pH 7.5) for 20 min at RT (Wimer-Mackin and Granger, 1996). All cells were washed twice with PBS, subsequently incubated in PBS containing 1% bovine serum albumin (BSA) (BSA/PBS) for 30 min at RT, and then incubated for 1 h with corresponding primary antibodies (1:100 dilution) in 1% BSA/PBS at RT. Cells were washed three times with PBS and then immunostained with fluorescein isothiocyanate- or tetramethylrhodamine B isothiocyanate (TRITC)-conjugated secondary antibodies (1:100 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA) or Alexa Fluor 488-conjugated secondary antibody (1:100 dilution; Molecular Probes, Eugene, OR) in 1% BSA/PBS for 30 min at RT. Cells were washed three times with PBS and mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Cells were observed and analyzed with a Fluoview FV300 confocal laser scanning microscope (Olympus, Tokyo, Japan).
Fluorescence Recovery after Photobleaching (FRAP) Analysis

ΔF508-BH4K cells infected with or without Ad-CNX were fixed in 1% glutaraldehyde for 30 min and postfixed in 1% osmium tetroxide for 30 min at 4°C. After dehydration in a graded ethanol series, the cells were embedded in epoxy resin. Ultrathin sections stained with uranyl acetate and lead citrate were observed with an H-7500 electron microscope (Hitachi, Tokyo, Japan).

RESULTS

Calnexin Overexpression Partially Attenuates the ERAD of ΔF508 CFTR

Previous reports have indicated that calnexin may target ΔF508 CFTR to the ERAD, but contradictory findings have been reported. To clarify the role of calnexin on the fate of ΔF508 CFTR, we examined the effect of calnexin overexpression. By coimmunoprecipitation using anti-CFTR antibody, we showed that calnexin overexpression increased the interaction between calnexin and ΔF508 CFTR (Figure 1A). If calnexin can promote the ERAD, calnexin overexpression would lead to a diminished expression of ΔF508 and wt CFTR. ΔF508- and CFTR-CHO cells infected with or without Ad-CNX were pulse labeled 48 h after infection. Radiolabeled CFTR was isolated from cell lysates after the indicated chase period by immunoprecipitation with anti-CFTR antibody and analyzed by SDS-PAGE. The right panel in C and the lower panels in D show the quantification of pulse-chase experiments in ΔF508- and CFTR-CHO cells, respectively. The intensity of the band indicating ΔF508 CFTR and wt CFTR was quantified by Image Gauge software (version 3.4, Fujifilm) and expressed as a percentage of the total material present at t = 0, respectively. The data shown are representative of three independent experiments. (E) Effect of calnexin-specific RNAi on the steady-state level of wt CFTR. Cell lysates from CFTR-CHO cells transfected with or without calnexin-specific siRNA (10 nM) were subjected to Western blotting with anti-CFTR (a), -calnexin (b), -hsc70 (c), -Bip (e), and -calreticulin (f) antibodies 48 h after transfection.
Calnexin overexpression formed IB-like structures where ΔF508 CFTR and calnexin accumulated. (A) All panels are fluorescence micrographs of BHK cells stably expressing ΔF508 CFTR-GFP (a, ΔF508-BHK) and wt CFTR-GFP (b, CFTR-BHK). The cells were infected with or without Ad-CNX at a MOI 50 (+CNX), and 48 h after infection cells were fixed and permeabilized, immunostained with anti-calnexin antibody, and visualized with TRITC-conjugated secondary antibody. Analysis was performed by confocal laser scanning microscopy. GFP fluorescence is shown in the left panels (a, ΔF508 CFTR; b, wt CFTR), TRITC fluorescence in the middle panels (CNX), and right panels show the overlay (merge). Note that calnexin overexpression formed IB-like structures and ΔF508 CFTR specifically accumulated in the structures. Arrowheads represent the regions in which wt CFTR slightly accumulated with calnexin. (B) Time course of IB-like structure formation. ΔF508-BHK cells were infected with Ad-CNX (MOI 50) and incubated for the times indicated. Note that formation of IB-like structures of ΔF508 CFTR coincides with that of calnexin. (C) ΔF508- (a) and CFTR-CHO (b) cells were infected with or without Ad-CNX (MOI 50) and 48 h after infection cells were fixed and permeabilized, immunostained with anti-CFTR and anti-calnexin antibodies, and visualized with fluorescein isothiocyanate- and TRITC-conjugated secondary antibodies. (D) CFBE410® cells (ΔF508 CFTR) and 16HBE14o- (wt CFTR) were infected with or without Ad-CNX (MOI 50), and 48 h after infection cells were fixed and permeabilized, immunostained with anti-CFTR antibody, and visualized with Alexa Flour488-conjugated secondary antibody. The cells were imaged so that the ER region was in focus, except for some images that were focused on the cell surface (cell surface). Bars, 10 μm.
ner (Figure 1B, b and b'). To further support these observations, pulse-chase analysis revealed that calnexin overexpression partially attenuated the degradation of immature ΔF508 CFTR and prolonged the half-life ($t_{1/2}$) about twofold (control, $t_{1/2} \sim 45$ min; CNX, $t_{1/2} \sim 90$ min) (Figure 1C). Similarly, calnexin overexpression moderately attenuated the ERAD of wt CFTR and prolonged the $t_{1/2}$ about threefold (control, $t_{1/2} \sim 45$ min; CNX, $t_{1/2} \sim 130$ min) (Figure 1D, immature CFTR). The reason why wt CFTR at the steady-state level is increased by CNX overexpression (Figure 1B) is maybe because wt CFTR is temporarily retained by calnexin in the ER and then goes to the cell surface through the Golgi. It is likely that the results of calnexin overexpression may partly reflect the physiological condition, because down-regulation of endogenous calnexin by using siRNA decreased the CFTR

