Calnexin Δ185–520 partially reverses the misprocessing of the ΔF508 cystic fibrosis transmembrane conductance regulator[1]

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Abstract Abnormal retention of ΔF508 CFTR (cystic fibrosis transmembrane conductance regulator) in the endoplasmic reticulum is a major cause of cystic fibrosis (CF). We show that calnexin Δ185–520 but not calnexin can partially reverse the mislocalization of ΔF508 CFTR. This 256-amino acid protein has neither the transmembrane domain nor the P domain of calnexin. Calnexin Δ185–520 interacted with CFTR directly, and was secreted into the extracellular compartment over time. Forty-eight hours after transfection into CHO cells, calnexin Δ185–520 increased the conversion of immature ΔF508 CFTR into mature ΔF508 CFTR. In immortalized human CF cell lines expressing ΔF508 CFTR, a halide efflux assay showed that calnexin Δ185–520 partially restored CFTR function. These data indicate that calnexin Δ185–520 may give a clue to develop the therapeutic way of cystic fibrosis with ΔF508 CFTR.

Key words: Cystic fibrosis; Cystic fibrosis transmembrane conductance regulator; Calnexin; Endoplasmic reticulum

1. Introduction

Cystic fibrosis transmembrane conductance regulator (CFTR) is a plasma membrane Cl^- channel regulated by cAMP-dependent phosphorylation and by intracellular ATP [1–4]. 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) [5–7]. 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 approximately 70% of CF chromosomes and results in a severe form of the disease; more than 90% of CF patients have at least one ΔF508 allele [8,9].

Clues as to how this mutation compromises CFTR function were first provided by Cheng et al. following studies of ΔF508 CFTR biosynthesis in transfected COS cells [10]. The failure of *N*-linked oligosaccharides processing beyond the immature high-mannose form suggested that this mutant was trapped within the endoplasmic reticulum (ER). Understanding the basis of this intracellular retention would be of great value, since ΔF508 CFTR is functionally competent in model situations where it is able to reach the plasma membrane. Functional Cl^- channel activity is found in the plasma membrane when mammalian cells expressing ΔF508 CFTR are grown at reduced temperatures [11] or when this mutant is expressed in amphibian and insect cells [2] or is purified and reconstituted in planar lipid bilayers [12]. Although some differences remain in the channel characteristics reported among the various groups, the primary effect of the ΔF508 mutation in mammalian cells is not functional impairment, but rather mislocalization of the protein product.

Overcoming the biosynthetic arrest of ΔF508 CFTR and promoting the trafficking of this mutant to the plasma membrane require knowledge of the retention mechanism. The ER quality control machinery participates in the ER localization of ΔF508 CFTR [13–16]. This machinery involves a variety of mechanisms to ensure that only correctly folded and assembled proteins are transported to the late secretory pathway, while non-native proteins are retained in the ER and eventually targeted for degradation [17,18].

Intracellular retention of mutant proteins has often been linked to prolonged interactions with molecular chaperones in the ER [17]. Chaperones associate transiently with many nascent proteins and are thought to promote productive folding by preventing the aggregation of folding intermediates. However, proteins that are unable to fold correctly or assemble into complete oligomeric complexes often exhibit prolonged association with chaperones, a property that may contribute to their retention in the ER. Calnexin (CNX), a lectin-like transmembrane protein located in the ER, has been shown to associate transiently with a large number of newly

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[1] The data on hamster calnexin Δ185–520, have been submitted to GenBank under accession number AF380341, and those on hamster calnexin under accession number AB071869.

Abbreviations: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; CNX, calnexin; ER, endoplasmic reticulum; GFP, green fluorescence protein

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synthesized proteins, including both transmembrane and secretory proteins [18,19]. In addition, calnexin displays a prolonged binding to certain misfolded proteins and to incompletely assembled forms of oligomeric proteins [18,19]. It was reported that calnexin associates specifically with newly synthesized CFTR and ΔF508 CFTR and that ΔF508 CFTR retains the association with calnexin throughout its life, whereas wild-type CFTR escapes the association and exits the ER [14]. The binding of calnexin to nascent proteins in vivo generally requires the formation of monoglucose oligosaccharides [19]. However, non-sugar-mediated interactions such as polypeptide-based interactions have also been reported [20,21]. Here we show that calnexin Δ185–520, which lacks calnexin's transmembrane domain and P domain, partially restores the function of ΔF508 CFTR. calnexin Δ185–520 interacted with CFTR directly, and was secreted into the extracellular compartment over time. Forty-eight hours after transfection into CHO cells, calnexin Δ185–520 increased the conversion of immature ΔF508 CFTR into mature ΔF508 CFTR. In immortalized human CF cell lines expressing ΔF508 CFTR, a halide efflux assay showed that calnexin Δ185–520 partially restored CFTR function.

