Calreticulin Negatively Regulates the Cell Surface Expression of Cystic Fibrosis Transmembrane Conductance Regulator

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Cystic fibrosis transmembrane conductance regulator (CFTR) is a cAMP-dependent Cl− channel at the plasma membrane, and its malfunction results in cystic fibrosis, the most common lethal genetic disease in Caucasians. Quality control of CFTR is strictly regulated by several molecular chaperones. Here we show that calreticulin (CRT), which is a lectin-like chaperone in the endoplasmic reticulum (ER), negatively regulates the cell surface CFTR. RNA interference-based CRT knockdown induced the increase of CFTR expression. Consistently, this effect was observed in vivo. CRT heterozygous (CRT+/−) mice had a higher endogenous expression of CFTR than the wild-type mice. Moreover, CRT overexpression induced cell surface expression of CRT, and it significantly decreased the cell surface expression and function of CFTR. CRT overexpression destabilized the cell surface CFTR by enhancing endocytosis, leading to proteasomal degradation. Deletion of the carboxyl domain of CRT, which results in its ER export, increased the negative effect and enhanced the interaction with CFTR. Thus, CRT in the post-ER compartments may act as a negative regulator of the cell surface CFTR.

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EXPERIMENTAL PROCEDURES

Materials—The following antibodies were used in this study: mouse monoclonal anti-CFTR (C terminus-specific) (clone 24-1, Genzyme/Technne, Cambridge, MA), anti-rabbit anti-CFTR (H182) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-rabbit anti-calnexin (anti-CNX, C terminus-specific; SPA-860, Stressgen Biotechnologies, Inc, San Diego, CA), rat monoclonal anti-Hsc70 (SPA-815, Stressgen), rabbit anti-calreticulin (anti-CRT; SPA-600, Stressgen), mouse monoclonal anti-Myc (Santa Cruz Biotechnology, Santa Cruz, CA), and rabbit anti-Myc (Upstate, Charlottesville, VA) antibodies. MG-132 was purchased from...
Calbiochem, and digitonin, lactacystin, brefeldin A, bafilomycin A1, forskolin, chlorophenylthio-cAMP, and cycloheximide were from Sigma.

**DNA Constructs and Recombinant Adenovirus—**Several CRT mutants tagged with c-Myc in the C terminus were constructed in pCMV-Tag5 vector (Stratagene, La Jolla, CA) with standard methods and verified by sequencing. In the case of full-length CRT, c-Myc tag was fused before the KDEL ER retrieval signal (amino acid residues 1–417) was synthesized by PCR-driven reaction using CRTΔKDEL as template and the following oligodeoxynucleotides with Xhol (primer 5′-Xhol-Myc+KDEL+S and 3′-Xhol-Myc+KDEL+S) restriction sites: 5′-Xhol-Myc+KDEL+S, 5′-TCGAGCAGAAAATCTCTCTGAAAGAGGATCAGGAAGCAGCA-GCTTGAGC-3′ and 3′-Xhol-Myc+KDEL+S, 5′-TCGAGCCTACAGCT-GTCTTTCAAGATCCTTCTACAGAAGATGTTTCTCG-3′. cDNA encoding the deletion of the KDEL sequence of the protein (amino acid residues 1–417) was synthesized by PCR-driven reaction using the following CRTΔ5′- HindIII and CRT1239-Xhol primers with 5′-flanking HindIII and Xhol, respectively; CRTΔ5′-HindIII, 5′-CCCAAGCCTATGCTGCTATTCCGTCG-3′, CRT1239-Xhol, 5′-CTACCGCTCTCGAGGCCCTGG-3′. cDNA encoding the deletion of the KDEL sequence and the C-domain of the protein (amino acid residues 1–307) was synthesized by PCR-driven reaction using the following CRTΔ5′-HindIII and CRT921-Xhol primers with 5′-flanking HindIII and Xhol, respectively; CRT921-Xhol, 5′-CGCCAAAGTTTCCGAGGGCATAGATACT-3′. Recombinant human CRT (Ad-CRT), calnexin (Ad-CNX), or LacZ (Ad-LacZ) based on adenovirus 5 (Ad5) was produced as described previously (16). Vesicular stomatitis virus (VSV)-G tsO45-CFP was driven reaction using CRTΔKDEL as template and the following oligodeoxynucleotides with Xhol (primer 5′-Xhol-Myc+KDEL+S and 3′-Xhol-Myc+KDEL+S) restriction sites: 5′-Xhol-Myc+KDEL+S, 5′-TCGAGCAGAAAATCTCTCTGAAAGAGGATCAGGAAGCAGCA-GCTTGAGC-3′ and 3′-Xhol-Myc+KDEL+S, 5′-TCGAGCCTACAGCT-GTCTTTCAAGATCCTTCTACAGAAGATGTTTCTCG-3′. cDNA encoding the deletion of the KDEL sequence of the protein (amino acid residues 1–417) was synthesized by PCR-driven reaction using the following CRTΔ5′-HindIII and CRT1239-Xhol primers with 5′-flanking HindIII and Xhol, respectively; CRTΔ5′-HindIII, 5′-CCCAAGCCTATGCTGCTATTCCGTCG-3′, CRT1239-Xhol, 5′-CTACCGCTCTCGAGGCCCTGG-3′. cDNA encoding the deletion of the KDEL sequence and the C-domain of the protein (amino acid residues 1–307) was synthesized by PCR-driven reaction using the following CRTΔ5′-HindIII and CRT921-Xhol primers with 5′-flanking HindIII and Xhol, respectively; CRT921-Xhol, 5′-CGCCAAAGTTTCCGAGGGCATAGATACT-3′. Recombinant human CRT (Ad-CRT), calnexin (Ad-CNX), or LacZ (Ad-LacZ) based on adenovirus 5 (Ad5) was produced as described previously (16). Vesicular stomatitis virus (VSV)-G tsO45-CFP was constructed as described previously (27).