![Image](https://example.com/image.png)

**Figure 3.** IB-like structures are not aggresomes. (A and B) Fluorescence micrographs of ΔF508-BHK cells infected with Ad-CNX at a MOI 50 (+CNX) or treated with 10 μM MG-132 (+MG-132) for 6 h. The cells were immunostained with anti-ubiquitin (Ub), anti-ubiquitinated protein (Ub-protein), anti-20S proteasome (20S), and anti-vimentin (Vim) antibodies, and visualized with TRITC-conjugated secondary antibodies. When 20S proteasome was immunostained, aggresomes were formed by heat shock (+heat shock, 42°C for 2 h and subsequently incubated at 37°C for 5 h). Note that aggresomes formed by MG-132 or heat shock are positive for aggresome markers, whereas IB-like structures formed by calnexin overexpression are negative. Bar, 10 μm. (C) Detergent solubility of IB-like structures. ∆F508-CHO cells were infected with Ad-CNX (MOI 50, CNX, lanes 2 and 5) or treated with MG-132 (2 μM, MG, lanes 3 and 6) for 24 h. Detergent-soluble (lanes 1–3) and -insoluble fractions (lanes 4–6) prepared from the cells were analyzed by Western blotting with anti-CFTR (a), anti-calnexin (b), and anti-Hsc70 antibodies (c). Note that calnexin overexpression increased the amount of the detergent-soluble ∆F508 CFTR.
expression without affecting the expression of hsc70, Bip, and calreticulin (Figure 1E). siRNA of calreticulin rather increased the CFTR expression without affecting the expression of calnexin, hsc70, and Bip in the present study (our unpublished data).

Calnexin Overexpression Induces the Formation of Inclusion Body (IB)-like Structures in Which ΔF508 CFTR Accumulates

To examine the effect of calnexin overexpression on the cellular localization of ΔF508 CFTR, immunocytochemical
analyses by using a confocal laser scanning microscope were performed in BHK cells stably expressing GFP-ΔF508 (ΔF508-BHK) or GFP-wt CFTR (CFTR-BHK). In agreement with a previous report (Loo et al., 1998), ΔF508 CFTR was located in the ER with calnexin (Figure 2A, a), whereas most wt CFTR was located on the cell surface in the steady state (Figure 2A, b). When calnexin was overexpressed, it formed IB-like structures in both CFTR- and ΔF508-BHK cells (Figure 2A, +CNX). Formation of IB-like structures was MOI-dependent (our unpublished data), and in many cases the structures emerged near the nucleus as a ring-shape. Interestingly, ΔF508 CFTR significantly accumulated in IB-like structures with calnexin (Figure 2A, a; +CNX). In contrast, most wt CFTR was dispersed in the cells and expressed at the cell surface, although a small amount of wt CFTR also accumulated in IB-like structures (Figure 2A, b; +CNX, arrowhead).