2. Materials and methods

2.1. cDNA cloning

Total RNA from hamster trachea was isolated with Isogen (Nippon Gene) according to the manufacturer's protocol. Total RNA was reverse transcribed (RT) to cDNA at 42°C for 30 min. The cDNA was denatured for 3 min at 95°C, followed by 40 cycles of amplification by polymerase chain reaction (PCR). Each cycle consisted of denaturation at 95°C for 30 s, annealing at 62°C for 30 s, and a primer extension at 72°C for 1.5 min. The RT-PCR product was denatured for 8 min at 95°C followed by 40 cycles of amplification by nested PCR. Each cycle consisted of denaturation at 95°C for 1 min, annealing at 62°C for 1 min, and primer extension at 72°C for 2 min. The RT-PCR and nested PCR primer sequences used were as follows: RT primer and hamster calnexin forward primer: 5′-GGTCCTCTCCTAGGACCACTCTTGC-3′; hamster calnexin reverse primer: 5′-GTCCCCGGGCAGGCTAGAGATCATGGA-3′; nested forward primer: 5′-CTCTCTTCGTGGCTTTCTGTT-3′; nested reverse primer: 5′-ATGGAAGGGAAGTGGTTGCTGTGT-ATG-3′. The nested PCR product was cloned into a TA cloning vector and the clones were sequenced.

2.2. Cell culture

16HBE14o$^-$ and CFBE41o$^-$ cells were grown in Eagle's minimum essential medium (MEM) with 10% fetal bovine serum and antibiotics. The cells were grown on 35-mm culture dishes coated with human fibronectin. CFTR-CHO cells, ΔF508-CHO cells, and BHK cells stably expressing a CFTR-green fluorescent protein (GFP) fusion have been described previously [22]. All cells were incubated at 37°C in an atmosphere of 5% CO_2–95% air.

2.3. Anti-calnexin Δ185–520 antibody production

Female BALB/c mice were immunized with the peptide QFHDKTPQPDVKEE. After the final immunization, the spleen was removed and fused with P3U1 myeloma cells. Antibody-positive hybridomas were subcloned using limiting dilution. The antibody was purified from the ascites of pristane-treated BALB/c mice.

2.4. Preparation of recombinant adenoviruses

Calnexin Δ185–520 and calnexin adenoviruses (both based on adenovirus 5) were produced by homologous recombination in 293 cells using a TaKaRa Adenovirus Expression Vector Kit (TaKaRa).

2.5. GFP fusion proteins

To construct GFP-CNX, GFP-calnexin Δ185–520, and GFP-calnexin Δ185–520 ΔS (a deletion mutant of the N-terminal 20 amino acid residues), cDNA (human calnexin, calnexin Δ185–520, and calnexin Δ185–520 ΔS cDNA in the TA cloning vector) was amplified by PCR. Each cDNA was denatured for 3 min at 95°C, followed by 40 cycles of amplification. Each cycle consisted of denaturation at 94°C for 1 min, annealing at 64°C for 1 min, and primer extension at 72°C for 1.5 min. PCR products were digested with *Eco*RI and *Bam*HI and ligated into the pEGFP vector (Clontech). The PCR primer sequences used were as follows: pEGFP-CNX and pEGFP-calnexin Δ185–520 forward primer: 5′-CAGAATTCGGCTTATGG-AAGGGAAGTGGTTGC-3′; pEGFP-calnexin Δ185–520 ΔS forward primer: 5′-CAAATGGGCGGTAGGCGTGTACGG-3′; pEGFP-CNX, pEGFP-calnexin Δ185–520 and pEGFP-calnexin Δ185–520 ΔS reverse primer: 5′-CGGGATCCCACTCTCTTCGTGGCTTT-CTGT-3′. Transfectam (Promega) was used to transfect 2.5 μg of pEGFP, pEGFP-CNX, pEGFP-calnexin Δ185–520, or pEGFP-calnexin Δ185–520 ΔS into ΔF508-CHO cells. Transfected cells were then incubated for 48 h in growth medium. Images were collected and analyzed by a Fluoview FV300 confocal laser scanning microscope (Olympus).