**Cell Lines and Transfection—**CFTR-BHK, DF508-BHK, CFTR-CHO, DF508-CHO, and 16HBE14o− cells have been described (16, 28). Transfection and adenovirus infection were performed as described previously (16). Cells were incubated with 1 mM forskolin, chlorophenylthio-cAMP, and cycloheximide were from Sigma. Cells were incubated with 1 mM (CFTR- and Transfection and adenovirus infection were performed as described previously (16). Cells were incubated with 1 mM (CFTR- and Transfection and adenovirus infection were performed as described previously (16). Cells were incubated with 1 mM (CFTR- and Transfection and adenovirus infection were performed as described previously (16). Cells were incubated with 1 mM (CFTR- and Transfection and adenovirus infection were performed as described previously (16).}

**siRNA Preparation and Transfection—**CRT-siRNA was designed as described previously (29). We used the 21-nucleotide sense strand (CAUGCCAGAAACACTGCAGAGACUCGdTdT) and the 21-nucleotide antisense strand (AGUCGCUAGGUAUGUCGACUdTdT) of CRT mRNA (GenBank™ accession number M84739). siRNA duplex was prepared as described previously (29). Transient transfection with siRNA was performed using TransIT-TKO (Mirus, Madison, WI) following the protocol recommended by the manufacturer.

**Western Blotting—**Western blotting experiments were performed as described previously (16).

**Detection of Endogenous CFTR Protein in CRT Heterozygous (CRTΔ+/−) Mice—**CRTΔ+/− mice were obtained from M. Michalak (30). After rapid excision, kidneys from a CRTΔ+/− mouse were homogenated in homogenate buffer (140 mM NaCl, 5 mM EDTA (pH 7.4)) containing 1% protease inhibitor mixture and centrifuged at 400 × g for 2 min. The supernatant was suspended in resuspension buffer (12 mM Tris-HCl (pH 7.4), 0.3 M mannitol, 10 mM KCl, 0.5 mM EDTA) containing 1% protease inhibitor mixture and centrifuged at 4,000 × g for 10 min. Furthermore, supernatant was ultracentrifuged at 40,000 × g for 60 min. The pellet (microsome fraction) was immediately solubilized in Laemmli sample buffer (60 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 0.1 M dithiothreitol, 0.1% bromphenol blue), incubated for 30 min at room temperature, and centrifuged at 8,000 × g for 2 min. The supernatant was analyzed by Western blot as described above.