We next examined the time course of IB-like structure formation after calnexin overexpression in ΔF508-BHK cells. IB-like structures had not emerged 24 h after infection but some occurred at 36 h (Figure 2B). Calnexin and ΔF508 CFTR simultaneously accumulated in IB-like structures (Figure 2B), indicating that calnexin participates in ΔF508 CFTR accumulation. To exclude the possibility that IB-like structure formation is an artifact of using GFP-tagged proteins, we performed double immunofluorescence staining with anti-CFTR and anti-calnexin antibodies in CFTR- and ΔF508-CHO cells. Similar results were obtained in these cells (Figure 2C). Moreover, calnexin overexpression formed IB-like structures in the cells in which neither CFTRs were ex-
pressed (our unpublished data), indicating that their formation is CFTR independent.

Finally, we determined whether calnexin overexpression induces the formation of IB-like structures in a human cystic fibrosis airway epithelial cell line, CFBE410− (Zeitlin et al., 1991), that endogenously expresses ΔF508 CFTR. Although the pattern of IB-like structures was slightly different in CFBE410− cells, calnexin overexpression induced the ΔF508 CFTR accumulation in structures around the nucleus in these cells, but not in a human airway epithelial cell line, 16HBE14o− (Cozens et al., 1994), that endogenously expressed wt CFTR (Figure 2D).

**IB-like Structures Are Not Aggresomes**

It has been reported that inhibition of proteasomal degradation leads to misfolded CFTR accumulation in the cytosol and aggresome formation (Johnston et al., 1998). Aggresomes consist of aggregated proteins, such as polyubiquitinated proteins, proteasomes, and Hsp70, and are surrounded by vimentin cages (Johnston et al., 1998; Wigley et al., 1999). Therefore, we tried to determine whether the IB-like structures are aggresomes. Immunocytochemical analysis showed that aggresome marker proteins such as ubiquitin, ubiquitinated proteins, and 20S proteasome did not accumulate in IB-like structures (Figure 3, A and B, ubiquitin, ubiquitinated proteins, and 20S proteasome did not accumulate in IB-like structures (Figure 3, A and B)).

Thus, the concentric membranes seemed to form a CM body of the ER (Ghaliyali, 1996). CM bodies of the ER differed from structures in which ER soluble and membrane proteins accumulated, such as the Russell body (Kopito and Sitta, 2000) and crystallloid ER (Yamamoto et al., 1996). In this study, we found that one or two CM bodies of the ER occurred in cells infected with Ad-CNX. However, their formation was not due to an artifact of recombinant adenovirus infection, because we did not find the structures in cells infected with adenovirus expressing calreticulin or β-galactosidase (our unpublished data). Moreover, the CM bodies were formed by calnexin overexpression by using cationic liposomes (our unpublished data). In immunocytochemical studies, IB-like structures formed by calnexin overexpression were seen as ring-shaped structures (Figure 2A) that were consistent with the morphological features of CM bodies of the ER.

To further characterize CM bodies of the ER, we performed FRAP analysis. ΔF508-BHK cells were infected with Ad-CNX at MOI 50 to form CM bodies. About 48 h after infection, a CM body (indicated by arrowhead) was photobleached by laser pulses, and fluorescence recovery was recorded by serial imaging after 1 mM cycloheximide treatment. In living cells, the fluorescence of a photobleached CM body was progressively recovered immediately after photobleaching, and CM bodies were recreated in the same place (Figure 4I). In contrast, fluorescence recovery did not occur in fixed cells (our unpublished data). Quantification analysis of FRAP showed that ~30% of the fluorescence in the CM body was recovered 10 min after photobleaching in living cells (Figure 4I). These results indicate that fluorescence recovery resulted from the lateral diffusion of ΔF508 CFTR-GFP localized in the ER. Therefore, CM bodies of the ER are dynamic and connected to the peripheral ER reticular domain.