2.6. Immunofluorescence assay

Cells were grown on glass-bottomed culture dishes, fixed in 3.7% paraformaldehyde for 20 min at room temperature, and permeabilized with 0.5% Triton X for 20 min at room temperature. Fixed cells were subsequently blocked for 30 min at room temperature with phosphate-buffered saline (PBS) containing 1.5% bovine serum albumin and incubated with anti-calnexin, anti-calreticulin (stressgen), and anti-calnexin Δ185–520 antibodies (1:100 dilution) for 1 h at room temperature. Cells were washed three times with PBS and then stained with fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (1:100 dilution, Jackson ImmunoResearch Laboratories) for 45 min at room temperature. Cells were washed three times with PBS and mounted with Vectashield® mounted medium (Vector Laboratories). Cells were observed and analyzed with a Fluoview FV300 confocal laser scanning microscope (Olympus).

2.7. Immunoprecipitation and Western blotting

CFTR-CHO cells and ΔF508-CHO cells were infected with calnexin Δ185–520, calnexin, or LacZ expression adenovirus (multiplicity of infection (MOI): 50). Forty-eight hours after infection, all cells were solubilized with RIPA buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mg/ml sodium deoxycholate and 1% NP-40) containing 1 mM phenylmethylsulfonyl fluoride (PMSF). An anti-CFTR (clone: 24-1; Genzyme/Techne) antibody was immobilized on protein G Sepharose beads (Pharmacia Biotech) and incubated with ΔF508-CHO cell lysates for 4 h at 4°C. Immune complexes were washed four times with RIPA buffer and eluted with 2×concentrated sodium dodecyl sulfate (SDS) loading buffer. Immunoprecipitated proteins were analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane (Millipore), and probed with the antibodies specified in the figure legends before chemiluminescent detection (ECL Western Blotting Detection Reagents, Amersham Pharmacia Biotech).

2.8. Metabolic labeling with [^{35}S]methionine

Pulse-chase analysis and immunoprecipitation were carried out as described previously [23]. CFTR-CHO and ΔF508-CHO cells were seeded on 60-mm dishes and grown to 70% confluence before being infected. Forty-eight hours after infection, cells were pre-incubated for 30 min in methionine- and cysteine-free αMEM and then labeled for 20 min with 100 μCi/ml of [^{35}S]methionine and [^{35}S]cysteine (>1000 Ci/mmol, Amersham). For chasing, cells were washed three times with αMEM and incubated with complete αMEM supplemented with 7% serum and 0.75 mM methionine for the time periods. The cells were washed with ice-cold PBS and lysed overnight at 4°C in 800 μl RIPA buffer containing 1 mM PMSF. Samples were centrifuged at 15 000×g for 10 min at 4°C, and the supernatant was incubated for 4 h at 4°C with anti-CFTR antibody. Immunoprecipitated proteins were analyzed by 6% SDS–PAGE, and visualized by autoradiography (BAS-1800II, Fujifilm). The radioactivity associated with CFTR was quantified by Image Gauge Ver. 3,4 software (Fujifilm).

2.9. ^{125}I efflux experiments

^{125}I efflux experiments were performed on cells grown on fibronectin-coated six-well plates. Confluent cells were rinsed four times with 1 ml of efflux buffer (140 mM NaCl, 3.3 mM KH_2PO_4, 0.83 mM