**Cell Surface Biotinylation—**CFTR-CHO cells were biotinylated in 1 mg/ml EZ-Link™ sulfo-NHS-SS biotin (Pierce) at 4 °C for 30 min and solubilized in radioimmune precipitation assay buffer (lysate) after washing three times with PBS. Biotinylated proteins were isolated by incubation with ImmunoPure immobilized streptavidin (Pierce) at 4 °C for 4 h. After washing four times with radioimmune precipitation assay buffer, biotinylated proteins were eluted with 2× SDS sample buffer (cell surface). Cell lysates (40 μg) and biotinylated proteins were analyzed by Western blotting.

**Endocytic Assay—**Cell surface CFTR was biotinylated at 4 °C for 30 min as described above. Internalization of biotinylated CFTR was initiated by adding prewarmed (37 °C) culture medium and then transferring the dishes to 37 °C followed, after the chase period (3–30 min), by arrest of internalization with ice-cold PBS. Biotin molecules remaining at the cell surface were stripped by reducing their disulfide bonds with the reducing agent 2-mercaptoethanesulfonic acid (MESNA). Cells were treated with stripping buffer (50 mM MESNA, 100 mM NaCl, 50 mM Tris/HCl (pH 8.6), 1 mM MgCl₂, 0.1 mM CaCl₂) for 45 min (three times for 15 min) at 4 °C. Biotinylated CFTR was isolated as described above and analyzed by Western blotting.
CRT Negatively Regulates Cell Surface Expression of CFTR

Pulse-Chase Analysis and Immunoprecipitation—Pulse-chase analysis and immunoprecipitation were carried out as described previously (16). To detect weak interaction, cells were lysed in 0.5% digitonin buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% digitonin) containing 1% protease inhibitor mixture, and the cell lysates were used for immunoprecipitation. The immunoprecipitants were washed with 0.1% digitonin buffer, (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% digitonin) containing 1% protease inhibitor mixture, and the cell lysates were used for immunoprecipitation. The cross-linking reaction was terminated by the addition of 1 M Tris-HCl (pH 7.4) to a final concentration of 50 mM and by incubation on ice for 5 min. After washing, cells were lysed in radioimmune precipitation assay buffer, and the cell lysates were subjected to immunoprecipitation as described above. The cross-linked proteins were dissociated by SDS-sample buffer containing a reducing agent prior to loading on SDS-PAGE.

Immunocytochemistry—Immunocytochemical analyses were performed as described previously (16).

125I Efflux Experiments—125I efflux experiments were performed as described previously (31). The percentage efflux was calculated as follows: % efflux (E) = (count secreted)/(total count remaining in the cells each minute) × 100. Data are expressed as mean values ± S.D. (n = 3). Data were evaluated for statistical differences by analysis of t test with Microsoft Excel.

RESULTS

CRT Knockdown Increases CFTR Expression—To investigate the role of CRT in the quality control of CFTR, we examined the effect of CRT knockdown on the expression of CFTR. Western blotting analysis showed that siRNA for CRT (CRT-siRNA) specifically decreased endogenous CRT expression by ~60% reduction but did not affect the expression of calnexin (Fig. 1A). Interestingly, RNAi-based CRT knockdown significantly increased CFTR expression by ~1.6-fold (Fig. 1A, WT), although it did not affect ΔF508 CFTR expression (Fig. 1A, ΔF). Moreover, we also determined that CRT knockdown increases the CFTR expression in vivo. Because CRT knock-out mice (CRT−/−) are embryonically lethal (30), we utilized CRT heterozygous mice (CRT+/−) (30). Consistent with the results in RNAi-based knockdown, endogenous CFTR expression in CRT heterozygous mice (CRT+/−) was significantly higher than in wild-type mouse (CRT+/+) (Fig. 1B, and C). In CRT+/− mice, in which there was a ~35% reduction of endogenous CFTR expression, we detected an ~1.4-fold increase of endogenous CFTR compared with that in CRT+/+ mice (Fig. 1B, and C).