**Calnexin Overexpression Attenuates Aggresome Formation**

Calnexin overexpression partially attenuated the ERAD of ΔF508 CFTR, leading to accumulation of ΔF508 CFTR in CM bodies of the ER. Moreover, the accumulated ΔF508 CFTR was not ubiquitinated. To confirm the possibility that calnexin overexpression attenuates aggresome forma-
tion, we examined the effect of calnexin overexpression on aggresome formation induced by MG-132. In ΔF508-BHK cells treated with MG-132 (10 μM), many aggresomes accumulating ubiquitinated ΔF508 CFTR were formed (Figure 5A). Aggresome formation by MG-132 was moderately decreased by calnexin overexpression (Figure 5A). In control, aggresomes were detected in ~49% of the total cells, whereas with calnexin overexpression only 7.5% of the cells had aggresome formation (Figure 5A, bottom). Because the ΔF508 CFTR accumulating in CM bodies was not ubiquitinated (Figure 3A), calnexin overexpression may inhibit the ubiquitination and retrotranslocation of ΔF508 CFTR. Indeed, Western blots show that calnexin overexpression attenuated the formation of HMW complex of both wt and ΔF508 CFTR induced by MG-132 (Figure 5A). The level of many CM bodies was decreased by calnexin overexpression (our unpublished data). This result may explain the fact that CFTR does not interact with BiP (Pind et al., 1994).

**CM Bodies of the ER Are Functional Compartments**

One of the ways to establish that CM bodies are functional is to prove that they are connected to the late secretory pathway. Wild-type CFTR, but not ΔF508 CFTR, could leave the CM bodies for the late secretory pathway. We tried to visualize the transport of wt CFTR from CM bodies to the Golgi complex. To retain wt CFTR in CM bodies, we treated the cells with BFA, an inhibitor of ER–Golgi transport. In BFA-treated cells, the ER–Golgi transport of wt CFTR was inhibited and wt CFTR accumulated in the CM bodies. Merged images show that calnexin overexpression inhibits ubiquitination. It is well known that when misfolded proteins accumulate in the ER, ER chaperones’ expression is induced by unfolded protein response (Kaufman, 1999). However, ER chaperones BiP and GRP94 were not increased in both ΔF508-CHO and ΔF508-BHK cells infected with Ad-CNX (our unpublished data). Rather, BiP and GRP94 were slightly decreased by calnexin overexpression (our unpublished data). This result may explain the fact that CFTR does not interact with BiP (Pind et al., 1994).

**DISCUSSION**

Calnexin is an ER chaperone that has a central role in the ER quality control. Although it has been thought that calnexin specifically retains misfolded proteins in the ER, leading them to the ERAD, the role of calnexin in the ERAD was controversial. In this study, we showed that calnexin overexpression partially attenuated the ERAD of misfolded CFTR and accumulated it in CM body of the ER. It is unlikely that formation of CM bodies is necessary for attenuation of the ERAD because calnexin overexpression at a low MOI (e.g., 12.5) in which there are few CM bodies, increased the steady-state level of ΔF508 CFTR (Figure 1A). ΔF508 CFTR in CM body was not ubiquitinated (Figure 3), and calnexin overexpression moderately attenuated the formation of aggresomes and HMW complexes induced by MG-132 (Figure 5B), indicating that interaction with calnexin might inhibit the ubiquitination and retrotranslocation of ΔF508 CFTR. Therefore, calnexin retains misfolded CFTR in the ER but does not lead it to the ERAD pathway. Other key molecules for ERAD such as ubiquitin ligases and MApp-lactin (Hosokawa et al., 2001; Meacham et al., 2002; Lenk et al., 2002; Molinari et al., 2002; Yoshida et al., 2002) seem to participate in the ERAD targeting of ΔF508 CFTR after dissociation from calnexin. It has been reported that after dis-
Association from calnexin, BiP, and PDI are mainly involved in determining the direction of ERAD substrates toward retrotranslocation and ERAD (Cabral et al., 2002; Molinari et al., 2002). Our finding that CM bodies of the ER localize in ER regions devoid of BiP and PDI (Figure 4) may partly explain one of the mechanisms by which calnexin overexpression attenuated ERAD.

Furthermore, assuming that CM body formation excludes other ER luminal proteins as well, it is predicted that further processing of oligosaccharides in the mutant CFTR should be arrested as long as it is sequestered in the CM bodies. This would prolong “off-time” duration of the CFTR from the reglucosylation-mediated calnexin cycle (Parodi, 2000; Cabral et al., 2001; Schrag et al., 2003).