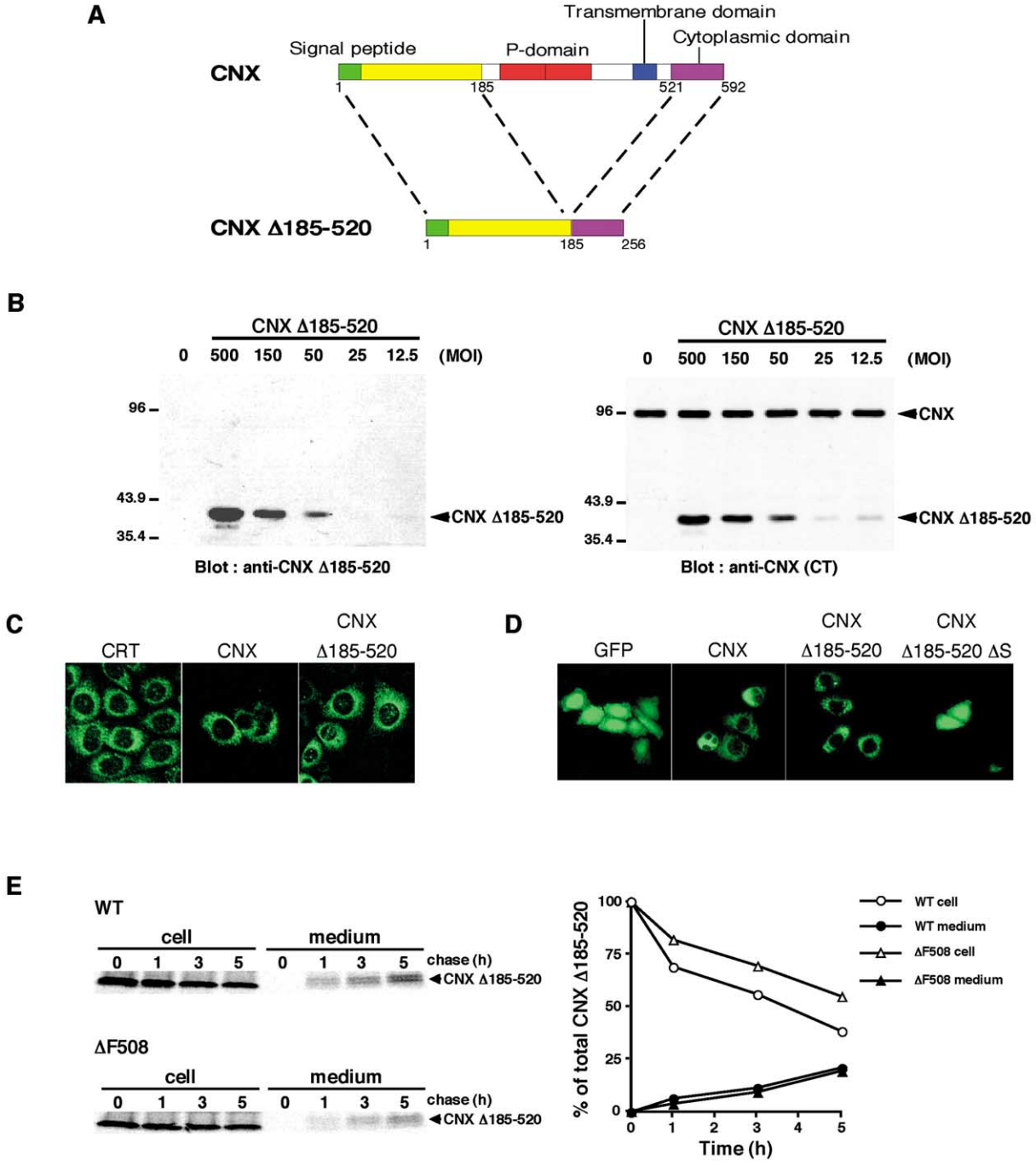

Fig. 1. Characterization of calnexin Δ185–520. A: Expression of calnexin Δ185–520 and calnexin after transfection of the calnexin Δ185–520 cDNA using an adenovirus vector. B: Forty-eight hours after infection, all cells were solubilized with RIPA buffer. Whole cell lysates (10 μg) were subjected to SDS–PAGE on a 7.5% polyacrylamide gel, transferred to PVDF membrane, and blotted with anti-calnexin Δ185–520 (left) or anti-calnexin (right) antibody. The expression of calnexin Δ185–520 by adenovirus was MOI-dependent. Calnexin Δ185–520 was specifically detected by an anti-calnexin Δ185–520 antibody. An anti-calnexin antibody also detected calnexin Δ185–520. The calnexin Δ185–520 size is approximately 38 kDa. C: ER localization of transfected calnexin Δ185–520, endogenous calnexin, and endogenous calreticulin (CRT). All panels are fluorescence micrographs of ΔF508-CHO cells fixed in 4% paraformaldehyde and permeabilized with 0.5% Triton X. Cells were labeled with anti-calnexin, anti-calreticulin, or anti-calnexin Δ185–520 antibodies, and visualized with a FITC-labeled secondary antibody. D: Expression of GFP-fused proteins. ΔF508-CHO cells were transfected with 2.5 μg of pEGFP, pEGFP-CNX, pEGFP-calnexin Δ185–520, and pEGFP-calnexin Δ185–520 ΔS. Transfected cells were then incubated for 48 h in growth medium. E: Kinetics of newly synthesized calnexin Δ185–520. Pulse-chase experiments with [^{35}S]methionine were performed, and calnexin Δ185–520 was immunoprecipitated after transfection of calnexin Δ185–520 adenovirus vector into CFTR-CHO (WT, wild-type) and ΔF508-CHO cells (ΔF508). We determined the content of labeled calnexin Δ185–520 in the cells and in the culture medium. Quantification data (right) are shown as percent of total calnexin Δ185–520. The newly synthesized calnexin Δ185–520 in the cells at 0 h was regarded as the total calnexin Δ185–520 content (100%).