CRT Overexpression Decreases Expression and Function of CFTR—To confirm whether CRT negatively regulates the CFTR expression, we examined the effect of CRT overexpression on the steady state level of CFTR. Western blotting analysis showed that the recombinant adeno-
CRT Negatively Regulates Cell Surface Expression of CFTR

FIGURE 3. CRT overexpression destabilizes the mature CFTR. A, CRT overexpression does not inhibit the maturation of CFTR. CFTR-CHO cells infected with or without Ad-CRT (m.o.i. 50) were pulse-labeled 48 h after infection. Radiolabeled CFTR was isolated from cell lysates after the indicated chase periods by immunoprecipitation with anti-CFTR antibody and analyzed by SDS-PAGE. Band B, immature CFTR; band C, mature CFTR. B, quantification of pulse-chase experiments in Fig. 3A. The intensity of the band indicating wild-type (wt) CFTR was quantified by Image Gauge software and expressed as a percentage of the total material present at t = 0. C, CRT overexpression does not affect the ER-Golgi transport of CFTR. CFTR-BHK cells infected with or without Ad-CRT (m.o.i. 50) were treated with 5 μg/ml BFA for 6 h (0 min). After BFA treatment, cells were incubated with the medium containing 1 μM cycloheximide (BFA washout) for the time periods indicated, fixed, and analyzed using a confocal laser scanning microscope. Bars, 10 μm. D, CRT overexpression destabilizes mature CFTR. Pulse-chase analysis was performed as in A. E, stability of mature CFTR upon CRT overexpression. The intensity of the band indicating mature wild-type CFTR (in D) was quantified by Image Gauge software and expressed as a percentage of the mature CFTR present at t = 1 h, respectively (n = 2).

virus expressing CRT (Ad-CRT) significantly increased CRT expression in an m.o.i.-dependent manner (Fig. 2A, lanes 3–5). At a m.o.i. of 100, an ~2.2-fold induction of CRT was observed (Fig. 2A, lane 4). There was no morphological change of the cells even if cells were infected with Ad-CRT at a m.o.i. of 800 (data not shown). As expected, CRT overexpression significantly decreased CFTR expression, especially the mature form (band C), in a m.o.i.-dependent manner (Fig. 2A, lanes 3–5). It is unlikely that decreased CFTR expression resulted from the cytotoxicity of adenovirus, because control adenovirus expressing β-galactosidase (Ad-LacZ) did not affect the CFTR expression (Fig. 2A, lanes 6 and 7), similar to our previous report (16). In contrast to the result in wild-type CFTR, CRT overexpression did not affect the expression of ΔF508 CFTR, which is the most common mutant of CFTR retained in the ER (Fig. 2A). The negative effect of CRT on CFTR expression seems to be CRT-specific, because CRT overexpression did not affect the expression of a viral transmembrane protein, VSV-G (Fig. 2B). Cell surface biotinylation analysis revealed that CRT overexpression decreased cell surface expression of CFTR (Fig. 2C). Interestingly, cell surface expression of CRT was observed upon CRT overexpression (Fig. 2C). This result was supported by immunocytochemical analysis using BHK cells stably expressing GFP-CFTR (CFTR-BHK cells, Fig. 2D and E). Fig. 2D and E, shows that the fluorescence intensity of GFP-CFTR at the plasma membrane was significantly decreased by CRT overexpression and that there was an inverse correlation in fluorescence intensity between CFTR and CRT (Fig. 2D and E, +Ad-CRT, arrowhead). Furthermore, we examined the effects of CRT overexpression on the endogenous CFTR in normal human airway epithelial cells (16HBE14o− cell). Because it was very difficult to detect the cell surface expression of endogenous CFTR by Western blotting analysis, we measured cAMP-activated halide efflux as a functional marker of CFTR (31). In control cells, cAMP-induced 125I efflux was observed, indicating that CFTR was activated (Fig. 2F). In the cells infected with Ad-CRT, cAMP-induced 125I efflux was significantly inhibited, although Ad-LacZ did not affect cAMP-induced 125I efflux (Fig. 2F). These results demonstrate that CRT overexpression decreases the cell surface expression and function of CFTR.

CRT Overexpression Destabilizes the Mature CFTR—Next, we tried to determine the mechanism by which CRT negatively regulates CFTR expression. Reverse transcription-PCR analysis revealed that CRT overexpression did not affect the mRNA level of CFTR (data not shown). Because CRT overexpression decreased the mature CFTR, but not ΔF508 CFTR, CRT overexpression could inhibit the maturation of CFTR. To clarify this possibility, we did pulse-chase analysis. In controls, ~40% of immature CFTR was converted to the mature form (band C) after a 3-h chase (Fig. 3, A and B, uninfected). Upon CRT overexpression, ~40% of the immature CFTR was also converted to the mature form after a 3-h chase (Fig. 3, A and B, +Ad-CRT), indicating that CRT overexpression did not inhibit the maturation of CFTR. This result was supported by immunocytochemical analyses showing that CRT overexpression did not affect the ER-Golgi transport of CFTR (Fig. 3C). Because CRT overexpression did not affect the maturation of CFTR, it is possible that CRT overexpression may destabilize the mature CFTR. Pulse-chase analysis showed that in controls, the half-life of mature CFTR was ~20 h (Fig. 3, D and E, uninfected), the same as in previous reports (6). Interestingly, the half-life of mature CFTR upon CRT overexpression was ~10 h (Fig. 3, D and E, +Ad-CRT), indicating that CRT overexpression destabilized mature CFTR resulting in the decrease of CFTR expression.