Figure 6. Concentric membranous body of the ER is connected to the late secretory pathway. (A) BFA treatment caused the accumulation of wt CFTR in CM bodies upon calnexin overexpression. CFTR-BHK cells infected with or without Ad-CNX (MOI 50) were treated with 5 µg/ml BFA for 6 h after 42 h after infection. Before fixation, cells were continuously treated with 1 mM cycloheximide for 3 h upon BFA treatment (A, +CHX). After fixing, cells were immunostained with anti-calnexin antibody and visualized with TRITC-conjugated secondary antibody. (B and C) Transport of wt CFTR from the CM bodies after BFA washout. (B) After BFA treatment for 6 h, cells were incubated with medium containing with 1 mM cycloheximide (BFA washout), fixed, and immunostained with anti-calnexin antibody. (C) After BFA washout, living cells were observed at 20-s intervals, for 33 min, at 37°C. The numbers in the panels show the incubation time periods (seconds) after BFA washout. (D) ΔF508 CFTR was retained in CM bodies. ΔF508-BHK cells infected with Ad-CN (MOI 50) were treated with 1 mM cycloheximide for 2 h before fixation (+CHX). The cells were imaged so that the ER region was in focus, except for one image that focused on the cell surface (B, cell surface). Bar, 10 µm.
and likely delay its mannose trimming to ManB-containing oligosaccharides, which is known to trigger ERAD (Jakob et al., 1998). Recently (during preparing this manuscript), it has been reported that Edem, a putative Man$_\text{p}$-lectin, functions as an acceptor of terminally misfolded glycoproteins released from calnexin (Molinari et al., 2003; Oda et al., 2003). Similar to other ERAD substrates, Edem may lead $\Delta F508$ CFTR to the ERAD pathway after release from CM bodies. Hence, we think that CM bodies may function as a certain type of kinetic trap for $\Delta F508$ CFTR, thus rendering it either more chance to fold in the calnexin complex or making a pause for disposal. As a result, ERAD efficiency seems to be reduced when calnexin is overexpressed. Similarly, this may explain why formation of CM bodies delayed ER exit of wt CFTR (Figure 1D). In either case, release of CFTR from CM bodies could occur at the off-phase of the equilibrium when glucosidase II “fixes” the status by deglucosylating the dissociated monoglucosylated CFTR (Zapun et al., 1997). Alternatively, one could argue that CFTR may be trapped in the CM body in a nonglycan-mediated manner (Ihara et al., 1999; Saito et al., 1999; Danilczyk and Williams, 2001). In this model, misfolded moiety of CFTR should be responsible for the association. Currently, we were unable to exclude the possibility, however, we think it unlikely because 1) CM body is a dynamic structure (Figures 4, 1 and 6) so that once-bound CFTR should be dissociated from the CM body; and 2) no cellular factor has been identified to release the substrate from calnexin other than glucosidase II, despite the extensive studies on the ER quality control (Ellgaard and Helenius, 2003; Schrag et al., 2003). It is interesting why calnexin overexpression formed the CM bodies of the ER (Figure 4). CM bodies of the ER are usually composed of paired membrane arrays (Ghadially, 1996). Sequestered at the center of the concentric profiles of such bodies lie a portion of the cell cytoplasm, often containing organelles and inclusions, typically lipid droplets and mitochondria (Ghadially, 1996). It has been known that normal cells such as cerebellar Purkinje cells and pathologically altered cells have CM bodies in their cytoplasm (Takei et al., 1996; Ghadially, 1996). Moreover, it is thought that formation of CM bodies may represent an adaptive response to nonphysiological conditions such as hypoxia (Takei et al., 1994). CM bodies were also induced by overexpression of inositol 1,4,5-trisphosphate receptor, an ER membrane protein (Takei et al., 1994). Thus, overexpression of an ER membrane protein may induce formation of CM bodies. However, we think that calnexin may be directly involved in formation of CM bodies because overexpression of ER membrane proteins, such as inositol 1,4,5-trisphosphate receptor that interacts with calnexin, induces formation of CM bodies. In contrast, overexpression of other ER membrane proteins, such as microsomal aldehyde dehydrogenase that is not known to interact with calnexin, formed crystalloid ER but not CM bodies (Yamamoto et al., 1996). In terms of the function, it may be reasonable to think that calnexin is directly involved in the formation of CM bodies.