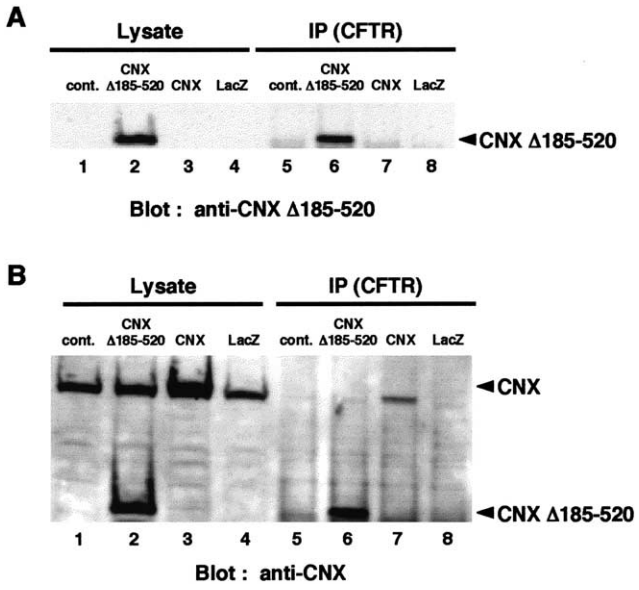

Fig. 2. The interaction of calnexin Δ185–520 with CFTR. ΔF508-CHO cells were infected with or without (control; lanes 1, 5) calnexin Δ185–520 (lanes 2, 6), calnexin (lanes 3, 7), or LacZ (lanes 4, 8) expression adenovirus (MOI: 50). Forty-eight hours after infection, all cells were solubilized with RIPA buffer. Anti-CFTR (C-terminus) antibody was immobilized on protein G Sepharose beads and mixed with ΔF508-CHO cell lysates. Bound proteins were recovered by brief centrifugation, resolved on SDS–PAGE, transferred to PVDF membranes. and blotted with anti-calnexin Δ185–520 (A) or anti-calnexin antibodies (B).

K_2HPO_4, 1 mM $CaSO_4$, 1 mM $MgSO_4$, 10 mM HEPES, 10 mM glucose, pH 7.4). After washing, 1 ml efflux buffer with 20 μCi/ml of ^{125}I (Amersham) was added to each well and incubated for 1 h at 37°C. After 1 h, cells were rinsed three times with 1 ml of efflux buffer, and 1 ml of efflux buffer was again added into each well. Samples were removed at 1.5-min intervals and replaced with fresh buffer. This process was repeated 15–26 times with cAMP treatment cocktail (500 μM CPT-cAMP, 1 mM isobutylmethylxanthine, 10 μM forskolin), beginning after the fourth efflux period. After the last efflux interval, cells were lysed with 0.1 M HNO_3 (1 ml) for 2 h at room temperature. All samples were counted with an autowell gamma counter (ARC-1000M, Aloka). Total counts equaled the sum of counts from lysed cells and from all efflux samples. Efflux is shown as the percent of the total count remaining at the end of each efflux period. The percentage efflux was calculated as follows: % efflux = (count secreted)/(total count remaining in the cells each minute) × 100.