CRT Overexpression Enhances the Internalization of Cell Surface CFTR—Previous reports showed that the amount of cell surface CFTR is regulated by the endocytosis pathway (32–35). Thus, it is possible that the destabilization of mature CFTR by CRT overexpression resulted
from enhanced endocytosis of cell surface CFTR. To clarify this possibility, we performed an endocytic assay using cell surface biotinylation by NHS-SS-biotin, a cleavable derivative of NHS-biotin (36). Following cell surface biotinylation at 4 °C, the temperature of the medium was shifted to 37 °C for a period of 3–30 min to allow internalization of cell surface CFTR. Biotin molecules remaining at the cell surface were stripped with the membrane-impermeable reducing agent MESNA (36). Immediately after cell surface biotinylation (0 min), biotinylated CFTR was not detected after stripping (Fig. 4A). However, after incubation at 37 °C, biotinylated CFTR was detected after stripping, indicating that cell surface CFTR was internalized (Fig. 4A). Quantification analysis showed that, similar to a previous report (36), ~20% of cell surface CFTR was internalized within 6 min followed by a gradual loss of internalized CFTR (Fig. 4, A and B, control), a result of recycling back to the plasma membrane (34, 35). Upon CRT overexpression, ~30% of cell surface CFTR was internalized within 6 min (Fig. 4, A and B, +Ad-CRT), indicating that CRT overexpression enhances the internalization of cell surface CFTR. Moreover, after 6 min, internalized CFTR was increased, and ~42% of cell surface CFTR was internalized after 30 min, indicating that CRT overexpression might also inhibit the recycling of internalized CFTR.

To clarify the possibility that CRT overexpression also inhibits endocytic recycling, we utilized a vacuolar ATPase inhibitor, bafilomycin A1 (BafA1), which inhibits the transport of vesicles out of the early endosome (37). After BafA1 treatment (1 μM for 3 h), CFTR was accumulated in the juxtanuclear region where an early endosome marker EE1A was co-localized (Fig. 4C). After BafA1 washout, CFTR accumulated in early endosome was rapidly moved and recycled back to the plasma membrane (Fig. 4D, control; supplemental Movie 1). However, in CRT-overexpressing cells, CFTR accumulated in the early endosome was immobilized (Fig. 4D, +Ad-CRT; supplemental Movie 2), indicating that CRT overexpression may inhibit the recycling of CFTR. These results indicate that CRT overexpression enhances the endocytosis of CFTR and inhibits recycling back to the plasma membrane.

**CRT Overexpression Directs CFTR to Proteasomal Degradation—**Our data showed that CRT overexpression enhances the endocytosis of cell surface CFTR and inhibits endocytic recycling to the plasma membrane. Thus, CRT may direct cell surface CFTR to lysosomal degradation, resulting in a decrease of CFTR expression. To clarify this possibility, we examined the effect of degradation inhibitors on the decrease of mature CFTR by CRT overexpression. Western blotting analysis showed that low concentration bafilomycin A1 treatment (200 nM, 24 h), which inhibits lysosomal degradation, did not block the decrease
CRT Negatively Regulates Cell Surface Expression of CFTR