ACKNOWLEDGMENTS

We thank Dr. J.R. Riordan for providing CFTR-CHO cells, $\Delta F508$-CHO cells, CFTR-BHK cells, and $\Delta F508$-BHK cells; Dr. D.C. Gruener for providing human airway cell lines; and Dr. Y. Ohtsuki for valuable discussion. The UHI antibody against Lamp-1 developed by Bruce L. Granger and Selvanayagam

Uthayakumar was obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the National Institute of Child Health and Human Development and maintained by The University of Iowa (Department of Biological Sciences, Iowa City, IA). We also thank K. Ueno for technical assistance. This work was supported by grants from the Ministry of Education, Science, Sport and Culture of Japan.

REFERENCES


Cohen-Doyle, M.F., Saito, Y., and Williams, D.B. (1999). CalnexinScript), it has been reported that Edem, a putative Man$_\text{p}$-lectin, functions as an acceptor of terminally misfolded glycoproteins released from calnexin (Molinari et al., 2003; Oda et al., 2003). Similar to other ERAD substrates, Edem may lead $\Delta F508$ CFTR to the ERAD pathway after release from CM bodies. Hence, we think that CM bodies may function as a certain type of kinetic trap for $\Delta F508$ CFTR, thus rendering it either more chance to fold in the calnexin complex or making a pause for disposal. As a result, ERAD efficiency seems to be reduced when calnexin is overexpressed. Similarly, this may explain why formation of CM bodies delayed ER exit of wt CFTR (Figure 1D). In either case, release of CFTR from CM bodies could occur at the off-phase of the equilibrium when glucosidase II “fixes” the status by deglucosylating the dissociated monoglucosylated CFTR (Zapun et al., 1997). Alternatively, one could argue that CFTR may be trapped in the CM body in a nonglycan-mediated manner (Ihara et al., 1999; Saito et al., 1999; Danilczyk and Williams, 2001). In this model, misfolded moiety of CFTR should be responsible for the association. Currently, we were unable to exclude the possibility, however, we think it unlikely because 1) CM body is a dynamic structure (Figures 4, 1 and 6) so that once-bound CFTR should be dissociated from the CM body; and 2) no cellular factor has been identified to release the substrate from calnexin other than glucosidase II, despite the extensive studies on the ER quality control (Ellgaard and Helenius, 2003; Schrag et al., 2003). It is interesting why calnexin overexpression formed the CM bodies of the ER (Figure 4). CM bodies of the ER are usually composed of paired membrane arrays (Ghadially, 1996). Sequestered at the center of the concentric profiles of such bodies lie a portion of the cell cytoplasm, often containing organelles and inclusions, typically lipid droplets and mitochondria (Ghadially, 1996). It has been known that normal cells such as cerebellar Purkinje cells and pathologically altered cells have CM bodies in their cytoplasm (Takei et al., 1996; Ghadially, 1996). Moreover, it is thought that formation of CM bodies may represent an adaptive response to nonphysiological conditions such as hypoxia (Takei et al., 1994). CM bodies were also induced by overexpression of inositol 1,4,5-trisphosphate receptor, an ER membrane protein (Takei et al., 1994). Thus, overexpression of an ER membrane protein may induce formation of CM bodies. However, we think that calnexin may be directly involved in formation of CM bodies because overexpression of ER membrane proteins, such as inositol 1,4,5-trisphosphate receptor that interacts with calnexin, induces formation of CM bodies. In contrast, overexpression of other ER membrane proteins, such as microsomal aldehyde dehydrogenase that is not known to interact with calnexin, formed crystalloid ER but not CM bodies (Yamamoto et al., 1996). In terms of the function, it may be reasonable to think that calnexin is directly involved in the formation of CM bodies.

ACKNOWLEDGMENTS

We thank Dr. J.R. Riordan for providing CFTR-CHO cells, $\Delta F508$-CHO cells, CFTR-BHK cells, and $\Delta F508$-BHK cells; Dr. D.C. Gruener for providing human airway cell lines; and Dr. Y. Ohtsuki for valuable discussion. The UHI antibody against Lamp-1 developed by Bruce L. Granger and Selvanayagam

Uthayakumar was obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the National Institute of Child Health and Human Development and maintained by The University of Iowa (Department of Biological Sciences, Iowa City, IA). We also thank K. Ueno for technical assistance. This work was supported by grants from the Ministry of Education, Science, Sport and Culture of Japan.