3. Results

3.1. Characterization of calnexin Δ185–520

First of all, bands of several sizes were detected on Western blots using an anti-calnexin antibody against hamster tissues (data not shown). Since this suggested that some isoforms of calnexin may exist, we performed a RT-PCR using hamster trachea mRNA. We cloned the cDNA of a truncated calnexin isoform (calnexin Δ185–520, GenBank accession number AF380341). Calnexin Δ185–520 was 100% homologous to the N- and C-termini of hamster calnexin (GenBank accession number AB071869) (Fig. 1A), and was 99% homologous to the the N- and C-termini of human calnexin. We found that the molecular weight of calnexin Δ185–520 expressed by recombinant adenovirus was approximately 38 kDa (Fig. 1A). Since the C-terminus of calnexin Δ185–520 has an acidic domain with a highly negative charge (like calnexin), the apparent molecular weight on SDS–PAGE is different from the calculated molecular weight (28.7 kDa). An immunocytochemical study indicated that the transfected calnexin Δ185–520 was localized in the ER, since calnexin Δ185–520 was colocalized with ER chaperones such as calnexin and calreticulin (Fig. 1B). Calnexin Δ185–520 includes the N-terminal ER localization signal sequence of calnexin (residues 1–20) [24]. Since calnexin Δ185–520 without the ER localization signal sequence was localized in the cytosol (Fig. 1C), the ER localization of calnexin Δ185–520 was due to the N-terminal ER-localizing signal sequence. The C-terminal ER retention signal (RKPRRE) of calnexin Δ185–520 might not be functional in the ER, since the ER retention signal of calnexin functions in the cytosol. Therefore, we speculated that some calnexin Δ185–520 may be secreted into the culture medium. To examine this possibility, pulse-chase experiments were performed in ΔF508-CHO and CFTR-CHO cells (Fig. 1D). In both cells, calnexin Δ185–520 progressively increased in the medium as it decreased in the cells, suggesting that calnexin Δ185–520 was secreted into the medium. The secreted calnexin Δ185–520 produced a doublet that was slightly larger than the cellular form. The identity and site of the modification were unclear.

3.2. Calnexin Δ185–520 can interact with CFTR

We determined whether calnexin Δ185–520, which lacks the P domain, could interact with CFTR. As shown in Fig. 2A, calnexin Δ185–520 expressed by a recombinant adenovirus vector was coprecipitated with ΔF508 CFTR in ΔF508-CHO cells. This interaction seemed to be specific since calnexin Δ185–520 was not coprecipitated by protein G Sepharose beads (data not shown). Although the efficiency of the interaction of calnexin Δ185–520 with ΔF508 CFTR seems to be stronger than that of calnexin (lanes 2 and 6 in Fig. 2B), it is difficult to conclude whether the binding is actually stronger because the nature of the association remains unclear.

3.3. Effect of calnexin Δ185–520 on the maturation of ΔF508 CFTR

To examine whether calnexin Δ185–520 affects the maturation of ΔF508 CFTR, we transfected calnexin Δ185–520 cDNA into ΔF508-CHO and CFTR-CHO cells with an adenovirus vector, and performed pulse-chase experiments (Fig. 3A). Newly synthesized CFTR has two oligosaccharide chains and migrates on SDS–polyacrylamide gels as a series of bands between 130 and 150 kDa in size (immature CFTR). It is converted to a mature form larger than 175 kDa in the Golgi apparatus [10]. In CFTR-CHO cells, mature CFTR was expressed at 3 h. In contrast, ΔF508 CFTR did not mature, and may have been mostly degraded by the ubiquitin–proteasome pathway [25,26]. However, in ΔF508-CHO cells transfected with calnexin Δ185–520, a 175-kDa band that seemed to be the mature form of ΔF508 CFTR appeared at a detectable level after a 3-h chase. Thus, overexpression of calnexin Δ185–520 might stimulate the maturation of ΔF508 CFTR, while maturation of wild-type CFTR was not inhibited by overexpression of calnexin Δ185–520 (Fig. 3A). To determine whether the mature form of ΔF508 CFTR induced by calnexin Δ185–520 was localized at the cell surface, the localization of ΔF508 CFTR tagged with GFP was examined. As shown in Fig. 3B, calnexin Δ185–520 partially alleviated the ER retention (perinuclear fluorescence) of GFP-fused ΔF508 CFTR, as