FIGURE 6. CRT negatively regulates the CFTR expression at the post-ER compartment. A, schematic model of CRT mutants. CRT mutants were tagged in the C terminus with c-Myc. In the case of CRT full-length (Full), c-Myc was tagged in front of the KDEL ER retrieval signal. N, N terminus; P, P-domain; C, C terminus. B, cellular localization of the CRT mutants. CFTR-BHK cells transfected with CRT deletion mutants tagged with c-Myc were immunostained with anti-Myc antibody and visualized with TRITC-conjugated secondary antibody. Bars, 10 μm. C, effect of CRT deletion mutants on the CFTR expression. Cell lysates from CFTR-CHO cells transfected with CRT mutants tagged with c-Myc were analyzed by Western blotting. CNX was used for loading controls. The data shown are representative of three independent experiments. Bands B and C, immature and mature CFTR, respectively. D, Quantification of panel C. The intensity of the mature CFTR was quantified with Image Gauge software and expressed as a percentage of band C in the control (mean value ± S.D., n = 3). *, p < 0.01; **, p < 0.05. E, deletion of CRT with the C terminus enhances the interaction with CFTR. CFTR-CHO cells transfected with CRT deletion mutants were lysed in 0.5% digitonin buffer. Cell lysates were immunoprecipitated with anti-CFTR antibody and the precipitants were analyzed by Western blotting. F, endogenous CRT has a weak interaction with CFTR. CFTR-CHO cells were treated with or without a cross-linker, DTSSP (100 μg/ml), for 30 min at room temperature and lysed in 0.5% digitonin buffer. Cell lysates were immunoprecipitated with anti-CFTR antibody, and the precipitants were analyzed by Western blotting with anti-CFTR and anti-CRT antibodies. G, CRT preferentially interacts with mature CFTR. CFTR-CHO cells infected with Ad-CRT were lysed in 0.5% digitonin buffer, and lysates were immunoprecipitated with anti-CRT antibody. Immunoprecipitants (IP) were analyzed by Western blotting.

of mature CFTR induced by CRT overexpression (Fig. 5a, lane 5). However, proteasome inhibitors MG-132 and lactacystin significantly blocked the negative effect of CRT (Fig. 5a, lanes 3 and 4). The same result was obtained from cell surface biotinylation analysis (Fig. 5b). These results indicate that CRT overexpression stimulates the proteasomal degradation of cell surface CFTR.

CRT Negatively Regulates CFTR Expression at the Post-ER Compartment—To examine which regions of CRT are required for CRT to exert its negative effect on CFTR expression, we constructed several CRT deletion mutants tagged with c-Myc (Fig. 6A). Immunocytochemical analysis of CFTR-BHK cells showed that deletion mutants of the ER retrieval signal (ΔKDEL) and the C terminus of CRT (ΔC) left the ER and localized in the Golgi (Fig. 6B; supplemental Fig. 1) similar to a previous report (38). Western blotting in CFTR-CHO cells showed that the ER-exported mutants of CRT, especially CRTΔC, decreased CFTR expression more than full-length CRT (Fig. 6C and D), indicating that the post-ER fraction of CRT exerts the negative effect. This result was supported by immunoprecipitation analysis; immunoprecipitation using anti-CFTR antibody revealed that CRTΔC interacted with CFTR (Fig. 6E, lane 8). There was a weak interaction between CFTR and CRT (Fig. 6E, lane 4), as detected by a cross-linker, DTSSP (Fig. 6F). Furthermore, immunoprecipitation using an anti-CRT antibody showed that CRT seems to interact preferentially with mature CFTR (Fig. 6G, band C). These results indicate that CRT interacts with mature CFTR and negatively regulates the CFTR expression at the post-ER compartment.

DISCUSSION

In this study, we demonstrate that CRT negatively regulates the cell surface expression of CFTR. RNAi-based CRT knockdown significantly increased mature CFTR (Fig. 1A), and endogenous CFTR expression was also increased in CRT+/− mice (Fig. 1, B and C). Thus, CRT seems to negatively regulate CFTR expression in vivo. This interpretation is supported by the results of overexpression experiments. CRT overexpression decreased the steady state level of CFTR, especially that of the mature form, in a dose-dependent manner (Fig. 2A). CRT overexpression also decreased the cell surface expression and function of CFTR (Fig. 2, C and F). However, CRT overexpression did not affect ΔF508 CFTR expression (Fig. 2A), indicating that CRT could affect the CFTR expression at the post-ER level. The negative effect of CRT may be
CFTR-specific, because CRT overexpression did not decrease the expression of VSV-G (Fig. 2B) and an ABC (ATP-binding cassette) transporter, ABCG5/8.4