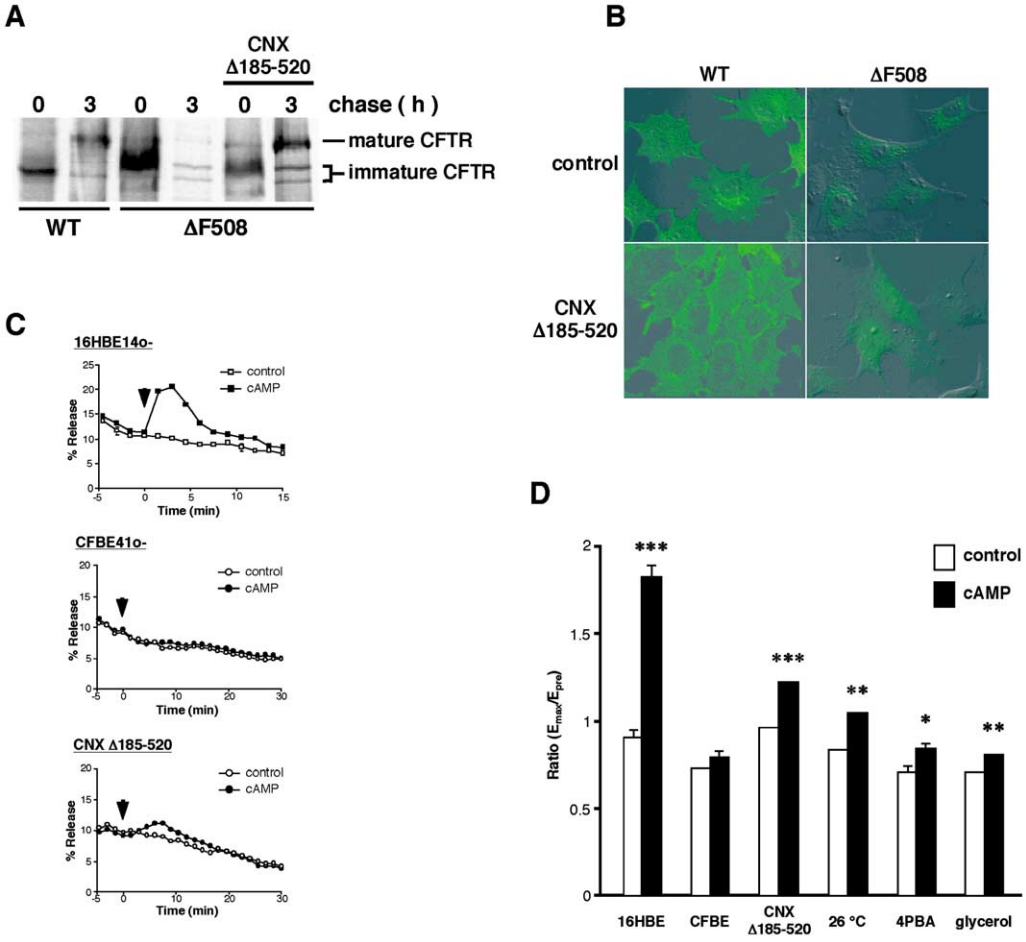

Fig. 3. The influence of calnexin Δ185–520 on the maturation of ΔF508 CFTR. A: CFTR-CHO and ΔF508-CHO cells were infected with or without adenovirus expressing calnexin Δ185–520 (MOI: 50). Cells were pre-incubated in methionine-free medium for 30 min, pulse-labeled with [^{35}S]methionine (100 μCi/ml) for 20 min, and then chased with methionine-containing medium for the indicated times. Cells were then solubilized with RIPA buffer and immunoprecipitated with an antibody against the CFTR C-terminus. Immunoprecipitates were analyzed by 6% SDS–PAGE and autoradiography. B: Cell surface and intracellular localization of wild-type and ΔF508 CFTR fused to GFP at the C-termini in BHK cells. A FITC filter was employed for fluorescence microscopy. GFP fused to wild-type CFTR (WT) is observed over the entire cell surface and also in a perinuclear distribution (seen as increased intensity below the uniform surface veil). ΔF508 CFTR fused to GFP (ΔF508) forms only perinuclear images. After calnexin Δ185–520 transfection, ΔF508 CFTR fused to GFP is observed over the cell surface. C: Calnexin Δ185–520 influences on halide efflux in human CF cells. ^{125}I efflux experiments were performed on 16HBE14o$^-$ and CFBE41o$^-$ cells infected with calnexin Δ185–520 expression adenovirus (MOI: 50). Confluent cells were loaded with ^{125}I (20 μCi/ml) for 1 h at 37°C. Samples were removed at 1.5-min intervals and their radioactivity was counted. The percentage efflux was calculated as follows: % efflux = (count secreted)/(total count remaining in the cells each minute) × 100. D: Effects on halide efflux in human CF cells. ^{125}I efflux experiments were performed on CFBE41o$^-$ cells infected with calnexin Δ185–520 expression adenovirus (MOI: 50), or incubated at 26°C for 72 h, or treated with 1 mM 4-PBA or 0.5 M glycerol for 48 h. The ratio was calculated as follows: ratio (E_{max}/E_{pre}) = (max % release after cAMP stimulation)/(% release at time 0) or (max % release after time 0)/(% release at time 0). Data are expressed as mean values ± S.E.M. Statistical differences are shown as $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ against control.

indicated by surface fluorescence (detectable as increased intensity at the perimeter, and a lighter tent-like veil over the entire surface). However, the surface fluorescence was slightly less intense than that of cells expressing the GFP-fused wild-type CFTR. The overexpression of calnexin Δ185–520 did not affect the localization of wild-type CFTR (Fig. 3B). Calnexin Δ185–520 also did not affect the localization of other ER membrane proteins such as the IP_3 receptor (data not shown).

3.4. Calnexin Δ185–520 partially restores the function of ΔF508 CFTR in human CF cells

Finally, we measured cAMP-activated halide efflux as a functional marker of CFTR using an immortalized human CF airway epithelial cell line (CFBE41o$^-$) with the ΔF508 mutation [27]. As illustrated in Fig. 3C, stimulation of normal human airway epithelial cells (16HBE14o$^-$ [28]) with a cAMP cocktail (10 μM forskolin, 1 mM isobutylmethylxanthine, 500 μM CPT-cAMP) resulted in a significant increase in efflux over the baseline. In contrast, cAMP stimulation did not increase the halide efflux in CFBE41o$^-$ cells. As expected, transfection of calnexin Δ185–520 significantly increased the cAMP-dependent halide efflux in CFBE41o$^-$ cells. Previous studies reported that lowering the growth temperature to 26°C [11], treatment with 1 mM sodium 4-phenylbutyrate (4-PBA) [29], and treatment with 0.5 M glycerol [30,31] all improved the processing of ΔF508 CFTR. In our study, the effect of calnexin Δ185–520 was comparable to these positive controls (Fig. 3C). Its effectiveness was found to be similar in ΔF508-

CHO cells (data not shown). Therefore, calnexin Δ185–520 can partially restore the function of ΔF508 CFTR in human CF cells.

4. Discussion

Our results indicate that calnexin Δ185–520 partially reverses the mislocalization and restores the function of ΔF508 CFTR. Based on the present and other findings, we have speculated on the mechanism of action of calnexin Δ185–520. The recent three-dimensional structural analysis reveals two unique domain structures of calnexin, a globular domain (lectin domain) and a long extended arm (P domain), supporting a dual role in protein folding [32]. Calnexin Δ185–520 seems not to possess the complete lectin domain and P domain, suggesting that calnexin Δ185–520 may not affect the folding of the normal protein through the ER machinery. Supporting this, overexpression of calnexin Δ185–520 did not affect the maturation of wild-type CFTR or the localization of the IP_3 receptor (data not shown). However, calnexin Δ185–520 directly interacted with ΔF508 CFTR without affecting the interaction between ΔF508 CFTR and calnexin (lanes 5 and 6 in Fig. 2B). Although it is conceivable that the observed coprecipitation of calnexin Δ185–520 with ΔF508 CFTR is responsible for the effects, the exact mechanisms remain unclear. After calnexin Δ185–520 binds to an unstable folding intermediate of ΔF508 CFTR, both might then be transported together to the late secretory pathway. We will need to perform further experiments to determine whether this actually happens.

It remains unclear whether calnexin Δ185–520 is a unique gene or a splicing variant of calnexin. Recently, the human calnexin gene sequence has become available (NCBI accession number NT_006932), indicating that the amino acid residues of calnexin at 185 and 520 resided in the middle of exon 7 and exon 13, respectively. Namely, we could not find the splicing sites that can produce calnexin Δ185–520 in human calnexin gene. However, we cannot exclude the possibility that calnexin Δ185–520 is produced by an unknown splicing mechanism.

In conclusion, calnexin Δ185–520 partially restored the maturation and function of ΔF508 CFTR, although not to wild-type levels. This provides a basis for specific rational approaches to develop a therapy based on the calnexin Δ185–520 sequence. Because the size of calnexin Δ185–520 cDNA (771 bp) is easier to handle than that of CFTR cDNA (6.5 kb), calnexin Δ185–520 may give us a clue for a good gene therapy candidate not only for CF (ΔF508 mutation), but also for other genetic diseases with protein trafficking defects in the ER, such as familial hypercholesterolemia (class 2 mutations) [33], emphysema with the Z mutation of the α1-antitrypsin gene [34], Tay–Sachs disease (one rare mutation) [35], and congenital sucrase-isomaltase deficiency (some mutations) [36].

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