Our data clarified one of the mechanisms by which CRT negatively regulates the cell surface CFTR expression. Upon CRT overexpression, CRT expressed at the plasma membrane (Fig. 2C) and interacted with mature CFTR (Fig. 6, F and G). Cell surface expression of CFTR may result from the binding of secreted CRT to cell surface CFTR because CRT was secreted to the extracellular domain upon CRT overexpression.5 Although CRT overexpression did not inhibit maturation (Fig. 3A), it destabilized mature CFTR (Fig. 3, D and E). CRT overexpression stimulated the internalization of cell surface CFTR and inhibited recycling back to the plasma membrane (Fig. 4, A, B, and D). A defect in recycling in CRT-overexpressing cells might be predominant, because the effect of CRT overexpression was stronger from 6 to 30 min after internalization (Fig. 4, A and B). Moreover, CRT seems to stimulate the proteasomal degradation of mature CFTR (Fig. 5). The proteasomal degradation of mature CFTR in the post-Golgi compartment has been reported previously (39). In regard to ΔF508 CFTR, we found that the function of cell surface ΔF508 CFTR rescued by incubation at 26 °C was enhanced by CRT knockdown.5 Moreover, it has been reported that cell surface ΔF508 CFTR is quickly internalized and degraded by proteosomes (35, 40) and that CRT expression is up-regulated in cystic fibrosis cells (41). Thus, we speculate that although CRT overexpression did not affect the expression of ΔF508 CFTR localized in the ER (Fig. 2A), CRT may be involved in the instability of ΔF508 CFTR at the cell surface.

Our interpretation that CRT negatively regulates CFTR expression at the post-ER level could be supported by the result that CRTΔC decreased the CFTR expression more than full-length CRT (Fig. 6, C and D). CRTΔC was exported from the ER (Fig. 6B; supplemental Fig. 1) and secreted to the extracellular domain (38). CRTΔC interacted with CFTR with higher affinity than full-length CRT; the interaction of CRT with CFTR was very weak (Fig. 6, E and F). It is unlikely that the enhanced affinity of CRTΔC was due simply to the inclusion of the two proteins into a misfolded aggregate, because the majority of CRTΔC appears to be in the native conformation and is functional for polypeptide binding (25). Moreover, in our experiments, we detected CRTΔC in the detergent-soluble fraction (Fig. 6E), proving that this mutant, under the conditions observed, did not misfold. It is reasonable to conclude that the affinity of CRTΔC with CFTR is higher than that of full-length CRT because the C-domain of CRT prevents the interaction between CRT and its substrates (25). Alternatively, because CRTΔC is secreted (38), it may interact efficiently with mature CFTR at the cell surface.

It has been known that CRT is an ER lectin-like chaperone protein, which selectively interacts with substrates having monoglucosylated oligosaccharides. In this study, we have shown that CRT interacts with mature CFTR, which has complex oligosaccharides. Because CRT cannot interact with complex oligosaccharides (42), CRT could interact with mature CFTR via polypeptide-based interaction. This interpretation may be supported by our finding that deletion of the CRT-C domain, which prevents polypeptide-based interaction (25), enhanced the interaction with CFTR (Fig. 6E). Moreover, we found that a CRT mutant with no lectin activity, CRT(Y109F), decreased CFTR expression and that CRT interacted with CFTR under tunicamycin treatment, which inhibits the oligosaccharide addition in ER.6 Therefore, the lectin function of CRT could not be involved in the negative regulation of CFTR.

A previous study showed that stresses such as heat shock and Ca2+ depletion induce conformational change in CRT and enhance CRT interaction with its substrates (25). It has also been reported that CRT overexpression stimulates the proteasomal degradation of SERCA (sarco/endoplasmic reticulum Ca2+ ATPase) under oxidative stress (43). Although further investigations may be needed to clarify this hypothesis, it is likely that under a stress condition in which CRT is up-regulated (25, 26), CRT may be expressed at the cell surface and interact with CFTR, which may stimulate the endocytosis of CFTR and direct it to proteasomal degradation. Recently (during the preparation of this manuscript), it was reported that oxidant stress suppresses CFTR expression (44). Therefore, CRT may enhance the degradation of CFTR by oxidant stress. Acknowledgments—We thank Dr. J. R. Riordan for providing CFTR-CHO, DF508-CHO, CFTR-BHK, and DF508-BHK cells, Dr. D. C. Gruener for providing the human airway cell line, 16HBE14o−, and Dr. M. Michalak for providing the CRT heterozygous mouse.

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